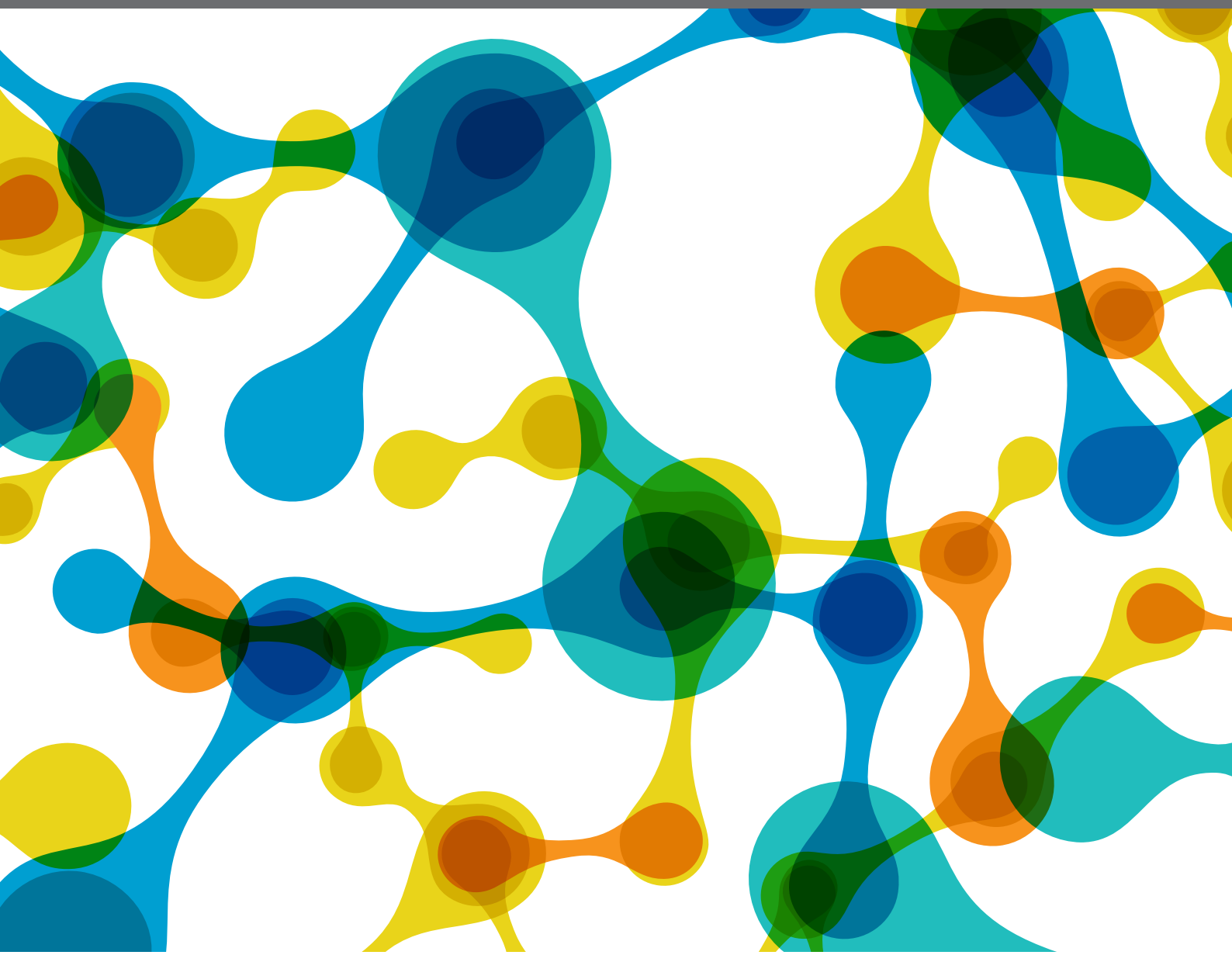
NEUROLOGICAL OUTCOMES IN PRETERM INFANTS – CURRENT CONTROVERSIES AND THERAPIES FOR BRAIN INJURY

EDITED BY: Carina Mallard, Mary Tolcos and Justin Dean

PUBLISHED IN: Frontiers in Physiology



frontiers Research Topics

frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-88963-494-1
DOI 10.3389/978-2-88963-494-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

NEUROLOGICAL OUTCOMES IN PRETERM INFANTS – CURRENT CONTROVERSIES AND THERAPIES FOR BRAIN INJURY

Topic Editors:
Carina Mallard, University of Gothenburg, Sweden
Mary Tolcos, RMIT University, Australia
Justin Dean, The University of Auckland, New Zealand

Preterm birth affects over 15 million newborns worldwide each year and is the main contributor of neonatal mortality and morbidity. While neonatal survival following preterm birth continues to improve, this has not been matched by a decline in neurological outcome. There is still a high prevalence of motor problems, executive dysfunction, and cognitive impairment in infants born preterm. Improved neuroimaging has helped to describe different types of neonatal brain injuries in this population and has given a better understanding of underlying pathogenesis. However, therapies are still lacking and there is a great need to find novel strategies to improve injury and functional outcome.

Citation: Mallard, C., Tolcos, M., Dean, J., eds. (2020). Neurological Outcomes in Preterm Infants – Current Controversies and Therapies for Brain Injury. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-494-1

Table of Contents

frontiers in Physiology

SYSTEMATIC REVIEW
published: 11 September 2018
doi: 10.3389/fphys.2018.01253

Chorioamnionitis Is a Risk Factor for Intraventricular Hemorrhage in Preterm Infants: A Systematic Review and Meta-Analysis

Eduardo Villamor-Martinez[1], *Monica Fumagalli*[2], *Owais Mohammed Rahim*[1], *Sofia Passera*[2], *Giacomo Cavallaro*[2], *Pieter Degraeuwe*[1], *Fabio Mosca*[2] *and Eduardo Villamor*[1]*

[1] *Department of Pediatrics, School for Oncology and Developmental Biology (GROW), Maastricht University Medical Center, Maastricht, Netherlands,* [2] *Neonatal Intensive Care Unit, Department of Clinical Sciences and Community Health, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, Italy*

OPEN ACCESS

Edited by:
Carina Mallard,
University of Gothenburg, Sweden

Reviewed by:
Adam John Watkins,
University of Nottingham, United Kingdom
Dean A. Myers,
University of Oklahoma Health Sciences Center, United States

***Correspondence:**
Eduardo Villamor
e.villamor@mumc.nl

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *30 May 2018*
Accepted: *20 August 2018*
Published: *11 September 2018*

Citation:
Villamor-Martinez E, Fumagalli M, Mohammed Rahim O, Passera S, Cavallaro G, Degraeuwe P, Mosca F and Villamor E (2018) Chorioamnionitis Is a Risk Factor for Intraventricular Hemorrhage in Preterm Infants: A Systematic Review and Meta-Analysis. Front. Physiol. 9:1253. doi: 10.3389/fphys.2018.01253

Although chorioamnionitis (CA) is a well-known risk factor for white matter disease of prematurity, the association with intraventricular hemorrhage (IVH) is controversial and has not been yet systematically reviewed. We performed a systematic review and meta-analysis of studies exploring the association between CA and IVH. A comprehensive literature search was conducted using PubMed/MEDLINE and EMBASE, from their inception to 1 July 2017. Studies were included if they examined preterm infants and reported primary data that could be used to measure the association between exposure to CA and the presence of IVH. A random-effects model was used to calculate odds ratios (OR) and 95% confidence intervals (CI). We found 1,284 potentially relevant studies, of which 85 met the inclusion criteria (46,244 infants, 13,432 CA cases). Meta-analysis showed that CA exposure was significantly associated with all grades IVH (OR 1.88, 95% CI 1.61–2.19), with grades 1–2 IVH (OR 1.69, 95% CI 1.22–2.34), and with grades 3–4 IVH (OR 1.62, 95% CI 1.42–1.85). Both clinical and histological CA were associated with an increased risk for developing IVH in very preterm infants. In contrast, the presence of funisitis did not increase IVH risk when compared to CA in the absence of funisitis (OR 1.22, 95% CI 0.89–1.67). Further meta-analyses confirmed earlier findings that CA-exposed infants have significantly lower gestational age (GA; mean difference [MD] −1.20 weeks) and lower birth weight (BW; MD −55 g) than the infants not exposed to CA. However, meta-regression and subgroup analysis could not demonstrate an association between the lower GA and BW and the risk of IVH in the CA-exposed infants. In conclusion, our data show that CA is a risk factor for IVH, but also a risk factor for greater prematurity and more clinical instability. In contrast to other complications of prematurity, such as patent ductus arteriosus, retinopathy of prematurity, or bronchopulmonary dysplasia, the effect of CA on IVH appears to be independent of CA as causative factor for very preterm birth.

Keywords: chorioamnionitis, intraventricular hemorrhage, very preterm infant, systematic review, meta-analysis

INTRODUCTION

Germinal matrix hemorrhage–intraventricular hemorrhage (GMH-IVH) is one of the most common complications of prematurity (Ballabh, 2010; Volpe, 2015; Inder et al., 2018). IVH typically initiates in the germinal matrix, which is a richly vascularized collection of neuronal-glial precursor cells in the developing brain and may disrupt the ependymal lining and extend into the lateral ventricle (Ballabh, 2010; Volpe, 2015; Inder et al., 2018). Severe IVH (grade 3–4) is associated with increased mortality as well as short- and long-term neurological morbidity, whilst the short-term and long-term outcomes of milder forms of IVH (grade 1–2) are less established, and they remain a significant research area (Volpe, 2015; Tortora et al., 2017; Inder et al., 2018).

As extensively reviewed by Inder et al. (2018) the pathogenesis of IVH is multifactorial and may involve intravascular, vascular, and extravascular factors. Intravascular factors relate to the regulation of blood flow, pressure, and volume in the microvascular bed of the germinal matrix as well as to platelet-capillary function and blood clotting capability (Inder et al., 2018). Vascular factors refer to the intrinsic fragility and vulnerability of germinal matrix blood vessels (Inder et al., 2018). Extravascular factors include the poor support of the extravascular space surrounding the germinal matrix capillaries, the postnatal decrease in extravascular tissue pressure, and an excessive fibrinolytic activity (Inder et al., 2018). As assessed by Inder et al. (2018) not all the pathogenetic factors are present in every IVH and the clinical circumstances determine which factors are most relevant in each infant. Among these clinical circumstances, very preterm birth, generally defined as birth before 32 completed weeks of gestation, is the most consistently associated with the development of IVH. However, a number of risk factors including, among others, absent antenatal corticosteroid (ACS) treatment, vaginal delivery, peri- and postnatal hypoxic-ischemic events, severe respiratory distress syndrome (RDS), pneumothorax, hypercapnia, hemodynamic disturbances (either systemic hypertension or hypotension), rapid volume expansion, decreased hematocrit, glucose and/or electrolyte disturbances, seizures, patent ductus arteriosus (PDA), thrombocytopenia, inherited thrombophilia, and infection may predispose to the development of IVH (Ballabh, 2010; Ramenghi et al., 2011; Volpe, 2015; Bermick et al., 2016; Romantsik et al., 2017; Inder et al., 2018; Poryo et al., 2018).

Several studies suggest that IVH is unequally distributed among the different leading causes of very preterm delivery (DiSalvo, 1998; Chevallier et al., 2017). An estimated 40% of very preterm births are associated with placental inflammation, which is often subclinical. This inflammation may be localized to the maternal placenta or membrane (chorioamnionitis) or may extend to the fetus, inducing an inflammatory response, which is evidenced by funisitis (Cornette, 2004; Gantert et al., 2010; Tita and Andrews, 2010; Thomas and Speer, 2011; Pugni et al., 2016; Jackson et al., 2017). Chorioamnionitis (CA) is not only a major risk factor for (very) preterm birth, but it is also considered a major risk factor for the morbidity and mortality associated with prematurity (Cornette, 2004; Gantert et al., 2010; Tita and Andrews, 2010; Thomas and Speer, 2011; Pugni et al., 2016; Jackson et al., 2017). The pathogenetic role of CA in the development of complications of prematurity, such as necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), PDA, retinopathy of prematurity (ROP), or cerebral palsy has been addressed in several systematic reviews (Wu and Colford, 2000; Hartling et al., 2012; Been et al., 2013; Mitra et al., 2014; Park et al., 2015; Behbodi et al., 2016; Villamor-Martinez et al., 2018a). Although intrauterine inflammation is a well-known risk factor for white matter disease of prematurity (Strunk et al., 2014), the association with IVH is controversial and has not been yet systematically reviewed. Moreover, a consideration with any analysis of CA as a risk factor for preterm morbidity, is accounting for the role of GA, birth weight (BW) and other baseline characteristics which differ between CA-exposed and CA-unexposed infants (Hartling et al., 2012; Mitra et al., 2014; Behbodi et al., 2016; Villamor-Martinez et al., 2018a). With this in mind, we aimed to perform a systematic review and meta-analysis of studies exploring the association between CA and IVH, as well as the role of potential confounding factors.

METHODS

The methodology followed the same structure as earlier meta-analyses on CA and ROP (Villamor-Martinez et al., 2018a), and CA and PDA (Behbodi et al., 2016). We developed a protocol a priori, which specified the objectives, inclusion criteria, method for evaluating study quality, included outcomes and covariates, and statistical methodology. We report the study according to the guidelines for Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (Moher et al., 2009).

Sources and Search Strategy

We performed a comprehensive literature search in the PubMed/MEDLINE and EMBASE databases from their inception to July 1, 2017. The search strategy involved combining the following keywords in various ways: "chorioamnionitis," "intrauterine infection," "intrauterine inflammation," "antenatal infection," "antenatal inflammation," "intraventricular hemorrhage," "risk factors," "outcome," "cohort," and "case-control." No studies were excluded based on language. In addition, we used the following strategies to identify additional studies: review of reference lists of previous systematic reviews on CA, and of articles included in the present review, and the use of the "cited by" tool in Web of Science and Google Scholar.

Study Selection

We included studies which evaluated infants who were preterm (<37 weeks) or low BW (<2,500g), as well as studies which used stricter inclusion criteria. Studies were included if they reported primary data on the association between CA-exposure and IVH. We included studies which reported the rate of IVH in infants with and without CA, and studies which reported the rate of CA in infants with and without IVH. The results of the total search were screened independently by two reviewers (O.M.R, E.V.), in several rounds: first by title only, second by title and abstract,

and thirdly by consulting the full text. The reviewers resolved discrepancies in inclusion through discussion and by consulting a third reviewer (P.D).

Data Extraction

Using a predetermined worksheet, two researchers (E.V.-M., O.M.R.) extracted data from the studies included. Another two investigators (P.D., E.V.) checked the extracted data for accuracy and completeness. We resolved discrepancies by discussion and through checking the primary report. The following data were extracted from each study: citation information, location of study, primary objective, criteria for inclusion/exclusion of infants, definitions used for CA and for IVH, infant baseline characteristics in the total group and the CA-exposed and CA-unexposed groups, and reported results on the outcomes of interest (including raw numbers, summary statistics and adjusted analyses on CA and IVH where available).

Quality Assessment

We used the Newcastle-Ottawa Scale (NOS) for cohort or case-control studies to assess the methodological quality of included studies. Three aspects of a study are evaluated by the NOS: selection, comparability and exposure/outcome, and these are scored individually and tallied up to a total of 9 points. Two researchers (E.V.-M. and E.V.) independently used the NOS to evaluate the quality of each study, and discrepancies were discussed and resolved by consensus.

Statistical Analysis

We combined and analyzed studies using COMPREHENSIVE META-ANALYSIS V 3.0 software (CMA, RRID:SCR_012779, Biostat Inc., Englewood, NJ, USA). We calculated the odds ratio (OR) and 95% confidence intervals (CI) for dichotomous outcomes from the data extracted from the studies. We calculated the mean difference (MD) and 95% CI for continuous outcomes. We used the method of Wan et al. (2014) to estimate the mean and standard deviation, when continuous variables were reported as median and range/interquartile range in studies. We used a random-effects model to calculate summary statistics, due to anticipated heterogeneity. This method accounts for both intra-study and inter-study variability.

A mixed-effects model was used for subgroup analyses (Borenstein et al., 2009a). This model is characterized by a random-effects model that combines studies within subgroups, and a fixed-effects model that combines subgroups together to create an overall effect. This model does not assume that study-to-study variance (tau-squared) is the same in all subgroups. We assessed statistical heterogeneity using the Cochran's Q statistic, which reflects the degree of variance, and the I^2-statistic, which describes the proportion of observed variance that is due to variance in true effect sizes rather than sampling error (Borenstein et al., 2009b). Visual inspection of funnel plots and Egger's regression test were used to evaluate evidence of publication bias.

We used univariate random-effects meta-regression (method of moments) to evaluate covariates which may affect the effect size (Borenstein et al., 2009c). We defined the following covariates a priori as potential sources of variability: CA type (clinical or histological), funisitis, differences in GA and BW between the infants with and without CA, use of ACS, mode of delivery, rate of preeclampsia, rate of small for gestational age (SGA), rate of premature rupture of membranes (PROM), rate of RDS, rate of PDA, rate of early onset sepsis (EOS), rate of late onset sepsis (LOS) and mortality. We considered probability values under 0.05 (0.10 for heterogeneity) as statistically significant.

RESULTS

Description of Studies

After removing duplicates, we found 1,284 potentially relevant studies, of which 85 (Morales, 1987; Yoon et al., 1995; Gray et al., 1997; Alexander et al., 1998; Watterberg et al., 1999; Dexter et al., 2000; Elimian et al., 2000; Kosuge et al., 2000; Hitti et al., 2001; Suarez et al., 2001; González-Luis et al., 2002; Ohyama et al., 2002; Fung et al., 2003; Holcroft et al., 2003; Linder et al., 2003; Ogunyemi et al., 2003; Vergani et al., 2004; Dempsey et al., 2005; Lau et al., 2005; Osmanagaoglu et al., 2005; Polam et al., 2005; Sarkar et al., 2005; Babnik et al., 2006; Kaukola et al., 2006; Mehta et al., 2006; Richardson et al., 2006; Rocha et al., 2006; Yanowitz et al., 2006; Kirchner et al., 2007; Baumert et al., 2008; Mu et al., 2008; Zanardo et al., 2008; Been et al., 2009; Soraisham et al., 2009, 2013; Suppiej et al., 2009; Austeng et al., 2010; Botet et al., 2010; Kallankari et al., 2010; Lee et al., 2010, 2011; Mestan et al., 2010; Alfiero Bordigato et al., 2011; Barrera-Reyes et al., 2011; Hendson et al., 2011; Lim et al., 2011; Ryckman et al., 2011; Sato et al., 2011; Wirbelauer et al., 2011; Ahn et al., 2012; Klebermass-Schrehof et al., 2012; Perrone et al., 2012; Poralla et al., 2012; Rong et al., 2012; Vaihinger et al., 2012; van Vliet et al., 2012; Xu et al., 2012; Adén et al., 2013; Erdemir et al., 2013; Gawade et al., 2013; Logan et al., 2013; Nasef et al., 2013; Salas et al., 2013; Seliga-Siwecka and Kornacka, 2013; Trevisanuto et al., 2013; Tsiartas et al., 2013; Arayici et al., 2014; Ecevit et al., 2014; Gagliardi et al., 2014; García-Muñoz Rodrigo et al., 2014; Kidokoro et al., 2014; Liu et al., 2014; Pappas et al., 2014; Shankaran et al., 2014; Bry et al., 2015; Dalton et al., 2015; Kim et al., 2015; Oh et al., 2015, 2018; Smit et al., 2015; Yamada et al., 2015; Bermick et al., 2016; Lu et al., 2016; Miyazaki et al., 2016; Rodríguez-Trujillo et al., 2016) met the inclusion criteria. **Figure 1** depicts the PRISMA flow diagram of the search. The included studies evaluated 46,244 infants, including 13,432 cases of CA. The characteristics of the included studies are summarized in **Supplementary Table 1**. Fifty-eight studies examined the outcomes of maternal CA and included IVH as one of the outcomes. Twenty-four studies evaluated risk factors for developing IVH and included maternal CA as of the risk factors. Five studies studied the association between CA and IVH as their primary outcome (De Felice et al., 2001; Sarkar et al., 2005; Babnik et al., 2006; Mehta et al., 2006; Zanardo et al., 2008). Fifty-four studies used a histological definition of CA and 24 studies used a clinical definition of CA. Only two studies (Hitti et al., 2001; Kirchner et al., 2007) examined microbiological CA and IVH. One study (Nasef et al., 2013) provided data on IVH and its association with histological and clinical CA separately.

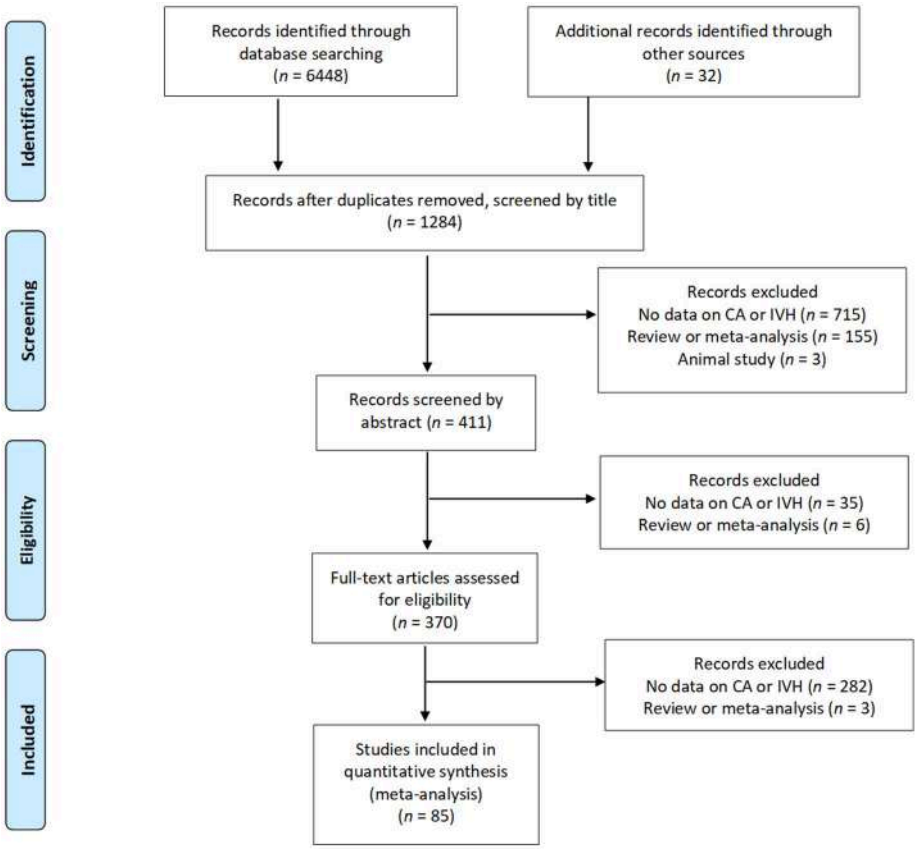


FIGURE 1 | Flow diagram of search process (Moher et al., 2009).

In four studies (Gray et al., 1997; Fung et al., 2003; Klebermass-Schrehof et al., 2012; Xu et al., 2012) infants were assigned to the CA group if they presented histological and/or clinical CA.

Quality Assessment

A summary of the quality assessment of each study using the NOS is shown in **Supplementary Table 2**. One study received a quality score of 5 points, 19 studies achieved a quality score of six points, 43 studies achieved a quality score of seven points, 11 studies achieved a quality score of eight, and 11 studies achieved the maximum score of 9 points. Studies were downgraded in quality for not adjusting the risk of IVH for confounders ($k = 62$), for not defining IVH clearly ($k = 9$), for only adjusting the risk of IVH for one confounding factor ($k = 7$), for not defining CA clearly ($k = 6$), and for losing a substantial portion of infants to follow-up ($k = 4$).

Analysis Based on Unadjusted Data

Meta-analysis showed that CA exposure was significantly associated with all grades IVH (**Figure 2A**), with grades 2–4 IVH (**Figure 2B**), with grades 1–2 IVH (**Figure 2C**), and with grades 3–4 IVH (**Figure 2D**). When the type of CA was analyzed separately, histological CA remained significantly associated with all grades IVH (**Figure 3**), with grades 2–4 IVH (**Figure 2B**), with grades 1–2 IVH (**Figure 2C**), and with grades 3–4 IVH (**Figure 4**). Clinical CA was significantly associated with all grades IVH (**Figure 5**) and with grades 3–4 IVH (**Figure 6**), but not with grades 1–2 IVH (**Figure 2C**). There was only one study providing data on the association of clinical CA and IVH grades 2–4 (**Figure 2B**). We could not find significant evidence of publication bias through visual inspection of the funnel plot (**Figure 7**), or through Egger's regression test.

Analysis of Covariates

To confirm findings from earlier reports (Behbodi et al., 2016; Villamor-Martinez et al., 2018a) on the differences in baseline and clinical characteristics between infants with and without CA, we carried out further meta-analyses. Infants exposed to CA had significantly lower GA and BW, as shown in **Table 1**. Moreover, infants with CA had significantly higher rates of exposure to ACS, significantly higher rates of PROM, significantly higher rates of EOS, significantly higher rates of LOS, and significantly higher rates of PDA (**Table 1**). Infants with CA also had significantly lower rates of cesarean delivery, significantly lower rates of small for gestational age (SGA) and significantly lower rates of preeclampsia (**Table 1**).

We carried out meta-regression analysis to determine the possible influence of GA and BW on the association between CA

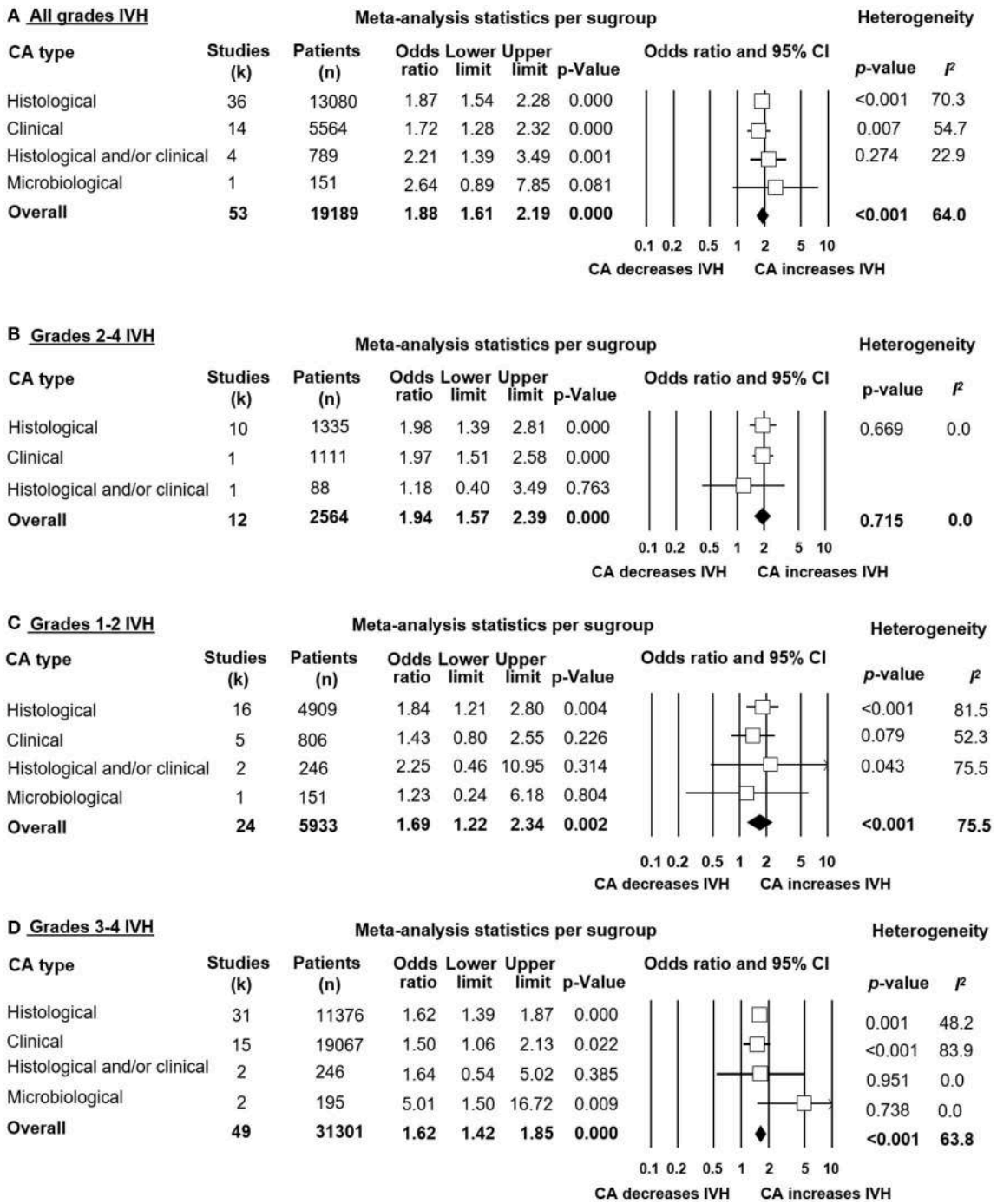


FIGURE 2 | Meta-analyses of the association between chorioamnionitis (CA) and intraventricular hemorrhage (IVH), according to definition of IVH. CI, confidence interval. **(A)** CA and all grades IVH; **(B)** CA and grades 2-4 IVH; **(C)** CA and grades 1–2 IVH; **(D)** CA and grades 3–4 IVH.

and IVH. As **Table 2** shows, meta-regression did not find that differences in GA or BW had a significant effect on the association between CA and IVH.

To further analyze the effect of GA on the risk of IVH, we carried out subgroup analyses. We found that in the group of studies where the CA-group did not differ significantly ($p > 0.05$) in GA from the control group, CA was a risk factor for all grades IVH but not for grades 3–4 IVH (**Table 3**). We analyzed a subgroup of studies where the CA-group had a MD in GA of $\leq$0.5 weeks, and we found that CA was a risk factor for all grades IVH

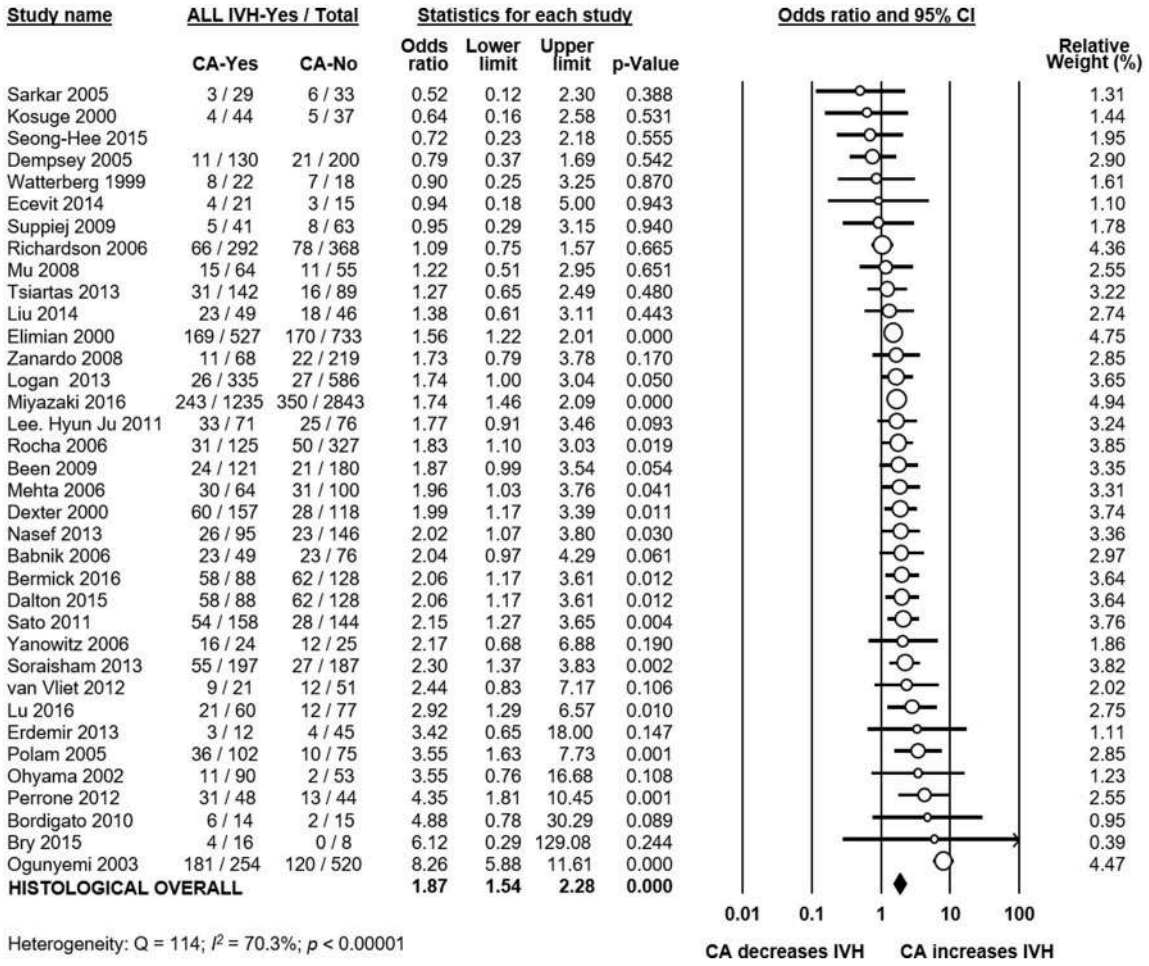


FIGURE 3 | Meta-analysis of the association between histological chorioamnionitis (CA) and all grades intraventricular hemorrhage (IVH). CI, confidence interval.

and for grades 3–4 IVH (**Table 3**). We also found that in studies where the CA-group had a MD in GA of less than 1 week, CA was a risk factor for all grades IVH and for grades 3–4 IVH (**Table 3**).

To evaluate the role of other prespecified covariates in the association between CA and IVH, we performed additional meta-regression analyses. Meta-regression could not find a significant difference in IVH risk between infants with clinical and infants with histological CA (**Table 4**). Meta-regression did find a significant association between the CA-associated risk of grades 3–4 IVH and the risk of preeclampsia (**Supplementary Figure 1**), mortality (**Supplementary Figure 2**), risk of LOS (**Supplementary Figure 3**) and risk of PDA (**Supplementary Figure 4**) Other meta-regressions could not find a significant association between the CA-associated risk of IVH and other covariates (**Table 4**).

Analysis of Funisitis

To evaluate the role of funisitis (i.e., fetal inflammatory response) in the development of IVH, we carried out further meta-analyses. Thirteen studies reported on IVH (Ohyama et al., 2002; Lau et al., 2005; Babnik et al., 2006; Richardson et al., 2006; Rocha et al., 2006; Been et al., 2009; Mestan et al., 2010; Logan et al., 2013; Trevisanuto et al., 2013; Tsiartas et al., 2013; Liu et al., 2014; Kim et al., 2015; Smit et al., 2015) in infants with histological CA with or without funisitis. As shown in **Figure 8**, meta-analysis could not show a significant difference in IVH risk between infants with funisitis and infants with CA without funisitis (OR all grades IVH: 1.22, 95% CI 0.89 to 1.67; grades 3-4 IVH: 1.17, 95% CI 0.74 to 1.85). Using meta-regression, we also found no significant difference in IVH risk between infants with funisitis, and infants with CA without funisitis (**Table 4**).

Analysis Based on Adjusted Data

Thirteen studies adjusted the association between CA and the risk of IVH for confounding factors. As shown in **Supplementary Tables 3, 4**, studies adjusted for different covariates. Meta-analysis pooling this adjusted data found that CA was significantly associated with a higher risk of all grades IVH (OR 1.25, 95% CI 1.02–1.53, **Supplementary Table 3**). This association became non-significant when only

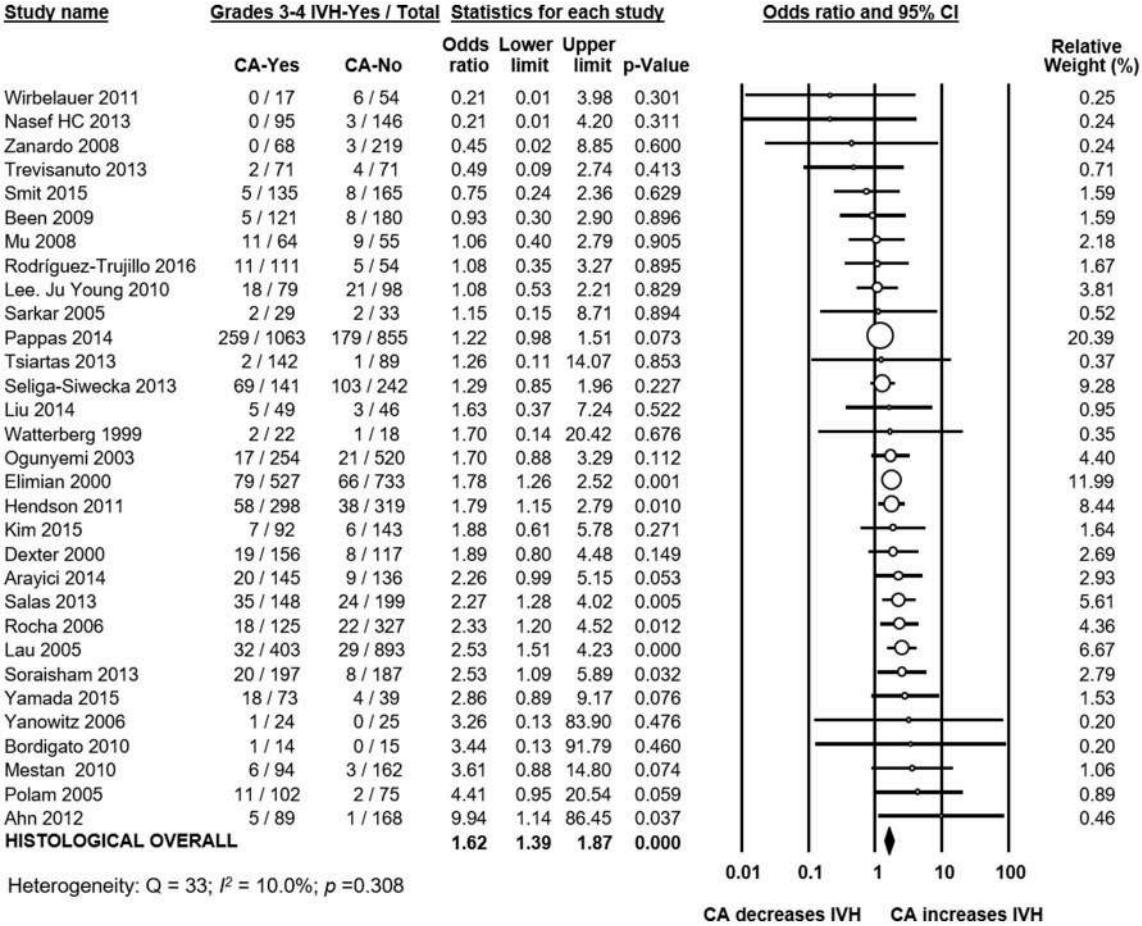


FIGURE 4 | Meta-analysis of the association between histological chorioamnionitis (CA) and grades 3–4 intraventricular hemorrhage (IVH). CI, confidence interval.

analyzing studies which used a histological definition of CA (**Supplementary Table 3**). Meta-analysis of adjusted data also found a significant association between CA and grades 3–4 IVH (OR 1.22, 95% CI 1.04–1.43, **Supplementary Table 4**). This association became non-significant when grouping studies by clinical or histological CA definition (**Supplementary Table 4**).

DISCUSSION

The current systematic review and meta-analysis demonstrates that both clinical and histological CA are associated with an increased risk for developing IVH in very preterm infants. In contrast, the presence of funisitis did not increase IVH risk when compared to CA in the absence of funisitis. We found through additional meta-analyses that CA-exposed infants had significantly lower GA and BW than infants not exposed to CA. However, meta-regression and subgroup analysis could not demonstrate an association between the lower GA and BW and the risk of IVH in the CA-exposed infants. This suggests that the effects of CA on IVH risk might be at least partially independent on the role of CA as an etiological factor for very preterm birth.

The association between CA and increased risk of IVH is biologically and clinically plausible. IVH generally occurs within the three first days of life and affects the infants with higher hemodynamic and respiratory instability, frequently associated with extreme prematurity and/or severe perinatal infections (Mohamed and Aly, 2010; Inder et al., 2018). Therefore, the clinical circumstances around birth and during the first days of life are critical for the development of IVH. Our study confirms previous reports showing that these clinical circumstances are different in CA-exposed and CA–unexposed very preterm infants (Hartling et al., 2012; Behbodi et al., 2016; Villamor-Martinez et al., 2018a). Thus, CA-exposed infants were born 1.2 weeks earlier, they were 55g lighter at birth, and they were more frequently exposed to ACS, PROM, vaginal delivery, early and late onset sepsis, and PDA. As mentioned in the introduction, some of these factors may have affected IVH risk.

The degree of prematurity is the most important predisposing factor for the occurrence of IVH (Ballabh, 2010; Volpe, 2015; Inder et al., 2018), as well as for other complications of prematurity such as BPD, ROP, NEC, or PDA (Hartling et al., 2012; Been et al., 2013; Mitra et al., 2014; Behbodi et al., 2016;

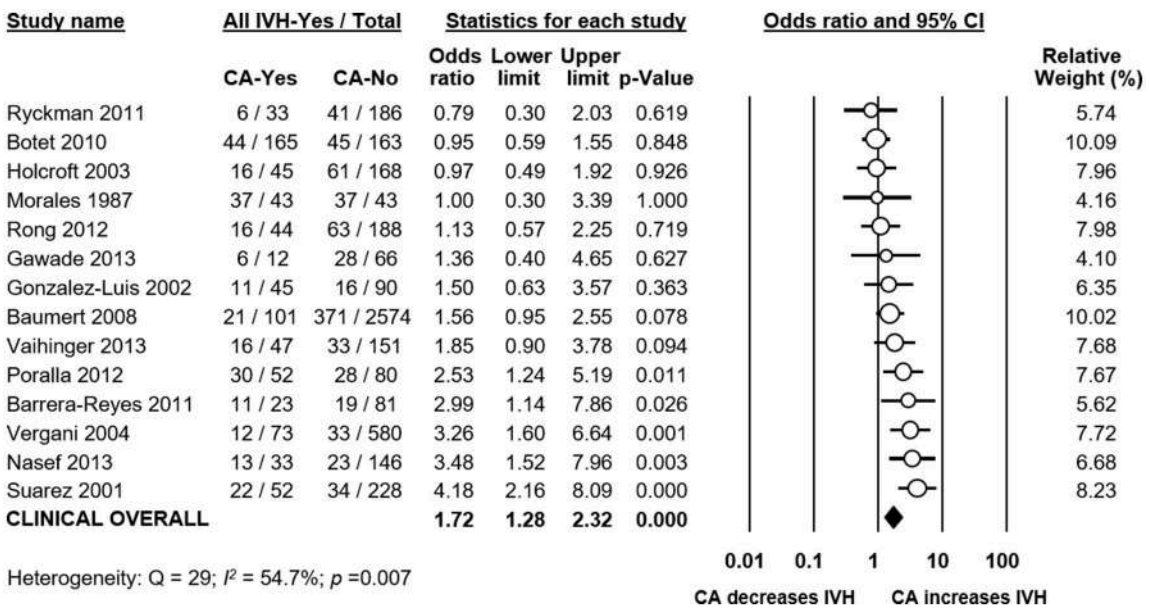


FIGURE 5 | Meta-analysis of the association between clinical chorioamnionitis (CA) and all grades intraventricular hemorrhage (IVH). CI, confidence interval.

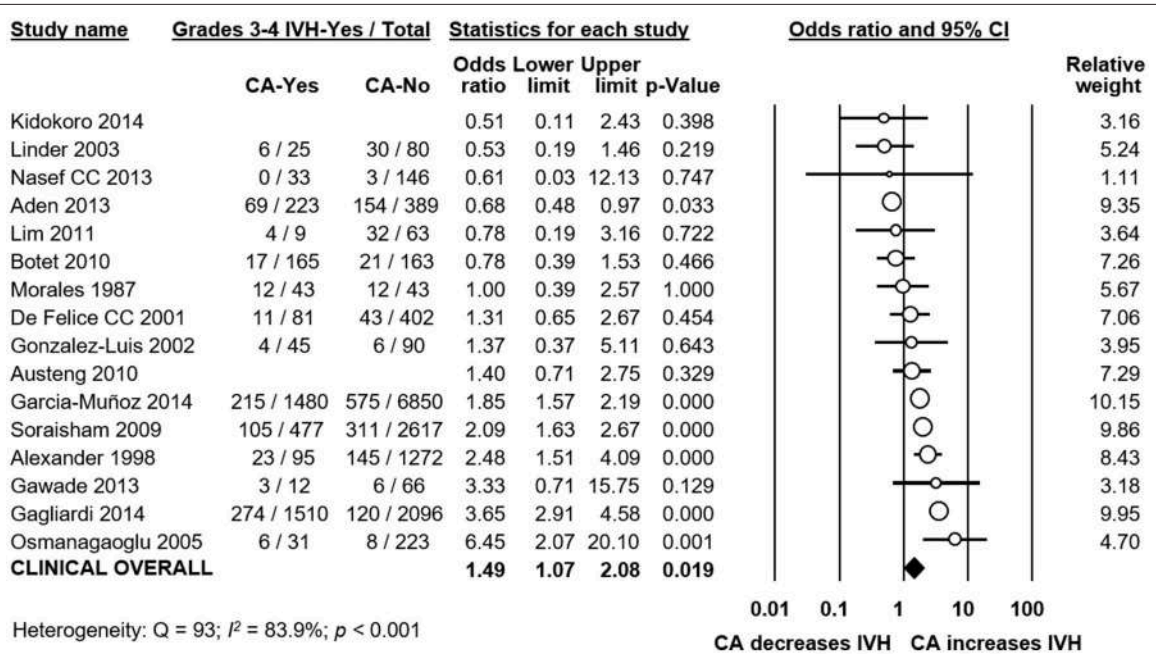


FIGURE 6 | Meta-analysis of the association between clinical chorioamnionitis (CA) and grades 3–4 intraventricular hemorrhage (IVH). CI, confidence interval.

Villamor-Martinez et al., 2018a). Nevertheless, very preterm birth is always a pathological event and very preterm infants have a morbidity and mortality risk associated with whichever condition led to their early delivery (McElrath et al., 2008; Wilcox et al., 2011; Gagliardi et al., 2013; Barros et al., 2015). Therefore, CA may affect infant morbidity through inducing very preterm birth or through the deleterious effects of infection/inflammation. Interestingly, previous meta-analyses showed an association between the lower gestational age of the CA-exposed group and the CA-associated risk of BPD (Hartling et al., 2012), PDA (Behbodi et al., 2016), and ROP (Mitra et al., 2014; Villamor-Martinez et al., 2018a). In contrast, our meta-regression could not show that the difference in GA between CA-exposed and CA-unexposed infants significantly correlated with IVH risk.

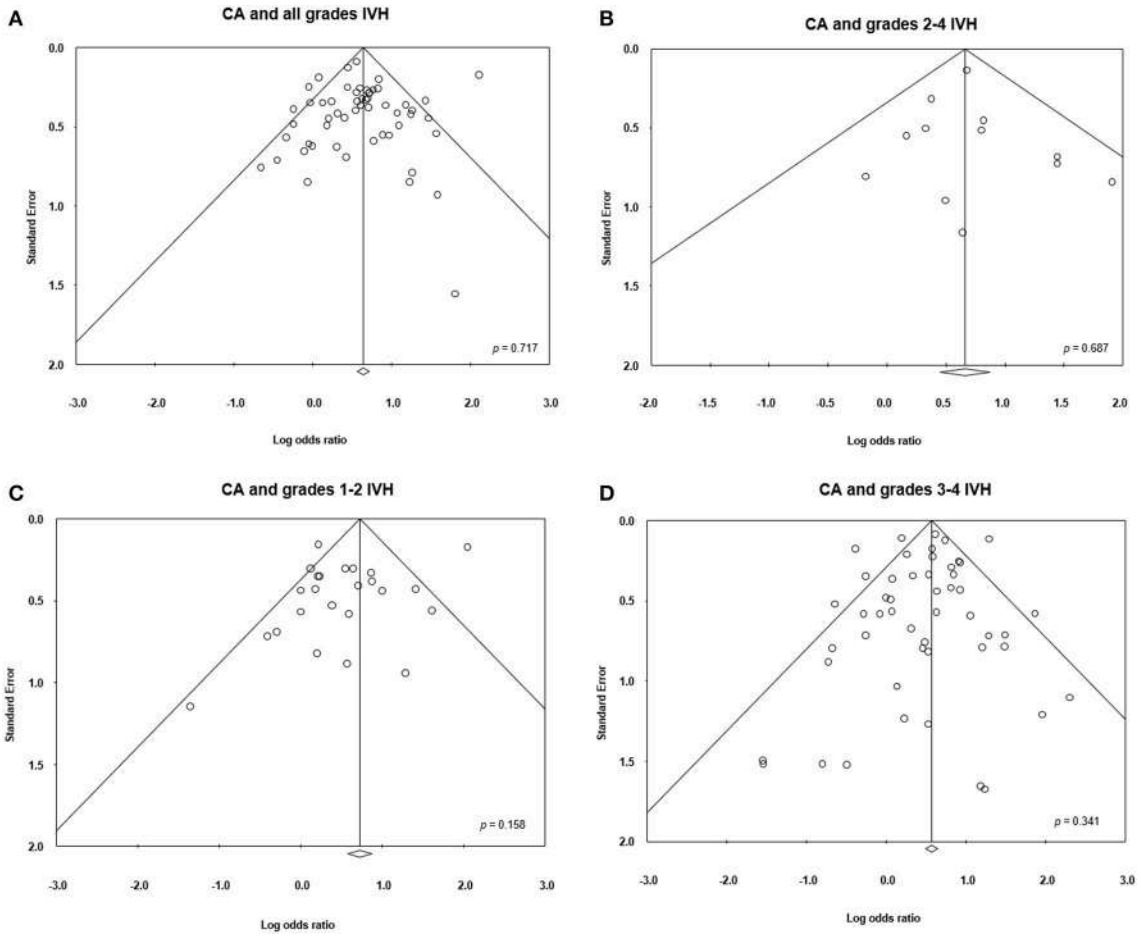


FIGURE 7 | Funnel plots assessing publication bias for the association between chorioamnionitis (CA) and intraventricular hemorrhage (IVH).

Moreover, we performed subgroup analyses in which we only included the studies showing small or no differences in GA between the CA-exposed and the control group and we observed that the significant IVH risk was maintained in this subgroup of studies. In contrast, this was not the case when the same subgroup analysis was performed for PDA (Behbodi et al., 2016) or ROP (Mitra et al., 2014; Villamor-Martinez et al., 2018a). Altogether this suggests that CA may increase complications such as PDA or ROP through GA-dependent mechanisms, whereas the effect on IVH may be mediated by GA-independent mechanisms.

Besides GA, several other factors potentially confound the association between CA and IVH. A number of studies provided data adjusted for confounding factors, but confounders accounted for in each model differed across studies. We performed separate analyses aggregating adjusted association measures. This reduced or made non-significant the association between CA and IVH (**Supplementary Tables 3, 4**). Earlier meta-analyses on the association between CA and cerebral palsy (Wu and Colford, 2000), BPD (Hartling et al., 2012), ROP (Villamor-Martinez et al., 2018a) also showed that the positive association found when unadjusted data were pooled, was reduced or became non-significant when only adjusted data were pooled. Moreover, in our previous meta-analysis on CA and PDA (Behbodi et al., 2016) we found that CA was risk factor for PDA when unadjusted data were pooled, and that CA was a protective factor for PDA when adjusted data were pooled.

Adjustment for potential confounders, particularly for GA and/or BW, is a common strategy used in observational studies analyzing predictors of outcomes of prematurity (Gagliardi et al., 2013). Quality assessment tools such as the NOS even downgrade studies for not adjusting for confounding factors. However, adjustment for GA and BW is controversial and can arguably lead to biased conclusions (Wilcox et al., 2011; Gagliardi et al., 2013). Preterm infants are at risk of adverse outcomes both due to their immaturity and due to the pathological conditions that led to their preterm birth (McElrath et al., 2008; Basso and Wilcox, 2010; Wilcox et al., 2011; Gagliardi et al., 2013). Very low GA is therefore both a risk factor for adverse outcomes, as well as a mediator in the causal pathway that links preterm birth to adverse outcomes (Wilcox et al., 2011; Gagliardi et al., 2013). The problem with adjusting for intermediate variables, such as GA, is that it may introduce bias unless each confounder is accounted

TABLE 1 | Meta-analysis of the association between chorioamnionitis and covariates.

Meta-analysis	Chorioamnionitis	*k*	Effect size	95% CI	*Z*	*p*	Heterogeneity		
							Q	*p*	I^2
Gestational age (weeks)	Clinical	11	MD −0.73	−1.16 to −0.30	−3.35	0.001	140	<0.001	92.9
	Histological	42	MD −1.27	−1.49 to −1.05	−11.42	<0.001	495	<0.001	91.7
	Any type	56	MD −1.20	−1.40 to −1.00	−11.66	<0.001	839	<0.001	93.4
Birth weight (g)	Clinical	11	MD −29.14	−77.66 to 19.39	−1.18	0.239	107	<0.001	90.6
	Histological	41	MD −70.21	−96.71 to −43.72	−5.19	<0.001	362	<0.001	89.0
	Any type	55	MD −55.00	−74.89 to −35.12	−5.42	<0.001	474	<0.001	88.6
Antenatal corticosteroids	Clinical	5	OR 1.10	0.76 to 1.60	0.52	0.605	55	<0.001	92.8
	Histological	31	OR 1.20	1.01 to 1.42	2.03	0.043	95	<0.001	68.3
	Any type	38	OR 1.19	1.02 to 1.38	2.28	0.023	155	<0.001	76.1
Cesarean section	Clinical	8	OR 0.53	0.30 to 0.93	−2.23	0.026	316	<0.001	97.8
	Histological	28	OR 0.33	0.25 to 0.45	−7.32	<0.001	177	<0.001	84.8
	Any type	36	OR 0.37	0.29 to 0.47	−8.06	<0.001	495	<0.001	92.9
SGA	Histological	15	OR 0.33	0.23 to 0.49	−5.53	<0.001	79	<0.001	82.3
	Any type	16	OR 0.34	0.23 to 0.50	−5.64	<0.001	80	<0.001	81.2
Preeclampsia	Clinical	3	OR 0.16	0.09 to 0.29	−5.98	<0.001	2	0.369	<0.001
	Histological	23	OR 0.15	0.11 to 0.20	−13.09	<0.001	65	<0.001	66.2
	Any type	27	OR 0.15	0.12 to 0.20	−15.25	<0.001	69	<0.001	62.2
PROM	Clinical	3	OR 5.02	2.71 to 9.31	5.12	<0.001	2	<0.001	18
	Histological	27	OR 3.14	2.54 to 3.87	10.63	<0.001	149	<0.001	82.6
	Any type	30	OR 3.29	2.70 to 4.02	11.76	<0.001	155	<0.001	81.3
Male sex	Clinical	8	OR 1.10	0.80 to 1.53	0.58	0.560	80	<0.001	91.2
	Histological	35	OR 0.99	0.89 to 1.11	−0.15	0.881	91	<0.001	62.8
	Any type	46	OR 1.00	0.90 to 1.12	0.07	0.941	193	<0.001	76.7
Maternal diabetes	Any type	9	OR 0.81	0.65 to 1.01	−1.92	0.055	5	0.725	0.0
EOS	Clinical	7	OR 4.41	3.58 to 5.42	14.08	<0.001	9	0.197	30.3
	Histological	18	OR 2.62	1.88 to 3.65	5.68	<0.001	48	<0.001	64.9
	Any type	25	OR 3.81	3.20 to 4.54	14.96	<0.001	87	<0.001	72.4
LOS	Clinical	5	OR 1.41	1.10 to 1.81	2.68	0.007	17	0.002	76.8
	Histological	30	OR 1.53	1.27 to 1.84	4.45	<0.001	134	<0.001	78.3
	Any type	37	OR 1.55	1.34 to 1.80	5.82	<0.001	174	<0.001	79.3
PDA	Clinical	4	OR 1.30	1.04 to 1.64	2.27	0.023	7	0.062	59.0
	Histological	26	OR 1.41	1.15 to 1.72	3.35	0.001	144	<0.001	82.6
	Any type	31	OR 1.60	1.35 to 1.80	6.00	<0.001	195	<0.001	84.6
RDS	Clinical	3	OR 2.01	0.48 to 8.41	0.95	0.341	11	0.004	82.0
	Histological	15	OR 1.09	0.81 to 1.45	0.55	0.582	89	<0.001	84.3
	Any type	21	OR 1.00	0.78 to 1.29	0.01	0.990	149	<0.001	86.6

CI, confidence interval; MD, mean difference; OR, odds ratio; SGA, small for gestational age; PROM, premature rupture of membranes; EOS, early-onset sepsis; LOS, late-onset sepsis; PDA, patent ductus arteriosus; RDS, respiratory distress syndrome.

for in the model (Hernández-Díaz et al., 2006; Basso and Wilcox, 2010; Wilcox et al., 2011; Gagliardi et al., 2013). As discussed by Gagliardi et al., "the difficulty of achieving—at least at the current level of knowledge of etiology of preterm birth—full control of all mediator–outcome confounders limits the possibility of causal interpretation of the associations found but not their descriptive

TABLE 2 | Random effects meta-regression of IVH risk in the chorioamnionitis group, and mean difference in gestational age and birth weight.

IVH grade	Meta-regression	*k*	CC	95% CI	*Z*	*p*	R^2
All grades IVH	Mean difference gestational age (per week)	35	−0.02	−0.19 to 0.16	−0.17	0.863	0.00
	Mean difference birth weight (per 100 g)	35	0.00	−0.001 to 0.001	0.07	0.942	0.00
Grades 1–2 IVH	Mean difference gestational age (per week)	20	0.05	−0.15 to 0.25	0.51	0.613	0.00
	Mean difference birth weight (per 100 g)	20	0.13	−0.07 to 0.33	1.27	0.203	0.54
Grades 3–4 IVH	Mean difference gestational age (per week)	37	−0.19	−0.43 to 0.04	−1.62	0.105	0.29
	Mean difference birth weight (per 100 g)	37	−0.10	−0.30 to 0.10	−1.01	0.312	0.00

IVH, intraventricular hemorrhage; k, number of included studies; CC, coefficient; CI, confidence interval.

TABLE 3 | Subgroup meta-analyses based on difference in gestational age (GA).

Subgroup of studies	IVH definition	*k*	OR	95% CI	*p*
Studies where CA-group did not differ significantly in GA from control ($p > 0.05$)	All grades IVH	15	1.59	1.20 to 2.10	0.001
	Grades 3–4 IVH	14	1.54	0.99 to 2.39	0.055
Studies where CA-group did differ significantly in GA from control ($p < 0.05$)	All grades IVH	20	1.71	1.46 to 2.01	< 0.001
	Grades 3–4 IVH	23	1.90	1.56 to 2.33	0.000
Studies where CA-group had a MD in GA of ≤0.5 weeks compared to control	All grades IVH	11	1.66	1.25 to 2.22	< 0.001
	Grades 3–4 IVH	13	1.36	1.03 to 1.80	0.028
Studies where CA-group had a MD in GA of >0.5 weeks compared to control	All grades IVH	24	1.68	1.43 to 1.98	< 0.001
	Grades 3–4 IVH	23	1.99	1.64 to 2.41	< 0.001
Studies where CA-group had a MD in GA of <1 weeks compared to control	All grades IVH	16	1.72	1.37 to 2.15	< 0.001
	Grades 3–4 IVH	18	1.52	1.21 to 1.92	< 0.001
Studies where CA-group had a MD in GA of ≥1 weeks compared to control	All grades IVH	19	1.65	1.37 to 1.97	< 0.001
	Grades 3–4 IVH	18	2.00	1.60 to 2.50	< 0.001

CA, chorioamnionitis; GA, gestational age; IVH, intraventricular hemorrhage; k, number of studies included; CI, confidence interval; MD, mean difference.

value" (121, p. 798). In this sense, by providing analysis of both the unadjusted and adjusted data, our study may be a valuable contribution to the understanding of CA as etiopathogenic factor of both prematurity and IVH.

Our data suggest that CA-exposed infants are not only younger but also more clinically unstable than the non-exposed infants. This is reflected in the higher mortality and the higher rate of sepsis and PDA in CA-exposed infants (**Table 1**). Of note, meta-regression showed a correlation between the effect size of the association between CA and grade 3-4 IVH and the effect sizes of the association between CA and PDA. As mentioned elsewhere, the presence of a hemodynamically relevant PDA has been correlated with the occurrence of IVH and the proposed mechanism is the disturbance of cerebral blood flow (Ballabh, 2010, 2014; Inder et al., 2018; Poryo et al., 2018). Our data support this association between IVH and PDA in CA-exposed infants.

The biological plausibility of the association between CA and IVH is supported by the direct and indirect effects of inflammatory mediators. Hemodynamic disturbances in preterm infants with CA have been correlated with elevated cord blood concentrations of proinflammatory cytokines such as IL-6, IL-1beta and TNF-alpha (Yanowitz et al., 2002). Cytokines can act directly on the vascular smooth muscle, producing vascular relaxation and hypotension or indirectly by increasing the production of endothelium-derived vasoactive mediators (Yanowitz et al., 2002). In addition, cytokines can eventually promote a neuro-inflammatory cascade in the fetal brain penetrating across the blood brain barrier or activating specific receptors such as CD14 and TLR4 which are constitutively expressed in the circumventricular organs, choroid plexus and leptomeninges (Rivest, 2003; McAdams and Juul, 2012). Inflamed glial or endothelial cells, challenged by external stimuli, enhance the release/expression of various chemoattractants and adhesion molecules which may promote the platelet and neutrophil activation and adhesion determining possible endothelial cell damage and changes in blood rheology and flow (Stanimirovic and Satoh, 2000; Molina-Holgado and Molina-Holgado, 2010). These changes, occurring inside the fragile germinal matrix capillaries or within the vascular connection between germinal matrix and the subependymal venous network, may increase the likelihood of IVH in preterm infants with CA.

TABLE 4 | Random effects meta-regression of IVH risk in the chorioamnionitis group, and predefined covariates.

IVH grade	Meta-regression	*k*	CC	95% CI	*Z*	*P*	R^2
All grades IVH	Chorioamnionitis type (histological/clinical)	49	−0.08	−0.45 to 0.29	−0.42	0.673	0.00
	Funisitis (CA+F+ vs. CA+F–)	9	−0.12	−0.62 to 0.38	−0.49	0.627	0.00
	ACS (log OR)	25	0.01	−0.46 to 0.49	0.05	0.964	0.00
	Cesarean section (log OR)	22	−0.07	−0.47 to 0.32	−0.36	0.717	0.00
	Maternal age (MD)	17	−0.08	−0.24 to 0.07	−1.09	0.276	0.00
	SGA (log OR)	11	0.39	−0.09 to 0.88	1.59	0.111	0.54
	PROM (log OR)	20	−0.40	−1.06 to 0.26	−1.19	0.233	0.08
	Preeclampsia (log OR)	16	0.03	−0.40 to 0.45	0.13	0.897	0.00
	Mortality (log OR)	26	0.18	−0.15 to 0.50	1.07	0.285	0.07
	Early onset sepsis (log OR)	14	0.13	−0.47 to 0.73	0.42	0.672	0.00
	Late onset sepsis (log OR)	24	−0.04	−0.39 to 0.32	−0.19	0.846	0.00
	PDA (log OR)	22	0.13	−0.21 to 0.48	0.75	0.453	0.00
	RDS (log OR)	21	0.02	−0.22 to 0.27	0.22	0.827	0.00
Grades 3–4 IVH	Chorioamnionitis type (histological/clinical)	46	−0.07	−0.44 to 0.30	−0.37	0.708	0.00
	Funisitis (CA+F+ vs. CA+F–)	8	0.07	−0.29 to 0.44	0.40	0.691	0.00
	ACS (log OR)	28	−0.11	−0.60 to 0.38	0.42	0.672	0.00
	Cesarean section (log OR)	27	0.08	−0.09 to 0.26	0.92	0.358	0.06
	Maternal age (MD)	18	−0.01	−0.25 to 0.22	−0.11	0.911	0.00
	SGA (log OR)	12	0.24	−0.19 to 0.67	1.08	0.280	0.22
	PROM (log OR)	22	−0.09	−0.35 to 0.17	−0.65	0.515	0.00
	Preeclampsia (log OR)	18	0.41	0.20 to 0.63	3.74	0.0004	1.00
	Mortality (log OR)	30	0.42	0.17 to 0.67	3.33	0.001	0.58
	Early onset sepsis (log OR)	20	0.11	−0.32 to 0.54	0.51	0.613	0.00
	Late onset sepsis (log OR)	26	0.35	0.01 to 0.70	1.99	0.047	0.22
	PDA (log OR)	21	0.40	0.04 to 0.76	2.18	0.029	0.48
	RDS (log OR)	27	−0.06	−0.34 to 0.21	−0.49	0.627	0.14

k, number of studies included; CA, chorioamnionitis; F, funisitis; CC, coefficient; CI, confidence interval; MD, mean difference; OR, odds ratio; ACS, antenatal corticosteroids; SGA, small for gestational age; PROM, premature rupture of membranes; PDA, patent ductus arteriosus; RDS, respiratory distress syndrome.

We have discussed the role of funisitis in earlier meta-analyses on CA and ROP (Villamor-Martinez et al., 2018a) and CA and PDA (Behbodi et al., 2016). It is worth noting that not all intraamniotic infections will induce an inflammatory response in the fetus (Revello et al., 2015). Funisitis is generally considered the histologic counterpart to fetal inflammatory response syndrome (Gantert et al., 2010; Revello et al., 2015). We found that exposure to funisitis did not significantly increase the risk of IVH, when compared to exposure to CA without funisitis. This is an argument against role of the fetal inflammatory response in the etiopathogenesis of IVH. We have previously reported that funisitis is not an additional risk factor for developing PDA (Behbodi et al., 2016) but the presence of funisitis significantly increased the risk of developing severe ROP (Villamor-Martinez et al., 2018a).

Our meta-analysis has several limitations that should be considered. Firstly, there was substantial heterogeneity in how CA was defined in studies. The definitions of clinical CA in particular varied substantially, and recent recommendations propose restricting the term CA to pathologic diagnosis (Higgins et al., 2016). Secondly, only 5 out of 85 included studies studied the association between CA and IVH as their main objective. However, this could also have reduced the effect of publication bias. Thirdly, only 13 out of the 85 included studies provided adjusted data, and they used different models and adjusted for different confounders. Finally, in this study and earlier studies on ROP (Villamor-Martinez et al., 2018a) and PDA (Behbodi et al., 2016), we had a much more limited number of studies to draw from for analyzing funisitis than when analyzing CA. The strengths of our study include: the use of a comprehensive search, duplication of screening, inclusion and data extraction to reduce bias, a large number of included studies, and an extensive analysis of confounding factors, through meta-analysis, meta-regression and the inclusion of adjusted data.

A significant limitation in any meta-analysis on IVH is the potential for heterogeneity in defining the condition. The grading system most commonly used for neonatal IVH was first reported by Papile et al. and later modified by Volpe and is based on the presence and amount of blood in the germinal matrix, lateral ventricles, and periventricular white matter (Volpe, 2015). Grade 1 represents germinal matrix hemorrhage only with no or minimal IVH (<10% of ventricular area on parasagittal view). When IVH occupies 10–50% of ventricular area on parasagittal view, it is defined as grade 2 (Volpe, 2015). Grade 3 is IVH with blood occupying more than 50%

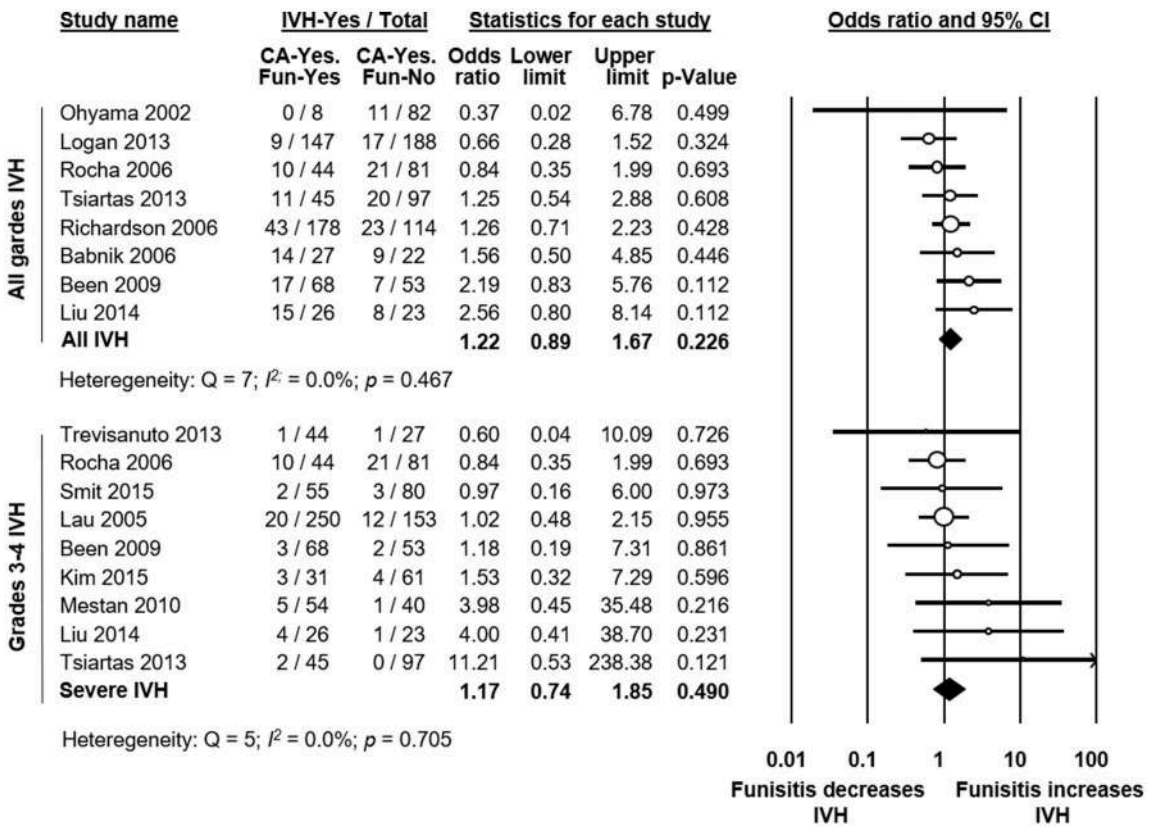


FIGURE 8 | Meta-analysis of the association between funisitis and intraventricular hemorrhage (IVH). Fun, funisitis; CI, confidence interval.

of the ventricular area on parasagittal view. Grade 4 represents severe IVH with associated periventricular echodensity (Volpe, 2015). Although grade 4 IVH is a periventricular hemorrhagic infarction rather than an extension of IVH *per se*, the 1–4 grading system remains pervasive in the literature and clinical setting despite debate regarding appropriate nomenclature (Leviton et al., 2007). In addition grade 3 and 4 IVHs are frequently grouped together as severe or high grade IVH (Leviton et al., 2007). Nevertheless, our meta-analysis shows a significant increased risk of both severe and less severe (grade 1–2) IVH in CA-exposed infants. Therefore, potential differences in IVH classification may not have affected the results.

CONCLUSION

IVH is a multifactorial complication that is more common in more preterm and more clinically unstable infants. We established for the first time through meta-analysis that CA is a risk factor for IVH. We also confirmed earlier findings that CA is a risk factor for being born more preterm and presenting more clinical instability. However, in contrast to other complications of prematurity, such as PDA, ROP, or BPD (Hartling et al., 2012; Mitra et al., 2014; Behbodi et al., 2016; Villamor-Martinez et al., 2018a), the effect of CA on IVH appears to be independent of CA as a causative factor for very preterm birth.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed for this study can be found in the Harvard Dataverse (Harvard Dataverse, 2018): https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi%3A10.7910%2FDVN%2FJ9RHUF.

A preprint version of this manuscript is made available to the scientific community on the preprint server bioRxiv (Villamor-Martinez et al., 2018b): https://www.biorxiv.org/content/early/2018/05/30/334375.

AUTHOR CONTRIBUTIONS

EV-M carried out data collection, carried out statistical analyses, assessed methodological quality, contributed to interpretation of results, drafted the initial manuscript, and reviewed and revised the manuscript. MF contributed to the design of the study, the statistical analysis and interpretation of results and reviewed and revised the manuscript. OM selected studies for inclusion, carried out data collection and carried out statistical analyses. SP contributed to interpretation of results and reviewed and revised the manuscript. GC contributed to interpretation of results and reviewed and revised the manuscript. PD carried out and supervised data collection and contributed to interpretation of results. FM contributed to interpretation of results and reviewed and revised the manuscript. EV conceptualized and

designed the study, carried out the search and selected studies for inclusion, supervised data collection, contributed to statistical analyses and interpretation of results, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01253/full#supplementary-material

REFERENCES

Adén, U., Lin, A., Carlo, W., Leviton, A., Murray, J. C., Hallman, M., et al. (2013). Candidate gene analysis: severe intraventricular hemorrhage in inborn preterm neonates. *J. Pediatr.* 163:1503-6. e1. doi: 10.1016/j.jpeds.2013.06.025

Ahn, H. M., Park, E. A., Cho, S. J., Kim, Y.-J., and Park, H.-S. (2012). The association of histological chorioamnionitis and antenatal steroids on neonatal outcome in preterm infants born at less than thirty-four weeks' gestation. *Neonatology* 102, 259–264. doi: 10.1159/000339577

Alexander, J. M., Gilstrap, L. C., Cox, S. M., McIntire, D. M., and Leveno, K. J. (1998). Clinical chorioamnionitis and the prognosis for very low birth weight infants. *Obstet. Gynecol.* 91(5 Part 1), 725–729.

Alfiero Bordigato, M., Piva, D., Di Gangi, I. M., Giordano, G., Chiandetti, L., and Filippone, M. (2011). Asymmetric dimethylarginine in ELBW newborns exposed to chorioamnionitis. *Early Hum. Dev.* 87, 143–145. doi: 10.1016/j.earlhumdev.2010.11.004

Arayici, S., Kadioglu Simsek, G., Oncel, M. Y., Eras, Z., Canpolat, F. E., Oguz, S. S., et al. (2014). The effect of histological chorioamnionitis on the short-term outcome of preterm infants≤ 32 weeks: a single-center study. *J. Matern. Fetal Neonatal Med.* 27, 1129–1133. doi: 10.3109/14767058.2013.850668

Austeng, D., Blennow, M., Ewald, U., Fellman, V., Fritz, T., Hellstrom-Westas, L., et al. (2010). Incidence of and risk factors for neonatal morbidity after active perinatal care: extremely preterm infants study in Sweden (EXPRESS). *Acta Paediatr.* 99, 978–992. doi: 10.1111/j.1651-2227.2010.01846.x

Babnik, J., Stucin-Gantar, I., Kornhauser-Cerar, L., Sinkovec, J., Wraber, B., and Derganc, M. (2006). Intrauterine inflammation and the onset of peri-intraventricular hemorrhage in premature infants. *Neonatology* 90, 113–121. doi: 10.1159/000092070

Ballabh, P. (2010). Intraventricular hemorrhage in premature infants: mechanism of disease. *Pediatr. Res.* 67, 1–8. doi: 10.1203/PDR.0b013e3181c1b176

Ballabh, P. (2014). Pathogenesis and prevention of intraventricular hemorrhage. *Clin. Perinatol.* 41, 47–67. doi: 10.1016/j.clp.2013.09.007

Barrera-Reyes, R., Ruiz-Macias, H., and Segura-Cervantes, E. (2011). [Neurodevelopment at one year of age (corrected) in preterm newborns with history of maternal chorioamnionitis]. *Ginecol. Obstet. Mex.* 79, 31–37.

Barros, F. C., Papageorghiou, A. T., Victora, C. G., Noble, J. A., Pang, R., Iams, J., et al. (2015). The distribution of clinical phenotypes of preterm birth syndrome: implications for prevention. *JAMA Pediatr.* 169, 220–229. doi: 10.1001/jamapediatrics.2014.3040

Basso, O., and Wilcox, A. (2010). Mortality risk among preterm babies: immaturity vs. underlying pathology. *Epidemiology* 21, 521–527. doi: 10.1097/EDE.0b013e3181debe5e

Baumert, M., Brozek, G., Paprotny, M., Walencka, Z., Sodowska, H., Cnota, W., et al. (2008). Epidemiology of peri/intraventricular haemorrhage in newborns at term. *J. Physiol. Pharmacol.* 59(Suppl. 4), 67–75.

Been, J. V., Lievense, S., Zimmermann, L. J., Kramer, B. W., and Wolfs, T. G. (2013). Chorioamnionitis as a risk factor for necrotizing enterocolitis: a systematic review and meta-analysis. *J. Pediatr.* 162, 236-42 e2. doi: 10.1016/j.jpeds.2012.07.012

Been, J. V., Rours, I. G., Kornelisse, R. F., Lima Passos, V., Kramer, B. W., Schneider, T. A., et al. (2009). Histologic chorioamnionitis, fetal involvement, and antenatal steroids: effects on neonatal outcome in preterm infants. *Am. Obstet. Gynecol.* 201, 587. e1-e8. doi: 10.1016/j.ajog.2009.06.025

Behbodi, E., Villamor-Martínez, E., Degraeuwe, P. L., and Villamor, E. (2016). Chorioamnionitis appears not to be a risk factor for patent ductus arteriosus in preterm infants: a systematic review and meta-analysis. *Sci. Rep.* 6:37967. doi: 10.1038/srep37967

Bermick, J., Dechert, R. E., and Sarkar, S. (2016). Does hyperglycemia in hypernatremic preterm infants increase the risk of intraventricular hemorrhage? *J. Perinatol.* 36, 729–732. doi: 10.1038/jp.2016.86

Borenstein, M., Hedges, L. V., Higgins, J., and Rothstein, H. R. (2009a). *Subgroup Analyses. Introduction to Meta-Analysis.* Chichester, UK: John Wiley & Sons.

Borenstein, M., Hedges, L. V., Higgins, J., and Rothstein, H. R. (2009b). *Identifying and Quantifying Heterogeneity. Introduction to Meta-Analysis.* Chichester, UK: John Wiley & Sons.

Borenstein, M., Hedges, L. V., Higgins, J., and Rothstein, H. R. (2009c). *Meta-Regression. Introduction to Meta-Analysis.* Chichester, UK: John Wiley & Sons.

Botet, F., Figueras, J., Carbonell-Estrany, X., Arca, G., and Group, C. S. (2010). Effect of maternal clinical chorioamnionitis on neonatal morbidity in very-low birthweight infants: a case-control study. *J. Perinat. Med.* 38, 269–273. doi: 10.1515/jpm.2010.029

Bry, K. J., Jacobsson, B., Nilsson, S., and Bry, K. (2015). Gastric fluid cytokines are associated with chorioamnionitis and white blood cell counts in preterm infants. *Acta Paediatr.* 104, 575–580. doi: 10.1111/apa.12947

Chevallier, M., Debillon, T., Pierrat, V., Delorme, P., Kayem, G., Durox, M., et al. (2017). Leading causes of preterm delivery as risk factors for intraventricular hemorrhage in very preterm infants: results of the EPIPAGE 2 cohort study. *Am. J. Obstet. Gynecol.* 216, 518. e1-. e12. doi: 10.1016/j.ajog.2017.01.002

Cornette, L. (2004). Fetal and neonatal inflammatory response and adverse outcome. *Semin. Fetal Neonatal Med.* 9, 459–470. doi: 10.1016/j.siny.2004.08.004

Dalton, J., Dechert, R. E., and Sarkar, S. (2015). Assessment of association between rapid fluctuations in serum sodium and intraventricular hemorrhage in hypernatremic preterm infants. *Am. J. Perinatol.* 32, 795–802. doi: 10.1055/s-0034-1396691

De Felice, C., Toti, P., Laurini, R. N., Stumpo, M., Picciolini, E., Todros, T., et al. (2001). Early neonatal brain injury in histologic chorioamnionitis. *J. Pediatr.* 138, 101–104. doi: 10.1067/mpd.2001.109605

Dempsey, E., Chen, M.-F., Kokottis, T., Vallerand, D., and Usher, R. (2005). Outcome of neonates less than 30 weeks gestation with histologic chorioamnionitis. *Am. J. Perinatol.* 22, 155–159. doi: 10.1055/s-2005-865020

Dexter, S. C., Pinar, H., Malee, M. P., Hogan, J., Carpenter, M. W., and Vohr, B. R. (2000). Outcome of very low birth weight infants with histopathologic chorioamnionitis. *Obstet. Gynecol.* 96, 172–177. doi: 10.1016/S0029-7844(00)00886-3

DiSalvo, D. (1998). The correlation between placental pathology and intraventricular hemorrhage in the preterm infant. The developmental epidemiology network investigators. *Pediatr. Res.* 43, 15–19.

Ecevit, A., Anuk-Ince, D., Yapak,çi, E., Kupana-Ayva, S., Kurt, A., Yanik, F. F., et al. (2014). Association of respiratory distress syndrome and perinatal hypoxia with histologic chorioamnionitis in preterm infants. *Turk. J. Pediatr.* 56, 56–61.

Elimian, A., Verma, U., Beneck, D., Cipriano, R., Visintainer, P., and Tejani, N. (2000). Histologic chorioamnionitis, antenatal steroids, and perinatal outcomes. *Obstet. Gynecol.* 96, 333–336. doi: 10.1016/S0029-7844(00)00928-5

Erdemir, G., Kultursay, N., Calkavur, S., Zekioglu, O., Koroglu, O. A., Cakmak, B., et al. (2013). Histological chorioamnionitis: effects on premature delivery and neonatal prognosis. *Pediatr. Neonatol.* 54, 267–274. doi: 10.1016/j.pedneo.2013.03.012

Fung, G., Bawden, K., Chow, P., and Yu, V. (2003). Long-term outcome of extremely preterm infants following chorioamnionitis 絨毛膜羊膜炎對極早早產兒的長影響. *HK J Paediatr (new series)* 8, 87–92.

Gagliardi, L., Rusconi, F., Bell,ù, R., Zanini, R., and Network, I. N. (2014). Association of maternal hypertension and chorioamnionitis with preterm outcomes. *Pediatrics* 134, e154–e61. doi: 10.1542/peds.2013-3898

Gagliardi, L., Rusconi, F., Da Fr,è, M., Mello, G., Carnielli, V., Di Lallo, D., et al. (2013). Pregnancy disorders leading to very preterm birth influence neonatal outcomes: results of the population-based ACTION cohort study. *Pediatr. Res.* 73, 794–801. doi: 10.1038/pr.2013.52

Gantert, M., Been, J. V., Gavilanes, A. W., Garnier, Y., Zimmermann, L. J., and Kramer, B. W. (2010). Chorioamnionitis: a multiorgan disease of the fetus? *J Perinatol* 30(Suppl.), S21–S30. doi: 10.1038/jp.2010.96

García-Muñoz Rodrigo, F., Galan Henriquez, G., Figueras Aloy, J., Garcia-Alix Perez, A., and Network, S. (2014). Outcomes of very-low-birth-weight infants exposed to maternal clinical chorioamnionitis: a multicentre study. *Neonatology* 106, 229–234. doi: 10.1159/000363127

Gawade, P. L., Whitcomb, B. W., Chasan-Taber, L., Pekow, P. S., Ronnenberg, A. G., Shah, B., et al. (2013). Second stage of labor and intraventricular hemorrhage in early preterm infants in the vertex presentation. *J. Matern. Fetal Neonatal Med.* 26, 1292–1298. doi: 10.3109/14767058.2013.783804

González-Luis, G., García, I. J., Rodríguez-Miguélez, J., Mussons, F. B., and Aloy, J. F. (2002). Patología neonatal en los menores de 1.500 gramos con relación al antecedente de corioamnionitis. *Anal. Pediatr.* 56, 551–555. doi: 10.1016/S1695-4033(02)77863-6

Gray, P. H., Hurley, T. M., Rogers, Y. M., O'Callaghan, M. J., Tudehope, D. I., Burns, Y. R., et al. (1997). Survival and neonatal and neurodevelopmental outcome of 24–29 week gestation infants according to primary cause of preterm delivery. *Austr. N. Z. J. Obstet. Gynaecol.* 37, 161–168. doi: 10.1111/j.1479-828X.1997.tb02245.x

Hartling, L., Liang, Y., and Lacaze-Masmonteil, T. (2012). Chorioamnionitis as a risk factor for bronchopulmonary dysplasia: a systematic review and meta-analysis. *Arch. Dis. Child. Fetal Neonatal Ed.* 97, F8–F17. . doi: 10.1136/adc.2010.210187

Harvard Dataverse (2018). *Chorioamnionitis and Intraventricular, Hemorrhage, Studies Included in Systematic Review [Internet]*. Available online at: doi: 10.7910/DVN/J9RHUF

Hendson, L., Russell, L., Robertson, C. M., Liang, Y., Chen, Y., Abdalla, A., et al. (2011). Neonatal and neurodevelopmental outcomes of very low birth weight infants with histologic chorioamnionitis. *J. Pediatr.* 158, 397–402. doi: 10.1016/j.jpeds.2010.09.010

Hernández-Díaz, S., Schisterman, E. F., and Hernán, M. A. (2006). The birth weight "paradox" uncovered? *Am. J. Epidemiol.* 164, 1115–1120. doi: 10.1093/aje/kwj275

Higgins, R. D., Saade, G., Polin, R. A., Grobman, W. A., Buhimschi, I. A., Watterberg, K., et al. (2016). Evaluation and management of women and newborns with a maternal diagnosis of chorioamnionitis: summary of a workshop. *Obstet. Gynecol.* 127, 426–436. doi: 10.1097/AOG.0000000000001246

Hitti, J., Tarczy-Hornoch, P., Murphy, J., Hillier, S. L., Aura, J., and Eschenbach, D. A. (2001). Amniotic fluid infection, cytokines, and adverse outcome among infants at 34 weeks' gestation or less. *Obstet. Gynecol.* 98, 1080–1088. doi: 10.1016/S0029-7844(01)01567-8

Holcroft, C. J., Blakemore, K. J., Allen, M., and Graham, E. M. (2003). Association of prematurity and neonatal infection with neurologic morbidity in very low birth weight infants. *Obstet. Gynecol.* 101, 1249–1253. doi: 10.1016/S0029-7844(03)00354-5

Inder, T. E., Perlman, J. M., and Volpe, J. J. (2018). *Preterm Intraventricular Hemorrhage/Posthemorrhagic Hydrocephalus Volpe's Neurology of the Newborn, 6th Edn*. Elsevier. Available online at: https://www.sciencedirect.com/book/9780323428767/volpes-neurology-of-the-newborn

Jackson, C. M., Wells, C. B., Tabangin, M. E., Meinzen-Derr, J., Jobe, A. H., and Chougnet, C. A. (2017). Pro-inflammatory immune responses in leukocytes of premature infants exposed to maternal chorioamnionitis or funisitis. *Pediatr. Res.* 81, 384–390. doi: 10.1038/pr.2016.232

Kallankari, H., Kaukola, T., Ojaniemi, M., Herva, R., Perhomaa, M., Vuolteenaho, R., et al. (2010). Chemokine CCL18 predicts intraventricular hemorrhage in very preterm infants. *Ann. Med.* 42, 416–425. doi: 10.3109/07853890.2010.481085

Kaukola, T., Herva, R., Perhomaa, M., Pääkkö, E., Kingsmore, S., Vainionpää, L., et al. (2006). Population cohort associating chorioamnionitis, cord inflammatory cytokines and neurologic outcome in very preterm, extremely low birth weight infants. *Pediatr. Res.* 59, 478–483. doi: 10.1203/01.pdr.0000182596.66175.ee

Kidokoro, H., Anderson, P. J., Doyle, L. W., Woodward, L. J., Neil, J. J., and Inder, T. E. (2014). Brain injury and altered brain growth in preterm infants: predictors and prognosis. *Pediatrics* 134, e444–e453. doi: 10.1542/peds.2013-2336

Kim, S. Y., Choi, C. W., Jung, E., Lee, J., Lee, J. A., Kim, H., et al. (2015). Neonatal morbidities associated with histologic chorioamnionitis defined based on the site and extent of inflammation in very low birth weight infants. *J. Korean Med. Sci.* 30, 1476–1482. doi: 10.3346/jkms.2015.30.10.1476

Kirchner, L., Helmer, H., Heinze, G., Wald, M., Brunbauer, M., Weninger, M. et al. (2007). Amnionitis with ureaplasma urealyticum or other microbes leads to increased morbidity and prolonged hospitalization in very low birth weight infants. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 134, 44–50. doi: 10.1016/j.ejogrb.2006.09.013

Klebermass-Schrehof, K., Czaba, C., Olischar, M., Fuiko, R., Waldhoer, T., Rona, Z., et al. (2012). Impact of low-grade intraventricular hemorrhage on long-term neurodevelopmental outcome in preterm infants. *Childs Nerv. Syst.* 28, 2085–2092. doi: 10.1007/s00381-012-1897-3

Kosuge, S., Ohkuchi, A., Minakami, H., Matsubara, S., Uchida, A., Eguchi, Y., et al. (2000). Influence of chorioamnionitis on survival and morbidity in singletons live-born at< 32 weeks of gestation. *Acta Obstet. Gynecol. Scand.* 79, 861–865. doi: 10.1034/j.1600-0412.2000.079010861.x

Lau, J., Magee, F., Qiu, Z., Houb,é, J., Von Dadelszen, P., and Lee, S. K. (2005). Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than chorioamnionitis displaying a maternal inflammatory response only. *Am. J. Obstet. Gynecol.* 193, 708–713. doi: 10.1016/j.ajog.2005.01.017

Lee, H. J., Kim, E.-K., Kim, H.-S., Choi, C. W., Kim, B. I., and Choi, J.-H. (2011). Chorioamnionitis, respiratory distress syndrome and bronchopulmonary dysplasia in extremely low birth weight infants. *J. Perinatol.* 31, 166–170. doi: 10.1038/jp.2010.113

Lee, J. Y., Kim, H. S., Jung, E., Kim, E. S., Shim, G. H., Lee, H. J., et al. (2010). Risk factors for periventricular-intraventricular hemorrhage in premature infants. *J. Korean Med. Sci.* 25, 418–424. doi: 10.3346/jkms.2010.25.3.418

Leviton, A., Kuban, K., and Paneth, N. (2007). Intraventricular haemorrhage grading scheme: time to abandon? *Acta Paediatr.* 96, 1254–1256. doi: 10.1111/j.1651-2227.2007.00379.x

Lim, W., Lien, R., Chiang, M., Fu, R., Lin, J., Chu, S., et al. (2011). Hypernatremia and grade III/IV intraventricular hemorrhage among extremely low birth weight infants. *J. Perinatol.* 31, 193–198. doi: 10.1038/jp.2010.86

Linder, N., Haskin, O., Levit, O., Klinger, G., Prince, T., Naor, N., et al. (2003). Risk factors for intraventricular hemorrhage in very low birth weight premature infants: a retrospective case-control study. *Pediatrics* 111, e590–e5. doi: 10.1542/peds.111.5.e590

Liu, Z., Tang, Z., Li, J., and Yang, Y. (2014). Effects of placental inflammation on neonatal outcome in preterm infants. *Pediatr. Neonatol.* 55, 35–40. doi: 10.1016/j.pedneo.2013.05.007

Logan, J. W., Westra, S. J., Allred, E. N., O'Shea, T. M., Kuban, K., and Paneth, N., et al. (2013). Antecedents of perinatal cerebral white matter damage with and without intraventricular hemorrhage in very preterm newborns. *Pediatr. Neurol.* 49, 88–96. doi: 10.1016/j.pediatrneurol.2013.03.018

Lu, H., Wang, Q., Lu, J., Zhang, Q., and Kumar, P. (2016). Risk factors for intraventricular hemorrhage in preterm infants born at 34 weeks of gestation or less following preterm premature rupture of membranes. *J. Stroke Cerebrovasc. Dis.* 25, 807–812. doi: 10.1016/j.jstrokecerebrovasdis.2015.12.011

McAdams, R. M., and Juul, S. E. (2012). The role of cytokines and inflammatory cells in perinatal brain injury. *Neurol. Res. Int.* 2012:561494. doi: 10.1155/2012/561494

McElrath, T. F., Hecht, J. L., Dammann, O., Boggess, K., Onderdonk, A., Markenson, G., et al. (2008). Pregnancy disorders that lead to delivery before the 28th week of gestation: an epidemiologic approach to classification. *Am. J. Epidemiol.* 168, 980–989. doi: 10.1093/aje/kwn202

Mehta, R., Nanjundaswamy, S., Shen-Schwarz, S., and Petrova, A. (2006). Neonatal morbidity and placental pathology. *Indian J. Pediatr.* 73, 25–28. doi: 10.1007/BF02758255

Mestan, K., Yu, Y., Matoba, N., Cerda, S., Demmin, B., Pearson, C., et al. (2010). Placental inflammatory response is associated with poor neonatal growth: preterm birth cohort study. *Pediatrics* 125, e891–e98. doi: 10.1542/peds.2009-0313

Mitra, S., Aune, D., Speer, C. P., and Saugstad, O. D. (2014). Chorioamnionitis as a risk factor for retinopathy of prematurity: a systematic review and meta-analysis. *Neonatology* 105, 189–199. doi: 10.1159/000357556

Miyazaki, K., Furuhashi, M., Ishikawa, K., Tamakoshi, K., Hayashi, K., Kai, A., et al. (2016). Impact of chorioamnionitis on short-and long-term outcomes in very low birth weight preterm infants: the neonatal research network Japan. *J. Matern. Fetal Neonatal Med.* 29, 331–337. doi: 10.3109/14767058.2014.1000852

Mohamed, M. A., and Aly, H. (2010). Transport of premature infants is associated with increased risk for intraventricular haemorrhage. *Arch. Dis. Child Fetal Neonatal Ed.* 95, F403– F407. doi: 10.1136/adc.2010.183236

Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G., and Group, P. (2009). Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* 6:e1000097. doi: 10.1371/journal.pmed.1000097

Molina-Holgado, E., and Molina-Holgado, F. (2010). Mending the broken brain: neuroimmune interactions in neurogenesis. *J. Neurochem.* 114, 1277–1290. doi: 10.1111/j.1471-4159.2010.06849.x

Morales, W. J. (1987). The effect of chorioamnionitis on the developmental outcome of preterm infants at one year. *Obstet. Gynecol.* 70, 183–186.

Mu, S.-C., Lin, C.-H., Chen, Y.-L., Ma, H.-J., Lee, J.-S., Lin, M.-I., et al. (2008). Impact on neonatal outcome and anthropometric growth in very low birth weight infants with histological chorioamnionitis. *J. Formos. Med. Assoc.* 107, 304–310. doi: 10.1016/S0929-6646(08)60091-1

Nasef, N., Shabaan, A. E., Schurr, P., Iaboni, D., Choudhury, J., Church, P., et al. (2013). Effect of clinical and histological chorioamnionitis on the outcome of preterm infants. *Am. J. Perinatol.* 30, 059–68. doi: 10.1055/s-0032-1321501

Ogunyemi, D., Murillo, M., Jackson, U., Hunter, N., and Alperson, B. (2003). The relationship between placental histopathology findings and perinatal outcome in preterm infants. *J. Matern. Fetal Neonatal Med.* 13, 102–109. doi: 10.1080/jmf.13.2.102.109

Oh, K. J., Park, J. Y., Lee, J., Hong, J.-S., Romero, R., and Yoon, B. H. (2018). The combined exposure to intra-amniotic inflammation and neonatal respiratory distress syndrome increases the risk of intraventricular hemorrhage in preterm neonates. *J. Perinat. Med.* 46, 9–20. doi: 10.1515/jpm-2016-0348

Oh, S.-H., Kim, J.-,j, Do, H.-,j, Lee, B. S., Kim, K.-S., and Kim, E. A.-R. (2015). Preliminary study on neurodevelopmental outcome and placental pathology among extremely low birth weight infants. *Korean J. Perinatol.* 26, 67–77. doi: 10.14734/kjp.2015.26.1.67

Ohyama, M., Itani, Y., Yamanaka, M., Goto, A., Kato, K., Ijiri, R., et al. (2002). Re-evaluation of chorioamnionitis and funisitis with a special reference to subacute chorioamnionitis. *Hum. Pathol.* 33, 183–190. doi: 10.1053/hupa.2002.31291

Osmanagaoglu, M. A., Ünal, S., and Bozkaya, H. (2005). Chorioamnionitis risk and neonatal outcome in preterm premature rupture of membranes. *Arch. Gynecol. Obstet.* 271, 33–39. doi: 10.1007/s00404-004-0644-8

Pappas, A., Kendrick, D. E., Shankaran, S., Stoll, B. J., Bell, E. F., Laptook, A. R., et al. (2014). Chorioamnionitis and early childhood outcomes among extremely low-gestational-age neonates. *JAMA Pediatr.* 168, 137–147. doi: 10.1001/jamapediatrics.2013.4248

Park, H. W., Choi, Y. S., Kim, K. S., and Kim, S. N. (2015). Chorioamnionitis and patent ductus arteriosus: a systematic review and meta-analysis. *PLoS ONE* 10:e0138114. doi: 10.1371/journal.pone.0138114

Perrone, S., Toti, P., Toti, M. S., Badii, S., Becucci, E., Gatti, M. G., et al. (2012). Perinatal outcome and placental histological characteristics: a single-center study. *J. Matern. Fetal Neonatal Med.* 25(Supp. 1), 110–113. doi: 10.3109/14767058.2012.664344

Polam, S., Koons, A., Anwar, M., Shen-Schwarz, S., Hegyi, T. (2005). Effect of chorioamnionitis on neurodevelopmental outcome in preterm infants. *Arch. Pediatr. Adolesc. Med.* 159, 1032–1035. doi: 10.1001/archpedi.159.11.1032

Poralla, C., Hertfelder, H.-J., Oldenburg, J., Müller, A., and Bartmann, P., Heep, A. (2012). Elevated interleukin-6 concentration and alterations of the coagulation system are associated with the development of intraventricular hemorrhage in extremely preterm infants. *Neonatology* 102, 270–275. doi: 10.1159/000341266

Poryo, M., Boeckh, J. C., Gortner, L., Zemlin, M., Duppr,é, P., Ebrahimi-Fakhari, D., et al. (2018). Ante-, peri-and postnatal factors associated with intraventricular hemorrhage in very premature infants. *Early Hum. Dev.* 116, 1–8. doi: 10.1016/j.earlhumdev.2017.08.010

Pugni, L., Pietrasanta, C., Acaia, B., Merlo, D., Ronchi, A., Ossola, M. W., et al. (2016). Chorioamnionitis and neonatal outcome in preterm infants: a clinical overview. *J. Matern. Fetal Neonatal Med.* 29, 1525–1529. doi: 10.3109/14767058.2015.1053862

Ramenghi, L. A., Fumagalli, M., Groppo, M., Consonni, D., Gatti, L., Bertazzi, P. A., et al. (2011). Germinal matrix hemorrhage: intraventricular hemorrhage in very-low-birth-weight infants: the independent role of inherited thrombophilia. *Stroke* 42, 1889–1893. doi: 10.1161/STROKEAHA.110.590455

Revello, R., Alcaide, M. J., Dudzik, D., Abehsera, D., and Bartha, J. L. (2015). Differential amniotic fluid cytokine profile in women with chorioamnionitis with and without funisitis. *J. Matern. Fetal Neonatal Med.* 29, 2161–2165. doi: 10.3109/14767058.2015.1077512

Richardson, B. S., Wakim, E., and Walton, J. (2006). Preterm histologic chorioamnionitis: impact on cord gas and pH values and neonatal outcome. *Am. J. Obstet. Gynecol.* 195, 1357–1365. doi: 10.1016/j.ajog.2006.03.053

Rivest, S. (2003). Molecular insights on the cerebral innate immune system. *Brain Behav. Immun.* 17, 13–19. doi: 10.1016/S0889-1591(02)00055-7

Rocha, G., Proença, E., Quintas, C., Rodrigues, T., and Guimarães, H. (2006). Chorioamnionitis and neonatal morbidity. *Acta Med. Port.* 19, 207–212.

Rodríguez-Trujillo, A., Cobo, T., Vives, I., Bosch, J., Kacerovsky, M., Posadas, D. E., et al. (2016). Gestational age is more important for short-term neonatal outcome than microbial invasion of the amniotic cavity or intra-amniotic inflammation in preterm prelabor rupture of membranes. *Acta Obstet. Gynecol. Scand.* 95, 926–933. doi: 10.1111/aogs.12905

Romantsik, O., Bruschettini, M., Calevo, M. G., Banzi, R., and Ley, D. (2017). Pharmacological pain and sedation interventions for the prevention of intraventricular hemorrhage in preterm infants on assisted ventilation - an overview of systematic reviews. *Cochrane Libr.* CD012706. doi: 10.1002/14651858.CD012706

Rong, Z., Liu, H., Xia, S., and Chang, L. (2012). Risk and protective factors of intraventricular hemorrhage in preterm babies in Wuhan, China. *Childs Nerv. Syst.* 28, 2077–2084. doi: 10.1007/s00381-012-1875-9

Ryckman, K. K., Dagle, J. M., Kelsey, K., Momany, A. M., and Murray, J. C. (2011). Replication of genetic associations in the inflammation, complement, and coagulation pathways with intraventricular hemorrhage in LBW preterm neonates. *Pediatr. Res.* 70, 90–95. doi: 10.1203/PDR.0b013e31821ceb63

Salas, A. A., Faye-Petersen, O. M., Sims, B., Peralta-Carcelen, M., Reilly, S. D., McGwin, G., et al. (2013). Histological characteristics of the fetal inflammatory response associated with neurodevelopmental impairment and death in extremely preterm infants. *J. Pediatr.* 163, 652-7. e2. doi: 10.1016/j.jpeds.2013.03.081

Sarkar, S., Kaplan, C., Wiswell, T. E., Spitzer, A. R. (2005). Histological chorioamnionitis and the risk of early intraventricular hemorrhage in infants born≤ 28 weeks gestation. *J. Perinatol.* 25, 749–752. doi: 10.1038/sj.jp.7211399

Sato, M., Nishimaki, S., Yokota, S., Seki, K., Horiguchi, H., An, H., et al. (2011). Severity of chorioamnionitis and neonatal outcome. *J. Obstet. Gynaecol. Res.* 37, 1313–1319. doi: 10.1111/j.1447-0756.2010.01519.x

Seliga-Siwecka, J. P., and Kornacka, M. K. (2013). Neonatal outcome of preterm infants born to mothers with abnormal genital tract colonisation and chorioamnionitis: a cohort study. *Early Hum. Dev.* 89, 271–275. doi: 10.1016/j.earlhumdev.2012.10.003

Shankaran, S., Lin, A., Maller-Kesselman, J., Zhang, H., O'shea, T. M., Bada, H. S., et al. (2014). Maternal race, demography, and health care disparities impact risk for intraventricular hemorrhage in preterm neonates. *J. Pediatr.* 164, 1005-11. e3. doi: 10.1016/j.jpeds.2014.01.036

Smit, A. L., Been, J. V., Zimmermann, L. J., Kornelisse, R. F., Andriessen, P., Vanterpool, S. F., et al. (2015). Automated auditory brainstem response in preterm newborns with histological chorioamnionitis. *J. Matern. Fetal Neonatal Med.* 28, 1864–1869. doi: 10.3109/14767058.2014.971747

Soraisham, A., Trevenen, C., Wood, S., Singhal, N., and Sauve, R. (2013). Histological chorioamnionitis and neurodevelopmental outcome in preterm infants. *J. Perinatol.* 33, 70–75. doi: 10.1038/jp.2012.49

Soraisham, A. S., Singhal, N., McMillan, D. D., Sauve, R. S., Lee, S. K., and Network, C. N. (2009). A multicenter study on the clinical outcome of

chorioamnionitis in preterm infants. *Am. J. Obstet. Gynecol.* 200, 372 e1–e6. doi: 10.1016/j.ajog.2008.11.034

Stanimirovic, D., and Satoh, K. (2000). Inflammatory mediators of cerebral endothelium: a role in ischemic brain inflammation. *Brain Pathol.* 10, 113–126. doi: 10.1111/j.1750-3639.2000.tb00248.x

Strunk, T., Inder, T., Wang, X., Burgner, D., Mallard, C., and Levy, O. (2014). Infection-induced inflammation and cerebral injury in preterm infants. *Lancet Infect. Dis.* 14, 751–762. doi: 10.1016/S1473-3099(14)70710-8

Suarez, R. D., Grobman, W. A., and Parilla, B. V. (2001). Indomethacin tocolysis and intraventricular hemorrhage. *Obstet. Gynecol.* 97, 921–925.

Suppiej, A., Franzoi, M., Vedovato, S., Marucco, A., Chiarelli, S., and Zanardo, V. (2009). Neurodevelopmental outcome in preterm histological chorioamnionitis. *Early Hum. Dev.* 85, 187–189. doi: 10.1016/j.earlhumdev.2008.09.410

Thomas, W., and Speer, C. P. (2011). Chorioamnionitis: important risk factor or innocent bystander for neonatal outcome? *Neonatology* 99, 177–187. doi: 10.1159/000320170

Tita, A. T., and Andrews, W. W. (2010). Diagnosis and management of clinical chorioamnionitis. *Clin. Perinatol.* 37, 339–354. doi: 10.1016/j.clp.2010.02.003

Tortora, D., Severino, M., Malova, M., Parodi, A., Morana, G., Sedlacik, J., et al. (2017). Differences in subependymal vein anatomy may predispose preterm infants to GMH–IVH. *Arch. Dis. Child Fetal Neonatal Ed.* 103,F59–F65. doi: 10.1136/archdischild-2017-312710

Trevisanuto, D., Peruzzetto, C., Cavallin, F., Vedovato, S., Cosmi, E., Visentin, S., et al. (2013). Fetal placental inflammation is associated with poor neonatal growth of preterm infants: a case-control study. *J. Matern. Fetal Neonatal Med.* 26, 1484–1490. doi: 10.3109/14767058.2013.789849

Tsiartas, P., Kacerovsky, M., Musilova, I., Hornychova, H., Cobo, T., Sävman, K., et al. (2013). The association between histological chorioamnionitis, funisitis and neonatal outcome in women with preterm prelabor rupture of membranes. *J. Matern. Fetal Neonatal Med.* 26, 1332–1336. doi: 10.3109/14767058.2013.784741

Vaihinger, M., Mazzitelli, N., Balanian, N., and Grandi, C. (2012). The relationship between placental lesions and early hemorrhagic-ischemic cerebral injury in very low birth weight infants. *Rev. Fac. Cienc. Med. Cordoba* 70, 123–133.

van Vliet, E. O., de Kieviet, J. F., van der Voorn, J. P., Been, J. V., Oosterlaan, J., and van Elburg, R. M. (2012). Placental pathology and long-term neurodevelopment of very preterm infants. *Am. J. Obstet. Gynecol.* 206, 489. e1-e7. doi: 10.1016/j.ajog.2012.03.024

Vergani, P., Locatelli, A., Doria, V., Assi, F., Paterlini, G., Pezzullo, J. C., et al. (2004). Intraventricular hemorrhage and periventricular leukomalacia in preterm infants. *Obstet. Gynecol.* 104, 225–231. doi: 10.1097/01.AOG.0000130838.02410.b7

Villamor-Martinez, E., Cavallaro, G., Raffaeli, G., Rahim, O. M. M., Gulden, S., Ghazi, A. M., et al. (2018a). Chorioamnionitis as a risk factor for retinopathy of prematurity: an updated systematic review and meta-analysis. *bioRxiv* [Preprint]. doi: 10.1101/291476

Villamor-Martinez, E., Fumagalli, M., Mohammed Rahim, O. M. M., Passera, S., Cavallaro, G., Degraeuwe, P., et al. (2018b). Chorioamnionitis is a risk factor for intraventricular hemorrhage in preterm infants: a systematic review and meta-analysis. *bioRxiv*. 1–30. doi: 10.1101/334375

Volpe, J. J. (2015). Impaired neurodevelopmental outcome after mild germinal matrix-intraventricular hemorrhage. *Pediatrics* 136, 1185–1187. doi: 10.1542/peds.2015-3553

Wan, X., Wang, W., Liu, J., and Tong, T. (2014). Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Med. Res. Methodol.* 14:135. doi: 10.1186/1471-2288-14-135

Watterberg, K. L., Gerdes, J. S., Gifford, K. L., and Lin, H.-M. (1999). Prophylaxis against early adrenal insufficiency to prevent chronic lung disease in premature infants. *Pediatrics* 104, 1258–1263. doi: 10.1542/peds.104.6.1258

Wilcox, A. J., Weinberg, C. R., and Basso, O. (2011). On the pitfalls of adjusting for gestational age at birth. *Am. J. Epidemiol.* 174, 1062–1068. doi: 10.1093/aje/kwr230

Wirbelauer, J., Thomas, W., and Speer, C. P. (2011). Response of leukocytes and nucleated red blood cells in very low-birth weight preterm infants after exposure to intrauterine inflammation. *J. Matern. Fetal Neonatal Med.* 24, 348–353. doi: 10.3109/14767058.2010.497568

Wu, Y. W., and Colford, J. M. Jr. (2000). Chorioamnionitis as a risk factor for cerebral palsy: a meta-analysis. *JAMA* 284, 1417–1424. doi: 10.1001/jama.284.11.1417

Xu, L. P., Ren, R., Zhu, S., Zhuang, H., Huang, Z., and Yang, H. (2012). Effect of chorioamnionitis on brain injury in preterm infants. *Zhongguo Dang Dai Er Ke Za Zhi* 14, 661–663.

Yamada, N., Sato, Y., Moriguchi-Goto, S., Yamashita, A., Kodama, Y., Sameshima, H., et al. (2015). Histological severity of fetal inflammation is useful in predicting neonatal outcome. *Placenta* 36, 1490–1493. doi: 10.1016/j.placenta.2015.10.021

Yanowitz, T. D., Jordan, J. A., Gilmour, C. H., Towbin, R., Bowen, A. D., Roberts, J. M., et al. (2002). Hemodynamic disturbances in premature infants born after chorioamnionitis: association with cord blood cytokine concentrations. *Pediatr. Res.* 51, 310–316. doi: 10.1203/00006450-200203000-00008

Yanowitz, T. D., Potter, D. M., Bowen, A. D., Baker, R. W., and Roberts, J. M. (2006). Variability in cerebral oxygen delivery is reduced in premature neonates exposed to chorioamnionitis. *Pediatr. Res.* 59, 299–304. doi: 10.1203/01.pdr.0000196738.03171.f1

Yoon, B. H., Romero, R., Kim, C. J., Jun, J. K., Gomez, R., Choi, J. -H., et al. (1995). Amniotic fluid interleukin-6: a sensitive test for antenatal diagnosis of acute inflammatory lesions of preterm placenta and prediction of perinatal morbidity. *Am. J. Obstet. Gynecol.* 172, 960–970. doi: 10.1016/0002-9378(95)90028-4

Zanardo, V., Vedovato, S., Suppiej, A., Trevisanuto, D., Migliore, M., Di Venosa, B., et al. (2008). Histological inflammatory responses in the placenta and early neonatal brain injury. *Pediatr. Dev. Pathol.* 11, 350–354. doi: 10.2350/07-08-0324.1

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Villamor-Martinez, Fumagalli, Mohammed Rahim, Passera, Cavallaro, Degraeuwe, Mosca and Villamor. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

CORRECTION
published: 15 February 2019
doi: 10.3389/fphys.2019.00102

Corrigendum: Chorioamnionitis Is a Risk Factor for Intraventricular Hemorrhage in Preterm Infants: A Systematic Review and Meta-Analysis

Eduardo Villamor-Martinez[1], *Monica Fumagalli*[2], *Owais Mohammed Rahim*[1], *Sofia Passera*[2], *Giacomo Cavallaro*[2], *Pieter Degraeuwe*[1], *Fabio Mosca*[2] *and Eduardo Villamor*[1]*

[1] Department of Pediatrics, School for Oncology and Developmental Biology (GROW), Maastricht University Medical Center, Maastricht, Netherlands, [2] Neonatal Intensive Care Unit, Department of Clinical Sciences and Community Health, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, Italy

Keywords: chorioamnionitis, intraventricular hemorrhage, very preterm infant, systematic review, meta-analysis

OPEN ACCESS

Edited and reviewed by:
Carina Mallard,
University of Gothenburg, Sweden

***Correspondence:**
Eduardo Villamor
e.villamor@mumc.nl

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *02 January 2019*
Accepted: *28 January 2019*
Published: *15 February 2019*

Citation:
Villamor-Martinez E, Fumagalli M, Mohammed Rahim O, Passera S, Cavallaro G, Degraeuwe P, Mosca F and Villamor E (2019) Corrigendum: Chorioamnionitis Is a Risk Factor for Intraventricular Hemorrhage in Preterm Infants: A Systematic Review and Meta-Analysis. Front. Physiol. 10:102. doi: 10.3389/fphys.2019.00102

A Corrigendum on

Chorioamnionitis Is a Risk Factor for Intraventricular Hemorrhage in Preterm Infants: A Systematic Review and Meta-Analysis

by Villamor-Martinez, E., Fumagalli, M., Mohammed Rahim, O., Passera, S., Cavallaro, G., Degraeuwe, P., et al. (2018). Front. Physiol. 9:1253. doi: 10.3389/fphys.2018.01253

In the original article, there was a mistake in **Supplementary Table 1** and **Supplementary Table 2** as published. The study by Vaihinger et al. was incorrectly given "8" points on the Newcastle Ottawa Scale instead of "9". The corrected **Supplementary Table 1** and **Supplementary Table 2** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

Copyright © 2019 Villamor-Martinez, Fumagalli, Mohammed Rahim, Passera, Cavallaro, Degraeuwe, Mosca and Villamor. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Supplementary Table 1 | Synoptic table of characteristics of all included studies.

First author, year	Location (s)	Cohort/ case-control[a]	Perspective[a]	Prosp/ Retro	Total infants (centers)	Mean BW (g)	Mean GA (wks)	Male (%)	ACS (%)	CA category[b]	Incidence of CA (%)	Definition of CA[c]	Incidence of all IVH (%)	Incidence of severe IVH (%)	Definition of IVH[d]	NO-S Quality score
Aden, 2013	Sweden, Finland, USA	Ca-co	IVH	Prosp	612 (27)	844	26,2	59	100	CCA	23	NoDes		37	NA	6
Ahn, 2012	Seoul, Korea	Cohort	CA	Prosp	257 (1)	1536	30,6	60		HCA	35	Ref		2	Ref	8
Alexander, 1998	Dallas, USA	Cohort	CA	Prosp	1367 (1)	1138	28,9			CCA	7	Des		12	Ref	7
Arayici, 2004	Ankara, Turkey	Cohort	CA	Retro	281 (1)	1173	28,9	55	71	HCA	52	Des		10	Ref	7
Austeng, 2010	Sweden	Cohort	CA	Prosp	468 (7)	767	24,9	55	71	CCA	17	NoDes		14	Ref	8
Babnik, 2006	Ljubljana, Slovenia	Cohort	CA-IVH	Prosp	125 (1)	1019	27	54	53	HCA & F	39	Des	41		Ref	7
Barrera-Reyes, 2011	Mexico, Mexico	Cohort	CA	Prosp	104 (1)	1071	30,0	52		CCA	22	Ref	29		NA	6
Baumert, 2008	Katowice, Poland	Ca-co	IVH	Prosp	2675 (1)	3351	38,3	51		CCA	4	NoDes	15		Ref	7
Been, 2009	Rotterdam, Nehterlands	Cohort	CA	Prosp	301 (1)	1143	29,1	51	70	HCA	40	Des	15	4	NA	7
Bermick, 2016	Michigan, USA	Cohort	IVH	Retro	216	764	25,5	51	81	HCA	41	NoDes	56	NA	Ref	8
Bordigato, 2010	Padova, Italy	Cohort	CA	Prosp	29 (1)	805	26,7	59	76	HCA	48	Ref	27	3	NA	7
Botet, 2010	Spain	Ca-co	CA	Prosp	328 (12)	1057	28,2	54		HCA	50	Ref	27	12	NA	8
Bry, 2015	Gothenburg, Sweden	Cohort	CA	Prosp	24 (1)	777	25,9	50	100	HCA	67	Ref	17		NA	6
Dalton, 2015	Michigan, USA	Cohort	IVH	Retro	216 (1)	764	25,5	51	81	HCA	41	NoDes	56		Ref	6
Dempsey, 2005	Montreal, Canada	Cohort	CA	Retro	330 (1)	987	27,0		63	HCA	39	Des	10		Ref	7
Dexter, 2000	Rhode Island, USA	Cohort	CA	Prosp	275 (1)	904	26,5	53	22.3	HCA	57	Des	32	10	NA	6
Ecevit, 2014	Ankara, Turkey	Cohort	CA	Retro	36 (1)	1524	29,7	59		HCA	58	Des	19		NA	6
Elimian, 2000	New York, USA	Cohort	CA	Prosp	1260 (1)	1183	29.0		42	HCA	42	Ref	27	12	Ref	9

(Continued)

Supplementary Table 1 | Continued

First author, year	Location (s)	Cohort/ case-control[a]	Perspective[a]	Prosp/ Retro	Total infants (centers)	Mean BW (g)	Mean GA (wks)	Male (%)	ACS (%)	CA category[b]	Incidence of CA (%)	Definition of CA[c]	Incidence of all IVH (%)	Incidence of severe IVH (%)	Definition of IVH[d]	NO-S Quality score
Erdemir, 2013	Izkir, Turkey	Cohort	CA	Prosp	57 (1)	1675	30,8	46	65	HCA and/or CCA	21	Des	12		NA	6
Fung, 2003	Clayton, Australia	Cohort	CA	Prosp	62 (1)	794	26,2	50	83	HCA and/or CCA	25	Des		24	NA	6
Gagliardi, 2014	Italian Neonatal Network	Cohort	CA	Retro	3606 (82)	938	27,4	50	84	CCA	42	NoDes		11	Ref	9
Garcia-Munoz Rodrigo, 2014	Spanish Network	Cohort	CA	Prosp	8330 (53)	1086	28,5	52	87	CCA	18	Des		10	NA	9
Gawade, 2013	Springfield, USA	Cohort	IVH	Retro	78 (1)	980	26,8	59	85	CCA	15	Nodes	44	12	Ref	6
Gonzalez-Luis, 2002	Barcelona, Spain	Ca-co	CA	Retro	135 (1)	1147	28,9			CCA	33	Des	20	7	NA	7
Gray, 1997	Brisbane, Australia	Cohort	IVH	Retro	158 (1)	955	27,0	57		HCA and/or CCA	10	Des	25	8	NA	7
Hendson, 2011	Edmonton, Canada	Cohort	CA	Prosp	617 (1)	930	26,9	48	83	HCA	48	Des		16	Ref	7
Hitti, 2001	Seattle, USA	Cohort	CA	Prosp	140 (2)	1699	29,3		51	Microbiol	17	Des	14	6	Ref	7
Holcroft, 2003	Baltimore, USA	Ca-Co	CA	Retro	213 (1)	1045	28,3			CCA	21	Des	36		NA	5
Kallankari, 2010	Oulu, Finland	Cohort	IVH	Prosp	163 (1)		92,2		86	HCA	39	Ref	14		Ref	7
Kaulkola,2006	Oulu, Finland	Cohort	IVH	Prosp	51 (1)	772	27	41	90	HCAA	49	Ref	22		Ref	7
Kidokoro	New Zealand, Australia, USA	Cohort	IVH	Prosp	325 (3)	959	27,5	47	86	CCA	22	Des	19	4	Ref	8
Kim, 2015	Seoul, Korea	Cohort	CA	Retro	235 (1)	1104	29,2	50	81	HCA & F	38	Ref		6	NA	7
Kirchner, 2007	Vienna, Austria	Cohort	CA	Retro	44 (1)		27,9	53	93	Microbiol	34	NoDes		9	Ref	7

(Continued)

Supplementary Table 1 | Continued

First author, year	Location (s)	Cohort/ case-control[a]	Perspective[a]	Prosp/ Retro	Total infants (centers)	Mean BW (g)	Mean GA (wks)	Male (%)	ACS (%)	CA category[b]	Incidence of CA (%)	Definition of CA[c]	Incidence of all IVH (%)	Incidence of severe IVH (%)	Definition of IVH[d]	NO-S Quality score
Klebermans-Schrehof	Vienna, Austria	Cohort	IVH	Retro	471 (1)	996	27,4	53	93	HCA and/or CCA	38	NoDes	32		Ref	7
Kosuge, 2000	Minamikawachi-machi, Japan	Cohort	CA	Retro	81 (1)	1181	28,1	68	17	HCA	54	Ref	11		NA	6
Lau, 2005	Vancouver, Canada	Cohort	CA	Prosp	1296 (1)	2068	33,2	55	47	HCA & F	31	Ref		5	Ref	7
Lee Hyun Ju, 2011	Seoul, Korea	Cohort	CA	Retro	147 (2)	791	27	55	67	HCA	48	Ref	40		Ref	7
Lee Ju Young, 2010	Seoul, Korea	Ca-Co	IVH	Retro	177 (2)	954	27,5	53		HCA	45	NoDes		22	Ref	8
Lim, 2011	Taiwan	Ca-Co	IVH	Retro	72 (1)	768	24,7	64		CCA	13	Des		50	Ref	9
Linder, 2003	Israel	Ca-Co	IVH	Retro	105 (1)	826	25,4	58	73	CCA	24	Des		34	Ref	9
Liu, 2014	Changhai, China	Cohort	CA	Prosp	95 (1)	1706	31,7	58	89	HCA	52	Ref	43	8	Ref	7
Logan, 2013	USA	Cohort	IVH	Retro	921 (14)			51	90	HCA	36	Des	6		NA	7
Lu, 2016	Jiangsu, China	Ca-Co	IVH	Retro	137 (1)	1205	31,9	58	50	HCA & CCA	44	Des	24		Ref	7
Mehta, 2006	New Brunswick, USA	Cohort	CA-IVH	Retro	164 (1)					HCA	39	Ref	37		NA	7
Mestan, 2010	Boston, USA	Cohort	CA	Prosp	256 (1)	1437	30,3	48	77	HCA	37	Ref		4	Ref	7
Miyazaki, 2016	Network database, Japan	Cohort	CA	Retro	4078 (54)	973	27,6	49	41	HCA	30	Ref	15		Ref	9
Morales, 1987	Orlando, USA	Ca-Co	Ca	Prosp	86 (1)	1178	29,2			HCA & CCA	50	Des	86	28	Ref	7
Mu, 2008	Taipei, Taiwan	Cohort	CA	Prosp	119 (1)	1108	28,6	54	45	HCA	54	Ref	22	17	Ref	8
Nasef, 2013	Toronto, Canada	Cohort	CA	Retro	274 (1)	952	27	55	85	HCA & CCA	47	Ref	23	1	Ref	7

(Continued)

Supplementary Table 1 | Continued

First author, year	Location (s)	Cohort/ case-control[a]	Perspective[a]	Prosp/ Retro	Total infants (centers)	Mean BW (g)	Mean GA (wks)	Male (%)	ACS (%)	CA category[b]	Incidence of CA (%)	Definition of CA[c]	Incidence of all IVH (%)	Incidence of severe IVH (%)	Definition of IVH[d]	NO-S Quality score
Ogunyemi, 2003	New jersey, USA	Cohort	CA	Retro	774 (1)	1313	29,4		53	HCA	33	Ref	39	5	NA	7
Oh, 2015	Seoul, Korea	Cohort	CA	Retro	175 (1)	765	27,1	55	60	HCA	25	Ref		NA	Ref	9
Oh, 2018	Seoul, Korea	Cohort	IVH	Retro	207	1269	29.7	48	75	HCA	44	Ref	7*	NA	Ref	9
Ohyama, 2002	Yokohama, Japan	Cohort	CA	Retro	143 (1)	1162	27,8			HCA & F	63	Ref	9		NA	6
Osmanagaoglu, 2005	Trabzon, Turkey	Cohort	CA	Retro	254 (1)	1828	32	56	65	CCA	12	Ref		6	NA	7
Pappas, 2014	USA	Cohort	CA	Prosp	1918 (16)		24,4	51	75	HCA	55	Ref		29	NA	6
Perrone, 2012	Siena, Italy	Cohort	CA	Prosp	92 (1)	998	26,3			HCA	49	Ref	48		Ref	7
Polam, 2005	New Brunswick, USA	Cohort	CA	Prosp	177 (1)	955	26,5	53	74	HCA	58	Des	26	7	NA	6
Poralla, 2012	Bonn, Germany	Cohort	IVH	Retro	132 (1)	714	25,5	50	87	CCA	39	Des	44		NA	6
Richardson, 2006	London Ontario, Canada	Cohort	CA	Retro	660 (1)	1602	30,1	55		HCA	44	Des	22		NA	6
Rocha, 2006 & Rocha, 2007	Porto, Portugal	Cohort	CA	Retro	452 (3)	1504	29,5	52	65	HCA	28	Ref	18	9	Ref	9
Rodríguez-Trujillo, 2016	Barcelona, Spain	Cohort	CA	Prosp	165 (1)	1721	30,2			HCA	67	NoDes		10	NA	7
Rong, 2012	Wuhan, China	Ca-co	IVH	Retro	232 (3)	1566	30,7	73	41	CCA	19	NoDes	34		NA	8
Ryckman, 2011	Iowa, USA	Cohort	IVH	Prosp	219			58		CCA	15	NoDes	22		Ref	6
Salas, 2013	Alabama, USA	Cohort	CA	Retro	347 (1)	829	26,1	50	62	HCA	43	Ref		17	Ref	7
Sarkar, 2005	New York, USA	Cohort	CA-IVH	Prosp	62 (1)	884	62,2	45	90	HCA	47	Ref	15	7	Ref	7

(Continued)

Supplementary Table 1 | Continued

First author, year	Location (s)	Cohort/ case-control[a]	Perspective[a]	Prosp/ Retro	Total infants (centers)	Mean BW (g)	Mean GA (wks)	Male (%)	ACS (%)	CA category[b]	Incidence of CA (%)	Definition of CA[c]	Incidence of all IVH (%)	Incidence of severe IVH (%)	Definition of IVH[d]	NO-S Quality score
Sato, 2011	Yokohama, Japan	Cohort	CA	Retro	302 (1)	938,4	26,3	52	62	HCA	52	Ref	27		NA	7
Seliga-Siwecka, 2013	Warsaw, Poland	Cohort	CA	Prosp	383 (1)	1338	29,2	56	84	HCA	37	Ref		45	Ref	9
Shankaran, 2014	USA and Sweden	Ca-co	IVH	Prosp	1111 (24)	817	26,0	56	53	CCA	29	NoDes	52		Ref	8
Smit, 2015	Veldhoven, Netherlands	Cohort	CA	Retro	300 (1)	1303	29,4	54	92	HCA & F	45	Ref		4	NA	7
Soraisham, 2009	Canadian Neonatal Network	Cohort	CA	Prosp	3094 (24)	1320	28,9	53	79	CCA	15	Des		14	Ref	9
Soraisham, 2013	Regional NICU Southern Alberta, Canada	Cohort	CA	Retro	384 (1)	885	26,3	51	86	HCA	51	Des	21	7	Ref	6
Suarez, 2001	Chicago, USA	Ca-co	IVH	Retro	280 (1)	1328	29,6	56		CCA	19	Des	20		Ref	7
Suppiej, 2009	Padova, Italy	Cohort	CA	Prosp	104 (1)	1078	28,5	46	87	HCA	39	Ref	13		Ref	6
Trevisanuto, 2010	Padua, Italy	Ca-co	CA	Prosp	142 (1)	1075	27,8	55	89	HCA	50	Ref		4	Ref	8
Tsiartas, 2013	Králove, Czech Republic	Cohort	CA	Retro	231 (1)	1975	33,0		56	HCA & F	61	Ref	20	1	Ref	7
Vaihinger, 2013	Buenos Aires, Argentina	Ca-co	IVH	Retro	198 (1)	1072	28,0	53	66	CCA	24	NoDes	25		Ref	9
van Vliet, 2012	Amsterdam, Netherlands	Cohort	CA	Prosp	72 (1)	1110	29,0	51	82	HCA	29	Ref	29		NA	6
Vergani, 2004	Monza, Italy	Cohort	IVH	Retro	653 (1)	1335	30,1	50	50	CCA	11	Des	7		Ref	7
Watterberg, 1999	Pennsylvania, USA	Cohort	CA	Prosp	40 (2)	751	25,3	38	85	HCA	55	NoDes	38	8	Na	7
Wirbelauer, 2011	Wuerzburg, Germany	Cohort	CA	Prosp	71 (1)	871	27,9	52	94	HCA & F	24	Ref		9	Ref	7
Xu, 2012	Hangzhou, China	Cohort	IVH	Prosp	88 (1)	1540	31,8	53		HCA and/or CCA	47	NoDes	25	8	Ref	7

(Continued)

Supplementary Table 1 | Continued

First author, year	Location (s)	Cohort/ case-control[a]	Perspective[a]	Prosp/ Retro	Total infants (centers)	Mean BW (g)	Mean GA (wks)	Male (%)	ACS (%)	CA category[b]	Incidence of CA (%)	Definition of CA[c]	Incidence of all IVH (%)	Incidence of severe IVH (%)	Definition of IVH[d]	NO-S Quality score
Yamada, 2015	Miyazaki, Japan	Cohort	CA	Prosp	212 (1)		25			HCA	65	Ref		20	NA	7
Yanowitz, 2006	Pittsburgh, USA	Cohort	CA	Prosp	49 (1)	1273	28,7	61		HCA	49	Ref	57	2	Ref	7
Yoon, 1995	Seoul, Korea	Cohort	CA	Prosp	50 (1)	1852	31,8			HCA	58	Ref	30		NA	7
Zanardo, 2008	Padua, Italy	Cohort	CA-IVH	Prosp	287 (1)	1146	29,3	48	80	HCA	24	Ref	12	1	Ref	7

CA, chorioamnionitis; IVH, intraventricular hemorrhage; NOS, Newcastle-Ottawa Scale; ACS, antenatal corticosteroids.

[a]Abbreviations for study design: Ca-Co, case-control study; Perspective, CA, study analyzed IVH as outcome of chorioamnionitis; Perspective, IVH, study analyzed chorioamnionitis as risk factor for IVH; Perspective, CA-IVH, study analyzed the association between chorioamnionitis and IVH as primary outcome. Prosp, prospective; Retro, retrospective;

[b]Chorioamnionitis category: CCA, clinical chorioamnionitis; HCA, histological chorioamnionitis; HCA & F, histological chorioamnionitis with funisitis mentioned separately. HCA and/or CCA, chorioamnionitis defined as positive when infants had histological or clinical chorioamnionitis; Microbiol, microbiological chorioamnionitis.

[c]Definition of chorioamnionitis: NoDes, no description; Des, clinical or histological description; Ref, defined according to cited article. [d]Definition of ROP: ICROP, International Classification of Retinopathy of Prematurity; Ref, defined according to cited article; Treat, laser treatment of ROP; NA, no diagnostic criteria mentioned.

Supplementary Table 2 | Newcastle-Ottawa Quality assessment of included studies.

First author, year	Perspective	Select.	Comp.	Outc.	Total	Reason for downgrade
Aden, 2013	IVH	4	0	2	6	No adjustment
Ahn, 2013	CA	4	1	3	8	Only adjusted for 1 factor
Alexander, 1998	CA	4	0	3	7	No adjustment
Arayici, 2004	CA	4	0	3	7	No adjustment
Austeng, 2010	CA	3	2	3	8	No CA definition
Babnik, 2006	CA-IVH	4	0	3	7	No adjustment
Barrera-Reyes, 2011	CA	4	0	2	6	No adjustment
Baumert, 2008	IVH	4	0	3	7	No adjustment
Been, 2009	CA	4	0	3	7	No adjustment
Bermick, 2016	IVH	3	2	3	8	No CA definition
Bordigato, 2010	CA	4	0	3	7	No adjustment
Botet, 2010	CA	4	1	3	8	Only adjusted for 1 factor
Bry, 2015	CA	4	0	2	6	No adjustment
Dalton, 2015	IVH	3	0	3	6	No CA definition
Dempsey, 2005	CA	4	0	3	7	No adjustment
Dexter, 2000	CA	4	0	2	6	Loss to follow up, no adjustment
Ecevit, 2014	CA	4	0	2	6	No IVH definition, no adjustment
Elimian, 2000	CA	4	2	3	9	
Erdemir, 2013	CA	4	0	2	6	No IVH definition, no adjustment
Fung, 2003	CA	4	0	2	6	No IVH definition, no adjustment
Gagliardi, 2014	CA	4	2	3	9	
Garcia-Munoz Rodrigo, 2014	CA	4	2	3	9	
Gawade, 2013	IVH	3	0	3	6	No CA definition, no adjustment
Gonzalez-Luis, 2002	CA	4	0	3	7	No adjustment
Gray, 1997	CA	4	0	3	7	
Hendson, 2011	CA	4	0	3	7	No adjustment
Hitti, 2001	CA	4	0	3	7	No adjustment
Holcroft, 2003	IVH	2	0	3	5	No IVH definition, no adjustment

(Continued)

Supplementary Table 2 | Continued

First author, year	Perspective	Select.	Comp.	Outc.	Total	Reason for downgrade
Kallankari, 2010	IVH	4	0	3	7	No adjustment
Kaulkola,2006	IVH	4	0	3	7	No adjustment
Kidokoro	IVH	4	1	3	8	Only adjusted for 1 factor
Kim, 2015	CA	4	0	3	7	No adjustment
Kirchner, 2007	CA	4	0	3	7	No adjustment
Klebermans-Schrehof	IVH	4	0	3	7	No adjustment
Kosuge, 2000	CA	4	0	2	6	No IVH definition, no adjustment
Lau, 2005	CA	4	0	3	7	No adjustment
Lee Hyun Ju, 2011	CA	4	0	3	7	No adjustment
Lee Ju Young, 2010	IVH	4	1	3	8	Only adjusted for 1 factor
Lim, 2011	IVH	4	2	3	9	
Linder, 2003	IVH	4	2	3	9	
Liu, 2014	CA	4	0	3	7	No adjustment
Logan, 2013	IVH	4	0	3	7	No adjustment
Lu, 2016	IVH	4	0	3	7	No adjustment
Mehta, 2006	CA-IVH	4	0	3	7	No adjustment
Mestan, 2010	CA	4	0	3	7	No adjustment
Miyazaki, 2016	CA	4	2	3	9	
Morales, 1987	CA	4	0	3	7	No adjustment
Mu, 2008	CA	4	1	3	8	Only adjusted for 1 factor
Nasef, 2013	CA	4	0	3	7	No adjustment
Ogunyemi, 2003	CA	4	0	3	7	No adjustment
Oh, 2015	IVH	4	2	3	9	
Oh, 2018	CA	4	2	3	9	
Ohyama, 2002	CA	4	0	2	6	No IVH definition, no adjustment
Osmanagaoglu, 2005	CA	4	0	3	7	No adjustment
Pappas, 2014	CA	4	0	2	6	No IVH definition, no adjustment
Perrone, 2012	CA	4	0	3	7	No IVH definition
Polam, 2005	CA	4	0	2	6	Loss to follow up, no adjustment
Poralla, 2012	IVH	4	0	2	6	No IVH definition
Richardson, 2006	CA	4	0	2	6	No IVH definition, no adjustment
Rocha, 2006 & Rocha, 2007	CA	4	2	3	9	
Rodríguez-Trujillo, 2016	CA	4	0	3	7	No adjustment
Rong, 2012	IVH	4	2	2	8	No CA definition
Ryckman, 2011	IVH	3	0	3	6	No CA definition, no adjustment
Salas, 2013	CA	4	0	3	7	No adjustment
Sarkar, 2005	CA-IVH	4	0	3	7	No adjustment
Sato, 2011	CA	4	1	2	7	No IVH definition, only adjusted for 1 factor
Seliga-Siwecka, 2013	CA	4	2	3	9	
Shankaran, 2014	IVH	4	2	2	8	
Smit, 2015	CA	4	0	3	7	No adjustment
Soraisham, 2009	CA	4	2	3	9	
Soraisham, 2013	CA	4	0	2	6	Loss to follow up, no adjustment
Suarez, 2001	IVH	4	0	3	7	No adjustment
Suppiej, 2009	CA	4	0	2	6	Loss to follow up, no adjustment
Trevisanuto, 2010	CA	4	1	3	8	Only adjusted for 1 factor
Tsiartas, 2013	CA	4	0	3	7	No adjustment
Vaihinger, 2013	IVH	4	2	3	9	
van Vliet, 2012	CA	4	0	2	6	No adjustment
Vergani, 2004	IVH	4	0	3	7	No adjustment
Watterberg, 1999	CA	4	0	3	7	No adjustment
Wirbelauer, 2011	CA	4	0	3	7	No adjustment
Xu, 2012	CA	4	0	3	7	No adjustment
Yamada, 2015	CA	4	0	3	7	No adjustment
Yanowitz, 2006	CA	4	0	3	7	No adjustment
Yoon, 1995	CA	4	0	3	7	No adjustment
Zanardo, 2008	CA-IVH	4	0	3	7	No adjustment

(Continued)

Select., selection; Comp., comparability; Outc., outcome; CA, chorioamnionitis; IVH, intraventricular hemorrhage.

frontiers in Physiology

ORIGINAL RESEARCH
published: 12 September 2018
doi: 10.3389/fphys.2018.01299

The Cerebrospinal Fluid Inflammatory Response to Preterm Birth

*James P. Boardman[1,2], Graeme Ireland[1], Gemma Sullivan[1], Rozalia Pataky[1], Bobbi Fleiss[3,4,5], Pierre Gressens[3,4,5] and Veronique Miron[1]**

[1] MRC Centre for Reproductive Health, The Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, United Kingdom, [2] Centre for Clinical Brain Sciences, Chancellor's Building, The University of Edinburgh, Edinburgh, United Kingdom, [3] Centre for the Developing Brain, Division of Imaging Sciences and Biomedical Engineering, King's College London, London, United Kingdom, [4] PROTECT, INSERM, Université Paris Diderot, Sorbonne Paris Cité, Paris, France, [5] PremUP, Paris, France

Background: Preterm birth is the leading risk factor for perinatal white matter injury, which can lead to motor and neuropsychiatric impairment across the life course. There is an unmet clinical need for therapeutics. White matter injury is associated with an altered inflammatory response in the brain, primarily led by microglia, and subsequent hypomyelination. However, microglia can release both damaging and trophic factors in response to injury, and a comprehensive assessment of these factors in the preterm central nervous system (CNS) has not been carried out.

Method: A custom antibody array was used to assess relative levels of 50 inflammation- and myelination-associated proteins in the cerebrospinal fluid (CSF) of preterm infants in comparison to term controls.

Results: Fifteen proteins differed between the groups: BDNF, BTC, C5a, FasL, Follistatin, IL-1β, IL-2, IL-4, IL-9, IL-17A, MIP-1α, MMP8, SPP1, TGFβ, and TNFβ ($p < 0.05$). To investigate the temporal regulation of these proteins after injury, we mined a gene expression dataset of microglia isolated from a mouse model of developmental white matter injury. Microglia in the experimental model showed dynamic temporal expression of genes encoding these proteins, with an initial and sustained pro-inflammatory response followed by a delayed anti-inflammatory response, and a continuous expression of genes predicted to inhibit healthy myelination.

Conclusion: Preterm CSF shows a distinct neuroinflammatory profile compared to term controls, suggestive of a complex neural environment with concurrent damaging and reparative signals. We propose that limitation of pro-inflammatory responses, which occur early after perinatal insult, may prevent expression of myelination-suppressive genes and support healthy white matter development.

Keywords: preterm birth, brain injury, inflammation, cerebrospinal fluid, microglia, myelination

OPEN ACCESS

Edited by:
Justin Dean,
University of Auckland, New Zealand

Reviewed by:
Angela Leigh Cumberland,
RMIT University, Australia
Rachel Anne Hill,
Monash University, Australia

****Correspondence:***
Veronique Miron
V.Miron@ed.ac.uk;
veronique.miron@ed.ac.uk

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *02 July 2018*
Accepted: *29 August 2018*
Published: *12 September 2018*

Citation:
Boardman JP, Ireland G, Sullivan G, Pataky R, Fleiss B, Gressens P and Miron V (2018) The Cerebrospinal Fluid Inflammatory Response to Preterm Birth. Front. Physiol. 9:1299. doi: 10.3389/fphys.2018.01299

INTRODUCTION

Preterm birth is closely associated with white matter injury and life course impairments including cerebral palsy, learning difficulty, autism spectrum disorder, and psychiatric disease (Volpe, 2009; Johnson and Marlow, 2017). A characteristic feature of white matter injury is oligodendrocyte dysmaturation, which is driven in part by immune dysregulation, and

results in hypomyelination (Back and Miller, 2014; Hagberg et al., 2015). Magnetic resonance imaging studies show that generalized atypical white matter tract development is often apparent in preterm infants at term-equivalent age (Batalle et al., 2017; Telford et al., 2017), which suggests that interventions to prevent injury and support normal myelination may need to be applied during the perinatal period. Therefore a priority is to better understand the immune mediators and receptors that drive preterm white matter injury in order to identify therapeutic targets that promote healthy white matter development.

Neuropathological analyses of post-mortem tissue have shown robust activation of central nervous system (CNS)-endogenous immune cells, microglia, which express pro-inflammatory markers (iNOS, TNFα, IL-1β, and IL-6) (Yoon et al., 1997; Haynes et al., 2009); and systemic inflammation due to co-morbidities of preterm birth such as chorioamnionitis and necrotizing enterocolitis, is associated with abnormal white matter on magnetic resonance imaging *in vivo* (Shah et al., 2008; Anblagan et al., 2016; Barnett et al., 2018).

Elevated levels of inflammatory proteins in blood or cerebrospinal fluid (CSF) are associated with perinatal brain injury and increased risk of adverse neurodevelopmental outcome (Yoon et al., 1996; Nelson et al., 1998; Savman et al., 1998; Bartha et al., 2004; Viscardi et al., 2004; Carlo et al., 2011; Armstrong-Wells et al., 2015; Basu et al., 2015). However, protein levels in plasma do not always correlate with those in the CSF in preterm infants with white matter injury, demonstrating that blood analyses may not reflect events in the CNS (Ellison et al., 2005; Rajkumar et al., 2018). Furthermore, a comprehensive assessment of inflammation-associated factors in preterm CSF has not been carried out. Here, we asked whether a large-scale measurement of inflammatory markers in preterm CSF, including measures of factors known to be detrimental or supportive of white matter development, could provide a broader understanding of the neuropathology of preterm brain injury.

MATERIALS AND METHODS

Participants

We recruited two groups of neonates from the Royal Infirmary of Edinburgh between June 2014 and September 2015 who required CSF sampling, usually for the evaluation of suspected meningitis: 17 preterm neonates with mean (SD) postmenstrual age (PMA) at birth 27.14 (2.14) weeks; and 20 term infants with mean (SD) PMA at birth 39.86 (1.86) weeks. The mean (SD) PMA at CSF sampling was 29.29 (2.86) weeks for preterm infants and 40.29 (2.0) weeks for term infants. There were no significant differences in the proportion of infants with CSF contaminated by blood defined as red blood cell count >1000 cells/mm^3 (50% versus 42%, $p = 0.73$). Methods for sampling and storage of CSF, and the clinical phenotype of participants including plasma C-Reactive Protein, full blood count, CSF total protein and glucose concentrations and CSF microscopy have been reported previously (Pataky et al., 2017). No infant in either group had meningitis; 10 out of 17 of the preterm infants and 8 out of 20 of the term infants were classified as having blood stream infection (BSI) at the time of CSF sampling, defined as either (1) blood culture grew a pathogenic bacterial species; or (2) the blood culture was negative or grew coagulase negative Staphylococcus (CoNS) *and* the infant had one or more signs of generalized infection (apnoea, temperature instability, feeding intolerance, worsening respiratory distress, or hemodynamic instability) *and* the attending neonatologist treated with IV antibiotics for $\geq$5 days. The difference in proportion of infants with BSI in each group was not statistically significant ($p = 0.33$).

This study was carried out in accordance with the recommendations of UK National Research Ethics Service with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the South East Scotland Research Ethics Committee (14/SS/044). Written parental informed consent was obtained for CSF sampling, and the study was approved by the UK National Research Ethics Service (14/SS/044).

Custom Antibody Microarray

A custom antibody array ("G-series" from Tebu-bio/RayBiotech) against 50 human analytes was generated to detect relative levels of: activin-A (INHBA), Brain-derived neurotrophic factor (BDNF), bone morphogenetic protein (BMP)2, BMP4, BMP7, betacellulin (BTC), cluster of differentiation (CD)200, Complement 5a (C5a), C-reactive protein (CRP), Fas ligand (FasL), follistatin, furin, Galectin-3 (Gal3), granulocyte macrophage colony-stimulating factor (GM-CSF), insulin-like growth factor-1 (IGF-1), interferon-gamma (IFNγ), insulin, interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, IL-17B, IL-17C, IL-17F, IL-18, monocyte chemoattractant protein-1 (MCP1), macrophage inflammatory protein 1-alpha (MIP1α), MIP1β, matrix metalloproteinase (MMP) 8, MMP-9, nerve growth factor (NGF)-β, neurotrophic factor 3 (NT3), Osteopontin (SPP1), placental growth factor (PLGF), regulated on activation, normal t cell expressed and secreted (RANTES), stem cell factor (SCF), tumor necrosis factor (TNF)-α, TNFβ, transforming growth factor-beta (TGFβ), urokinase-type plasminogen-activator (uPA), vascular endothelial growth factor-C (VEGF-C). Arrays were carried out according to the manufacturer's instructions. Briefly, antibodies printed onto sub-arrays were dried at room temperature (RT) for 2 h, then blocked for 30 min. Fifty microliters of CSF from each case was incubated with one sub-array for 2 h at RT, then washed with gentle rocking. Sub-arrays were then incubated with biotin-conjugated sandwich antibodies for 2 h at RT, washed thoroughly, then incubated with streptavidin-Cy3. Following washes in water, slides were read at 532 nm excitation frequency.

Antibody Array Data Analysis

Detected values of Cy3 intensity were normalized to an internal median background level on each slide. Analytes of interest were defined as those where the median value of the preterm group was outside the interquartile range of the controls. For analyses designed to generate hypotheses about group differences

for gene expression studies, the distribution of values according to gestation category (preterm versus term) was investigated using independent samples Mann–Whitney *U* test, individual test *p*-values are reported, and a threshold of <0.05 was used to select proteins for microglia gene expression analysis. Analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, United States).

Animal Protocol

Experimental protocols were approved by the Bichat-Robert Debre (France) ethical committee under the reference 2011-14/676-0053, and met the guidelines for the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals (NIH, Bethesda, MD, United States). We housed the OF1 strain mice (Charles River; L'Arbresle, France) under a 12 h light-dark cycle with *ad libitum* food and water. On P1 pups were sexed, all males were kept but litters were maintained at 9–11 pups. Assessments of injury and outcomes were made only in male animals as females do not display white matter injury in response to this paradigm. The preponderance to injury in males is similar to what is observed in preterm born infants (O'Driscoll et al., 2018). Neonatal received twice a day (bid) from P1 to P4 and once on P5 a 5 μl intra-peritoneal (ip) injection of 10 μg/kg/injection recombinant mouse IL-1β in phosphate buffered saline (PBS; R&D Systems, Minneapolis, MN, United States) or PBS alone (control). IL-1β exposure, as reported previously, sets up a complex systemic inflammatory response (Favrais et al., 2011) and then a complex central neuroinflammatory response (Krishnan et al., 2017; Van Steenwinckel et al., 2018). This leads to microgliosis, oligodendrocyte maturation arrest, hypomyelination and cognitive deficits (Favrais et al., 2011; Schang et al., 2014; Krishnan et al., 2017; Van Steenwinckel et al., 2018) reminiscent of what is observed in preterm born infants (Billiards et al., 2008; Verney et al., 2012; Caldinelli et al., 2017; Spittle et al., 2017).

Neural Tissue Dissociation and Magnetic-Activated Cell Sorting

At P1, P5, and P10, we collected brains for cell dissociation and CD11b-positive cell enrichment using a magnetic coupled antibody extraction technique (MACS), as previously described (Schang et al., 2014; Krishnan et al., 2017) and according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, we pooled brains ($n = 4$ at P1, $n = 3$ at P5, and $n = 2$ at P10) and after removing the cerebellum and olfactory bulbs they were dissociated using the Neural Tissue Dissociation Kit. A total of six samples per group and per time point were generated with at least four independent litters per group. Using anti-CD11b MicroBeads we captured the CD11b+ cells and after elution, we centrifuged the isolated cells for 5 min at 600 g and then conserved them at −80°C. The purity of MACSed CD11B+ fraction has been validated using FACS analysis of CD11B fluorescence, and with RT-qPCR of the positive and negative cell fractions as previously described (Schang et al., 2014; Krishnan et al., 2017) and revealed the negative fraction has gene expression levels 98% lower than found in the respective primary cultures of astrocytes, neurons, and oligodendrocytes.

Microarray Analysis

As previously published, Miltenyi Biotec (France) performed microarrays (Mouse Agilent Whole Mouse Genome Oligo Microarrays, 8 × 60K) on 6 samples per time point per group for CD11b enriched cell samples from P1, P5, and P10 mice exposed to IL-1β or PBS; a total of 24 samples (Krishnan et al., 2017). Preparation of samples for array analysis has been previously described (Husson et al., 2005; Chhor et al., 2013; Krishnan et al., 2017). The Agilent feature extraction software was used to process microarray image files. We only included signal intensities above background. Signal intensity values were background subtracted and uploaded following instructions by Miltenyi Biotec GmbH (Stefan Tomiuk) and PerkinElmer (Matt Hudson) into GeneSifter Analysis Edition v4.0[1] for further analysis as previously described (Gustavsson et al., 2007). The pre-processed signal intensity values were median normalized, and the gene expression in neuroinflammatory and PBS controls were compared at P1, P5, and P10 using *t*-test ($p < 0.05$) with Benjamini-Hochberg multiple testing correction.

RESULTS

Differential Levels of Inflammation-Associated Proteins in Pre-term vs. Term Infant Cerebrospinal Fluid

To conduct a comprehensive assessment of CNS inflammatory state in preterm infants and associate this with factors affecting myelination, we designed an antibody array assessing expression of 50 proteins with functions in regulating inflammation and myelination (**Table 1**). This approach was optimal to assess relative protein levels in neonatal CSF because it allowed high-content simultaneous screening of low volumes of fluid with high sensitivity and a broad range of detection.

We found that 28 protein levels had a median value in CSF from preterm infants that was outside the interquartile range of the controls (**Table 1**, bold type); these represented proteins with known functions in enhancing or resolving inflammation, as well impairing or supporting myelination (**Table 1**). The distribution of 15 proteins differed in preterm CSF from that of the controls (p-value <0.05), and 5 analytes were increased in preterm CSF at a threshold of $p < 0.01$: C5a, interleukin (IL)-9, osteopontin (SPP1), Fas ligand (FasL), and follistatin. These data demonstrate that preterm CSF has a distinct inflammatory profile compared to term controls that includes both pro-inflammatory and anti-inflammatory proteins, as well as those which can support or impair myelination.

[1] http://jhu.genesifter.net/login

TABLE 1 | Median (IQR) normalized fluorescence intensity of 50 cerebrospinal fluid analytes from term control infants and preterm infants.

Analyte	Function	Control			Preterm		*p*-Value
		Median	IQR	Q1–Q3	Median	IQR	
C5a (*Hc*)	⊙	**1378.5**	**1382.5**	**680.8–2063.3**	**3592.6**	**2540.3**	**0.001****
CRP	⊙	28446.4	20206.4	15120.2–35326.6	35217.1	18532.5	0.133
GM-CSF (*Csf2*)	⊙	**255.6**	**91.2**	**212.2–303.5**	**330.2**	**284.2**	**0.125**
IFNγ	⊙O	403.2	127.0	328.4–455.4	402.4	159.3	0.916
IL-1α	⊙	**490.5**	**81.4**	**450.6–532.0**	**581.9**	**546.13**	**0.220**
IL-1β	⊙O	**52.1**	**73.3**	**10.0–83.3**	**108.8**	**338.8**	**0.014***
IL-2	⊙	**413.1**	**77.8**	**367.7–445.5**	**520.5**	**298.0**	**0.030***
IL-6	⊙■	580.0	3259.5	413.8–3673.3	706.2	8133.4	0.869
IL-8	⊙	19466.8	55521.7	16192.7–71714.4	18370.4	79637.3	0.707
IL-9	⊙O	**188.8**	**76.1**	**136.9–213.1**	**242.6**	**233.23**	**0.005****
IL-12p40	⊙	62.5	87.3	22.8–110.1	83.3	206.6	0.167
IL-12p70	⊙	**164.3**	**80.0**	**110.1–190.0**	**220.1**	**219.8**	**0.177**
IL-17A	⊙■	**202.1**	**45.4**	**182.5–227.9**	**296.4**	**225.6**	**0.028***
IL-17B	⊙	104.6	20.0	96.3–116.3	107.0	125.4	0.798
IL-17C	⊙	508.8	99.8	456.7–556.5	546.2	246.9	0.052
IL-17F	⊙	72.5	73.2	22.8–96.0	62.6	214.0	0.619
IL-18	⊙	**80.9**	**49.6**	**50.0–81.0**	**101.8**	**129.0**	**0.326**
MCP-1 (Ccl2)	⊙	116104.0	10004.9	112697.7–122702.6	109752.5	40956.0	0.283
MIP1α (Ccl3)	⊙	**211.5**	**282.6**	**118.0–400.6**	**1054.5**	**4168.5**	**0.015***
MIP1β (Ccl4)	⊙	**6715.4**	**17113.7**	**5549.0–22562.7**	**26868.7**	**31793.7**	**0.133**
PIGF	⊙	**593.2**	**375.6**	**438.0–813.6**	**948.0**	**1180.7**	**0.056**
RANTES	⊙	182.8	1590.6	115.7–1706.3	736.4	14673.8	0.244
TNFα	⊙■O	**1095.9**	**167.4**	**1036.8–1204.2**	**1242.3**	**935.8**	**0.074**
TNFβ	⊙	**452.6**	**68.8**	**404.4–473.3**	**504.0**	**380.4**	**0.011***
uPA	⊙	8733.4	8969.4	4470.1–13439.5	12527.5	18004.0	0.149
CD200	♦	98.9	61.3	58.4–119.7	113.6	163.9	0.326
IL-4	♦	**402.7**	**80.7**	**340.7–421.4**	**430.6**	**176.6**	**0.042***
IL-5	♦	299.9	58.6	271.5–330.1	301.9	258.2	0.798
IL-10	♦■	481.6	787.9	429.1–1216.9	917.7	6406.5	0.341
IL-13	♦	382.8	93.4	336.2–429.5	416.3	330.8	0.104
Activin-A (INHBA)	■♦	**238.2**	**62.7**	**204.9–267.6**	**271.1**	**314.6**	**0.257**
BDNF	■	**101.6**	**18.6**	**95.3–113.9**	**145.8**	**191.5**	**0.024***
BTC	■	**304.7**	**49.3**	**273.3–322.6**	**354.0**	**256.7**	**0.045***
β-NGF	■	**206.4**	**169.2**	**119.1–288.2**	**339.6**	**550.56**	**0.117**
NT-3	■	**85.7**	**55.20**	**49.9–105.1**	**106.7**	**147.0**	**0.283**
Furin	■O	360.4	580.5	189.6–770.1	464.7	995.9	0.537
Galectin-3	■	1598.1	1419.0	859.2–2278.2	1069.9	4638.26	0.892
IGF-1	■	2005.4	574.4	1685.3–2259.6	2048.0	662.5	0.812
Insulin (*Ins1*)	■	**180.6**	**70.3**	**149.0–219.3**	**253.8**	**271.8**	**0.074**
SCF (*Kitl*)	■	346.7	287.7	230.9–518.6	419.4	631.8	0.326
SPP1	■⊙	**1209.4**	**1828.0**	**766.5–2594.4**	**3064.4**	**4333.1**	**0.007****
TGFβ	■♦	**706.2**	**122.0**	**640.7–762.7**	**919.0**	**600.0**	**0.013***
VEGF-C	■	250.6	100.1	209.6–309.7	233.7	312.5	0.641
BMP2	O	191.5	168.6	100.5–269.1	140.3	301.7	0.752
BMP4	O	116.1	90.1	72.4–162.5	106.0	173.1	0.845
BMP7	O	**196.5**	**211.8**	**156.2–232.0**	**246.0**	**221.5**	**0.104**
FasL	O	**290.1**	**159.6**	**203.1–362.7**	**967.0**	**1484.7**	**0.001****
Follistatin (*Fst*)	O	**920.6**	**286.0**	**754.3–1040.3**	**1683.0**	**1192.8**	**0.007****
MMP8	O⊙	**319.7**	**187.2**	**219.5–406.7**	**416.7**	**1319.90**	**0.026***
MMP-9	⊙	**156.4**	**126.2**	**110.8–237.0**	**244.8**	**1378.6**	**0.125**

*Bold type highlights analytes with median concentration in CSF from preterm infants that was outside the IQR of analyte concentration from controls; *$p < 0.05$, **$p < 0.01$. ⊙Pro-inflammatory function; ♦ anti-inflammatory function; ■supporting oligodendrocyte lineage responses and myelination; O impairing oligodendrocyte lineage responses and myelination.*

Mapping of Dynamic Regulation of Inflammatory and Myelination-Associated Proteins During Experimental Developmental Brain Injury

To better understand how the proteins elevated in preterm infant CSF are regulated after injury, we mined an existing dataset in which dynamic changes in microglia gene expression were measured in an experimental model of developmental white matter injury. In this model, damage was induced by intraperitoneal injections of recombinant IL-1β (10 μg/ml) prior to the onset of myelination (twice daily from postnatal day [P]1 to P4 and one injection at P5) (**Figure 1A**). This paradigm mimics the pathophysiology of human perinatal brain injury by chronically impairing oligodendrocyte differentiation and myelination, as evidenced by immunostaining of myelin proteins, the oligodendrocyte lineage and electron microscopy (Favrais et al., 2011; Krishnan et al., 2017).

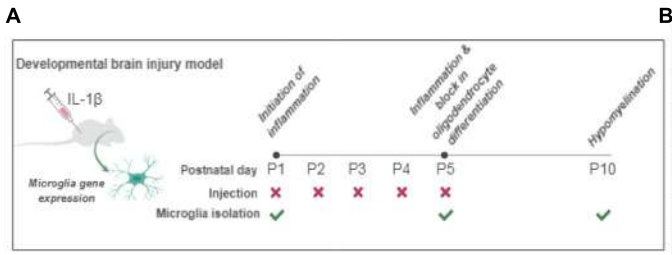
We analyzed microglia gene expression at the time of inflammation initiation (P1), during the subsequent phase when oligodendrocyte differentiation is impaired (P5), and when hypomyelination is observed (P10). Eleven of 15 proteins we found to be altered in human preterm CSF were significantly regulated at the mRNA level by microglia during the course of white matter injury (**Figure 1B**). At P1, microglia from IL-1β-treated mice showed an upregulation of pro-inflammatory genes *Il1b* and *Tnf* and concomitant downregulation of anti-inflammatory gene *Il4* (**Figure 1C**). *Tgfb1*, which supports oligodendrocyte lineage survival, proliferation, and differentiation (Dutta et al., 2014; Palazuelos et al., 2014), was upregulated simultaneously with *Mmp8*, which is associated with myelin damage (Folgueras et al., 2008; **Figure 1C**). At P5, microglia upregulated pro-inflammatory genes C5a (*Hc*), *Il1b*, *Il2*, *Il17a*, and showed a sustained downregulation of *Il4* (**Figure 1D**). Also upregulated were genes predicted to inhibit oligodendrocyte health and differentiation: *Mmp8* (Folgueras et al., 2008), *Fasl* (associated with oligodendrocyte death) (Wosik et al., 2003), and *Fst* (which would inhibit activin-A-driven oligodendrocyte differentiation) (Dillenburg et al., 2018; **Figure 1D**). At P10, *Il1b*, *Tnf*, and *Fasl* were still upregulated, however, concomitant upregulation of *Il4* and pro-myelination gene *Spp1* (Osteopontin) may indicate late attempts to resolve inflammation and counter white matter damage (**Figure 1E**). *Il1b* and *Il4*, prototypical pro- and anti-inflammatory cytokines respectively, were found to be regulated in expression throughout injury, with a sustained upregulation of *Il1b* and a delayed *Il4* response (**Figure 1F**). With regards to genes regulating myelination, although pro-survival/myelination genes *Tgfb1* and *Spp1* were slightly upregulated early and late in injury, respectively, genes whose products are predicted to impair white matter health were upregulated at all time points (**Figure 1G**). This data suggest that the proteins enriched in human preterm CSF are dynamically expressed by microglia following developing white matter insult, with an initial and sustained pro-inflammatory response followed by a delayed anti-inflammatory response, and a continuous expression of genes predicted to inhibit healthy myelination.

DISCUSSION

In this study, we identified a distinct inflammatory signature in preterm CSF relative to term controls. A comprehensive and sensitive measure of CSF protein levels by antibody array identified 15 factors which were relatively increased in preterm samples, and which have been previously associated with regulating inflammation and myelination. This revealed a complex preterm neural environment, with concurrent pro- and anti-inflammatory responses, and pro- and anti-myelination factors. Data-mining of microglia transcriptomes in the context of experimental perinatal brain injury revealed that microglia can express the majority of these factors following insult, and they are dynamically regulated over time, mirroring the complexity of inflammatory and myelination-regulating factors measured in the human samples. Although some of these genes are expressed by microglia in healthy developing brain (*Il1b, Ccl3, Tnf, Spp1,* and *Tgfb1*) (Zhang et al., 2014), the rapid increase of pro-inflammatory gene expression in microglia in this experimental model shortly after the first injection of IL-1β, concomitant with sustained expression of genes predicted to impair myelination, highlight the importance of early intervention to limit damage to the developing white matter.

Five proteins were increased in preterm CSF vs. controls at the threshold $p < 0.01$: C5a, IL-9, SPP1, FasL, and Follistatin. C5a is a component of the complement cascade which we previously showed to be increased in human preterm CSF by enzyme-linked immunoabsorbant assay (Pataky et al., 2017), validating our novel approach of using antibody array to identify differentially expressed proteins in CSF samples. Although C5a has been associated with normal brain development (Benard et al., 2008) and neuroprotection (Biggins et al., 2017), it may have damaging functions as inhibition of its receptor C5aR attenuates excitotoxic perinatal brain injury (Pedroni et al., 2013) and C5a is increased in the CSF of children with demyelinating disease (Horellou et al., 2015). In addition, we identified IL-9 as a novel preterm birth-associated CNS cytokine in humans. Although we found it is not regulated by microglia in the IL-1β injury model, it may be expressed by other cell types such as Th9 lymphocytes. In experimental models it has roles in mast cell activation and excitotoxicity (Patkai et al., 2001), neonatal cortical neuronal apoptosis (Fontaine et al., 2008), autoimmune demyelination (Li et al., 2011), and regulation of astrocyte chemokine production (Ding et al., 2015); our data support a role for IL9 in the human inflammatory response to preterm birth. In addition, its expression can be driven by TGFβ (Beriou et al., 2010), which when overexpressed by microglia is associated with hypomyelination (Nobuta et al., 2012). IL-9 may also have direct actions on oligodendrocyte lineage cells, as these express the IL-9 receptor and IL-9 treatment inhibits their differentiation *in vitro*, although, notably, it can encourage differentiation if co-supplied with IFN-γ (Ding et al., 2015).

The remaining three highly enriched proteins have been implicated in regulating the oligodendrocyte lineage and myelination. Osteopontin (SPP1) in particular has been suggested as a blood biomarker for neonatal encephalopathy (Graham et al., 2018) and it is highly induced by hypoxic



B

Gene	Function	P1		P5		P10	
		Fold change	p value	Fold change	p value	Fold change	p value
Hc	⊙	1.200	0.486	5.784	**0.00461****	1.321	0.471
Il1b	⊙O	2.082	**0.000008*****	1.583	**0.0000004*****	2.068	**0.00531****
Il2	⊙	-1.008	0.322	2.948	**0.0124***	-1.094	0.552
Il9	⊙O■	1.052	0.287	1.102	0.472	-1.084	0.353
Il17a	⊙■	-1.000	0.890	2.419	**0.0133***	-1.002	0.371
Ccl3	⊙	3.698	0.579	-1.322	0.677	1.194	0.482
Tnf	⊙■O	2.004	**0.0000879*****	-1.572	**0.0000103*****	1.847	**0.000536*****
Il4	◆	-1.786	**0.00992****	-1.633	**0.00111****	1.434	**0.00258****
Bdnf	■	-1.137	0.959	-1.001	0.974	1.23	0.161
Btc	■	-1.002	0.886	-1.003	0.472	1.004	0.417
Spp1	■⊙	1.061	0.406	-1.091	0.539	2.273	0.0107
Tgfb1	■◆	1.116	**0.00464****	1.099	0.109	1.077	0.128
Fasl	O	1.004	0.705	3.031	**0.000581*****	4.020	**0.00676****
Fst	O	-1.116	0.431	2.590	**0.00772****	1.545	0.259
Mmp8	O⊙	2.173	**0.000324*****	47.980	**<1x10^{-5}*****	2.203	0.927

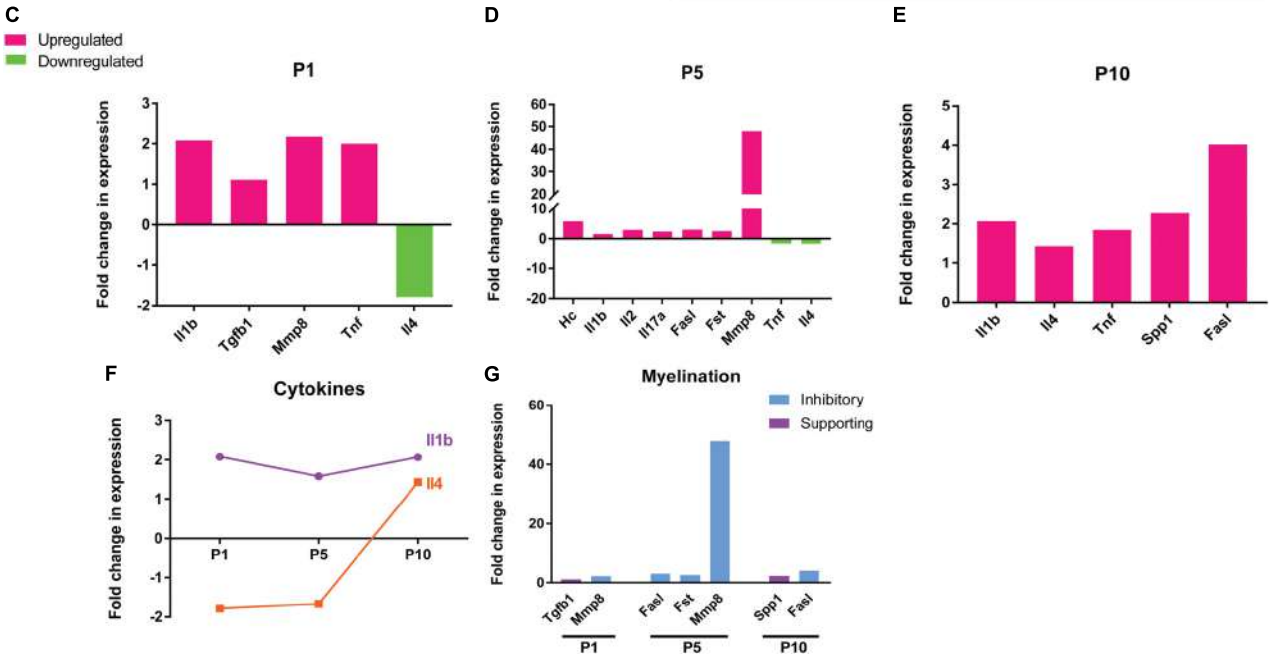


FIGURE 1 | Dynamic expression of human preterm CSF signature proteins at the gene expression level in microglia following experimental brain injury. **(A)** Mouse model of developmental white matter injury. **(B)** Fold change in gene expression in microglia isolated from IL-1β mouse model of developmental white matter injury, values at postnatal (P) day 1, 5, and 10 indicated, with *p*-values **p*<0.05, ***p* < 0.01, and ****p* < 0.001; ⊙ pro-inflammatory function, ◆ anti-inflammatory function, ■ supporting oligodendrocyte lineage responses and myelination, O impairing oligodendrocyte lineage responses and myelination. **(C–E)** Fold change in expression of genes in microglia isolated from IL-1β injury model compared to vehicle control, at postnatal days (P) 1, 5, and 10. Significantly upregulated genes shown in magenta, significantly downregulated genes shown in green. **(F)** Dynamic regulation of *Il1b* and *Il4* over the course of injury. **(G)** Fold change in expression over vehicle control for genes associated with impairing myelination (blue) and those supporting myelination (purple).

injury, where a protective role is suggested by decreased oligodendrogenesis in a knockout mouse subjected to hypoxic-ischemic injury (van Velthoven et al., 2011). This beneficial role may be dependent on mode of neural injury or age, as in adult mice SPP1 exacerbates autoimmune-mediated demyelination and is not required for regeneration of myelin on previously myelinated axons (Zhao et al., 2008). Nonetheless, it can directly increase myelin protein expression and myelination *in vitro* (Selvaraju et al., 2004). Another protein we detected in preterm CSF which may directly modulate myelination is follistatin, which sequesters activin-A to prevent its binding to activin receptors. This would be predicted to impair myelination, as we have recently shown these receptors to be required for oligodendrocyte differentiation and myelin maturation in healthy white matter development and following injury (Dillenburg et al., 2018). Lastly, the increase in Fas ligand in preterm CSF may indicate direct targeting of oligodendrocytes, as it has been associated with induction of oligodendrocyte death in a variety of neurological disorders (Austin and Fehlings, 2008).

Our study has some limitations. Although no infant in the study group had meningitis, a proportion of preterm and term infants had BSI at the time of CSF sampling. Therefore, it is possible that systemic inflammation contributed to observed alterations in the CSF inflammatory profile, although this potential confounding effect is likely to be balanced across the groups.

Our study was not designed to investigate the effect of astrocyte-mediated cytokine production, which could contribute to neuroinflammation as has been suggested by some experimental perinatal white matter injury models

(Nobuta et al., 2012; Shiow et al., 2017). In future work, investigating gene expression in other cell types including astrocytes and neurones may be informative.

Altogether, these findings identify a CSF signature in response to preterm birth, which reflects a complex environment that can both drive injury and support myelination. The pathological outcome of preterm birth may thus reflect a balance between damaging and reparative factors, which implies that effective therapies may need to operate on multiple targets. We propose that early therapeutic intervention could pre-empt the robust pro-inflammatory response and boost pro-repair mechanisms to support healthy myelination.

DATA AVAILABILITY

All human CSF data generated or analyzed during this study are included in this published article. The microglia gene expression datasets analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

JB co-designed the study, analyzed the patient data, and contributed to writing the manuscript. GI and GS analyzed the patient samples. BF and PG co-designed the study, carried out the animal experiments, and generated the microglial gene expression dataset. RP collected the human CSF samples. VM co-designed the study, analyzed the microglia gene expression dataset, and co-wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by Theirworld (www.theirworld.org), the MRC Centre for Reproductive Health (MRC G1002033), and a Career Development Award from the Medical Research Council (V.E.M.; MR/M020827/1). The funding body had no role in the design of the study or sample collection, analysis, and interpretation of data, or in writing the manuscript.

ACKNOWLEDGMENTS

The authors are grateful to the parents and carers who consented to take part in the study, and to clinical colleagues at the Neonatal Intensive Care Unit of the Royal Infirmary of Edinburgh for referring patients. The diagram in **Figure 1A** was created with BioRender.

REFERENCES

Anblagan, D., Pataky, R., Evans, M. J., Telford, E. J., Serag, A., Sparrow, S., et al. (2016). Association between preterm brain injury and exposure to chorioamnionitis during fetal life. *Sci. Rep.* 6:37932. doi: 10.1038/srep37932

Armstrong-Wells, J., Donnelly, M., Post, M. D., Manco-Johnson, M. J., Winn, V. D., and Sébire, G. (2015). Inflammatory predictors of neurologic disability after preterm premature rupture of membranes. *Am. J. Obstet. Gynecol.* 212, 212.e1–212.e9. doi: 10.1016/j.ajog.2014.09.016

Austin, J. W., and Fehlings, M. G. (2008). Molecular mechanisms of fas-mediated cell death in oligodendrocytes. *J. Neurotrauma* 25, 411–426. doi: 10.1089/neu.2007.0436

Back, S. A., and Miller, S. P. (2014). Brain injury in premature neonates: a primary cerebral dysmaturation disorder? *Ann. Neurol.* 75, 469–486. doi: 10.1002/ana.24132

Barnett, M. L., Tusor, N., Ball, G., Chew, A., Falconer, S., Aljabar, P., et al. (2018). Exploring the multiple-hit hypothesis of preterm white matter damage using diffusion MRI. *Neuroimage Clin.* 17, 596–606. doi: 10.1016/j.nicl.2017.11.017

Bartha, A. I., Foster-Barber, A., Miller, S. P., Vigneron, D. B., Glidden, D. V., Barkovich, A. J., et al. (2004). Neonatal encephalopathy: association of cytokines with MR spectroscopy and outcome. *Pediatr. Res.* 56, 960–966. doi: 10.1203/01.Pdr.0000144819.45689.Bb

Basu, S., Agarwal, P., Anupurba, S., Shukla, R., and Kumar, A. (2015). Elevated plasma and cerebrospinal fluid interleukin-1 beta and tumor necrosis factor-alpha concentration and combined outcome of death or abnormal neuroimaging in preterm neonates with early-onset clinical sepsis. *J. Perinatol.* 35, 855–861. doi: 10.1038/jp.2015.86

Batalle, D., Hughes, E. J., Zhang, H., Tournier, J. D., Tusor, N., Aljabar, P., et al. (2017). Early development of structural networks and the impact of prematurity on brain connectivity. *Neuroimage* 149, 379–392. doi: 10.1016/j.neuroimage.2017.01.065

Benard, M., Raoult, E., Vaudry, D., Leprince, J., Falluel-Morel, A., Gonzalez, B. J., et al. (2008). Role of complement anaphylatoxin receptors (C3aR, C5aR) in the development of the rat cerebellum. *Mol. Immunol.* 45, 3767–3774. doi: 10.1016/j.molimm.2008.05.027

Beriou, G., Bradshaw, E. M., Lozano, E., Costantino, C. M., Hastings, W. D., Orban, T., et al. (2010). TGF-beta induces IL-9 production from human Th17 cells. *J. Immunol.* 185, 46–54. doi: 10.4049/jimmunol.1000356

Biggins, P. J. C., Brennan, F. H., Taylor, S. M., Woodruff, T. M., and Ruitenberg, M. J. (2017). The alternative receptor for complement component 5a, C5aR2, conveys neuroprotection in traumatic spinal cord injury. *J. Neurotrauma* 34, 2075–2085. doi: 10.1089/neu.2016.4701

Billiards, S. S., Haynes, R. L., Folkerth, R. D., Borenstein, N. S., Trachtenberg, F. L., Rowitch, D. H., et al. (2008). Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia. *Brain Pathol.* 18, 153–163. doi: 10.1111/j.1750-3639.2007.00107.x

Caldinelli, C., Froudist-Walsh, S., Karolis, V., Tseng, C. E., Allin, M. P., Walshe, M., et al. (2017). White matter alterations to cingulum and fornix following very preterm birth and their relationship with cognitive functions. *Neuroimage* 150, 373–382. doi: 10.1016/j.neuroimage.2017.02.026

Carlo, W. A., McDonald, S. A., Tyson, J. E., Stoll, B. J., Ehrenkranz, R. A., Shankaran, S., et al. (2011). Cytokines and neurodevelopmental outcomes in extremely low birth weight infants. *J. Pediatr.* 159, 919.e3–925.e3. doi: 10.1016/j.jpeds.2011.05.042

Chhor, V., Le Charpentier, T., Lebon, S., Oré, M. V., Celador, I. L., Josserand, J., et al. (2013). Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. *Brain Behav. Immun.* 32, 70–85. doi: 10.1016/j.bbi.2013.02.005

Dillenburg, A., Ireland, G., Holloway, R. K., Davies, C. L., Evans, F. L., Swire, M., et al. (2018). Activin receptors regulate the oligodendrocyte lineage in health and disease. *Acta Neuropathol.* 135, 887–906. doi: 10.1007/s00401-018-1813-3

Ding, X., Cao, F., Cui, L., Ciric, B., Zhang, G. X., and Rostami, A. (2015). IL-9 signaling affects central nervous system resident cells during inflammatory stimuli. *Exp. Mol. Pathol.* 99, 570–574. doi: 10.1016/j.yexmp.2015.07.010

Dutta, D. J., Zameer, A., Mariani, J. N., Zhang, J., Asp, L., Huynh, J., et al. (2014). Combinatorial actions of Tgfbeta and activin ligands promote oligodendrocyte development and CNS myelination. *Development* 141, 2414–2428. doi: 10.1242/dev.106492

Ellison, V. J., Mocatta, T. J., Winterbourn, C. C., Darlow, B. A., Volpe, J. J., and Inder, T. E. (2005). The relationship of CSF and plasma cytokine levels to cerebral white matter injury in the premature newborn. *Pediatr. Res.* 57, 282–286. doi: 10.1203/01.pdr.0000148286.53572.95

Favrais, G., van de Looij, Y., Fleiss, B., Ramanantsoa, N., Bonnin, P., Stoltenburg-Didinger, G., et al. (2011). Systemic inflammation disrupts the developmental program of white matter. *Ann. Neurol.* 70, 550–565. doi: 10.1002/ana.22489

Folgueras, A. R., Fueyo, A., García-Suárez, O., Cox, J., Astudillo, A., Tortorella, P., et al. (2008). Collagenase-2 deficiency or inhibition impairs experimental autoimmune encephalomyelitis in mice. *J. Biol. Chem.* 283, 9465–9474. doi: 10.1074/jbc.M709522200

Fontaine, R. H., Cases, O., Lelièvre, V., Mesplès, B., Renauld, J. C., Loron, G., et al. (2008). IL-9/IL-9 receptor signaling selectively protects cortical neurons against developmental apoptosis. *Cell Death Differ.* 15, 1542–1552. doi: 10.1038/cdd.2008.79

Graham, E. M., Everett, A. D., Delpech, J. C., and Northington, F. J. (2018). Blood biomarkers for evaluation of perinatal encephalopathy: state of the art. *Curr. Opin. Pediatr.* 30, 199–203. doi: 10.1097/mop.0000000000000591

Gustavsson, M., Mallard, C., Vannucci, S. J., Wilson, M. A., Johnston, M. V., and Hagberg, H. (2007). Vascular response to hypoxic preconditioning in the immature brain. *J. Cereb. Blood Flow Metab.* 27, 928–938. doi: 10.1038/sj.jcbfm.9600408

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Haynes, R. L., Folkerth, R. D., Trachtenberg, F. L., Volpe, J. J., and Kinney, H. C. (2009). Nitrosative stress and inducible nitric oxide synthase expression in periventricular leukomalacia. *Acta Neuropathol.* 118, 391–399. doi: 10.1007/s00401-009-0540-1

Horellou, P., Wang, M., Keo, V., Chrétien, P., Serguera, C., Waters, P., et al. (2015). Increased interleukin-6 correlates with myelin oligodendrocyte glycoprotein antibodies in pediatric monophasic demyelinating diseases and multiple sclerosis. *J. Neuroimmunol.* 289, 1–7. doi: 10.1016/j.jneuroim.2015.10.002

Husson, I., Rangon, C. M., Lelièvre, V., Bemelmans, A. P., Sachs, P., Mallet, J., et al. (2005). BDNF-induced white matter neuroprotection and stage-dependent neuronal survival following a neonatal excitotoxic challenge. *Cereb. Cortex* 15, 250–261. doi: 10.1093/cercor/bhh127

Johnson, S., and Marlow, N. (2017). Early and long-term outcome of infants born extremely preterm. *Arch. Dis. Child.* 102, 97–102. doi: 10.1136/archdischild-2015-309581

Krishnan, M. L., Van Steenwinckel, J., Schang, A. L., Yan, J., Arnadottir, J., Le Charpentier, T., et al. (2017). Integrative genomics of microglia implicates DLG4 (PSD95) in the white matter development of preterm infants. *Nat. Commun.* 8:428. doi: 10.1038/s41467-017-00422-w

Li, H., Nourbakhsh, B., Cullimore, M., Zhang, G. X., and Rostami, A. (2011). IL-9 is important for T-cell activation and differentiation in autoimmune inflammation of the central nervous system. *Eur. J. Immunol.* 41, 2197–2206. doi: 10.1002/eji.201041125

Nelson, K. B., Dambrosia, J. M., Grether, J. K., and Phillips, T. M. (1998). Neonatal cytokines and coagulation factors in children with cerebral palsy. *Ann. Neurol.* 44, 665–675. doi: 10.1002/ana.410440413

Nobuta, H., Ghiani, C. A., Paez, P. M., Spreuer, V., Dong, H., Korsak, R. A., et al. (2012). STAT3-mediated astrogliosis protects myelin development in neonatal brain injury. *Ann. Neurol.* 72, 750–765. doi: 10.1002/ana.23670

O'Driscoll, D. N., McGovern, M., Greene, C. M., and Molloy, E. J. (2018). Gender disparities in preterm neonatal outcomes. *Acta Paediatr.* doi: 10.1111/apa.14390 [Epub ahead of print].

Palazuelos, J., Klingener, M., and Aguirre, A. (2014). TGFbeta signaling regulates the timing of CNS myelination by modulating oligodendrocyte progenitor cell cycle exit through SMAD3/4/FoxO1/Sp1. *J. Neurosci.* 34, 7917–7930. doi: 10.1523/jneurosci.0363-14.2014

Pataky, R., Howie, F. A., Girardi, G., and Boardman, J. P. (2017). Complement C5a is present in CSF of human newborns and is elevated in association with preterm birth. *J. Matern. Fetal Neonatal Med.* 30, 2413–2416. doi: 10.1080/14767058.2016.1251896

Patkai, J., Mesples, B., Dommergues, M. A., Fromont, G., Thornton, E. M., Renauld, J. C., et al. (2001). Deleterious effects of IL-9-activated mast cells and neuroprotection by antihistamine drugs in the developing mouse brain. *Pediatr. Res.* 50, 222–230. doi: 10.1203/00006450-200108000-200108010

Pedroni, S. M., Gonzalez, J. M., Wade, J., Jansen, M. A., Serio, A., Marshall, I., et al. (2013). Complement inhibition and statins prevent fetal brain cortical abnormalities in a mouse model of preterm birth. *Biochim. Biophys. Acta* 1842, 107–115. doi: 10.1016/j.bbadis.2013.10.011

Rajkumar, R., Bhaya, B., Mamilla, D., Czech, T., Kisseih, E., Saini, A., et al. (2018). A preliminary evaluation of glial cell line-derived neurotrophic factor (GDNF) levels in cerebrospinal fluid across various gestational ages and clinical conditions of the neonate. *Int. J. Dev. Neurosci.* 65, 61–65. doi: 10.1016/j.ijdevneu.2017.10.001

Savman, K., Blennow, M., Gustafson, K., Tarkowski, E., and Hagberg, H. (1998). Cytokine response in cerebrospinal fluid after birth asphyxia. *Pediatr. Res.* 43, 746–751. doi: 10.1203/00006450-199806000-00006

Schang, A. L., Van Steenwinckel, J., Chevenne, D., Alkmark, M., Hagberg, H., Gressens, P., et al. (2014). Failure of thyroid hormone treatment to prevent inflammation-induced white matter injury in the immature brain. *Brain. Behav. Immun.* 37, 95–102. doi: 10.1016/j.bbi.2013.11.005

Selvaraju, R., Bernasconi, L., Losberger, C., Graber, P., Kadi, L., Avellana-Adalid, V., et al. (2004). Osteopontin is upregulated during in vivo demyelination and remyelination and enhances myelin formation in vitro. *Mol. Cell. Neurosci.* 25, 707–721. doi: 10.1016/j.mcn.2003.12.014

Shah, D. K., Doyle, L. W., Anderson, P. J., Bear, M., Daley, A. J., Hunt, R. W., et al. (2008). Adverse neurodevelopment in preterm infants with postnatal sepsis or necrotizing enterocolitis is mediated by white matter abnormalities on magnetic resonance imaging at term. *J. Pediatr.* 153, 170.e1–175.e1. doi: 10.1016/j.jpeds.2008.02.033

Shiow, L. R., Favrais, G., Schirmer, L., Schang, A. L., Cipriani, S., Andres, C., et al. (2017). Reactive astrocyte COX2-PGE2 production inhibits oligodendrocyte maturation in neonatal white matter injury. *Glia* 65, 2024–2037. doi: 10.1002/glia.23212

Spittle, A. J., Walsh, J. M., Potter, C., Mcinnes, E., Olsen, J. E., Lee, K. J., et al. (2017). Neurobehaviour at term-equivalent age and neurodevelopmental outcomes at 2 years in infants born moderate-to-late preterm. *Dev. Med. Child Neurol.* 59, 207–215. doi: 10.1111/dmcn.13297

Telford, E. J., Cox, S. R., Fletcher-Watson, S., Anblagan, D., Sparrow, S., Pataky, R., et al. (2017). A latent measure explains substantial variance in white matter microstructure across the newborn human brain. *Brain Struct. Funct.* 222, 4023–4033. doi: 10.1007/s00429-017-1455-6

Van Steenwinckel, J., Schang, A. L., Krishnan, M. L., Degos, V., Delahaye-Duriez, A., Bokobza, C., et al. (2018). Loss of the Wnt/β-catenin pathway in microglia of the developing brain drives pro-inflammatory activation leading to white matter injury. *bioRxiv* [Preprint]. doi: 10.1101/334359

van Velthoven, C. T., Heijnen, C. J., van Bel, F., and Kavelaars, A. (2011). Osteopontin enhances endogenous repair after neonatal hypoxic-ischemic brain injury. *Stroke* 42, 2294–2301. doi: 10.1161/strokeaha.110.608315

Verney, C., Pogledic, I., Biran, V., Adle-Biassette, H., Fallet-Bianco, C., and Gressens, P. (2012). Microglial reaction in axonal crossroads is a hallmark of noncystic periventricular white matter injury in very preterm infants. *J. Neuropathol. Exp. Neurol.* 71, 251–264. doi: 10.1097/NEN.0b013e3182496429

Viscardi, R. M., Muhumuza, C. K., Rodriguez, A., Fairchild, K. D., Sun, C. C., Gross, G. W., et al. (2004). Inflammatory markers in intrauterine and fetal blood and cerebrospinal fluid compartments are associated with adverse pulmonary and neurologic outcomes in preterm infants. *Pediatr. Res.* 55, 1009–1017. doi: 10.1203/01.pdr.0000127015.60185.8a

Volpe, J. J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 8, 110–124. doi: 10.1016/S1474-4422(08)70294-1

Wosik, K., Antel, J., Kuhlmann, T., Brück, W., Massie, B., and Nalbantoglu, J. (2003). Oligodendrocyte injury in multiple sclerosis: a role for p53. *J. Neurochem.* 85, 635–644. doi: 10.1046/j.1471-4159.2003.01674.x

Yoon, B. H., Romero, R., Kim, C. J., Koo, J. N., Choe, G., Syn, H. C., et al. (1997). High expression of tumor necrosis factor-alpha and interleukin-6 in periventricular leukomalacia. *Am. J. Obstet. Gynecol.* 177, 406–411. doi: 10.1016/S0002-9378(97)70206-0

Yoon, B. H., Romero, R., Yang, S. H., Jun, J. K., Kim, I. O., Choi, J. H., et al. (1996). Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. *Am. J. Obstet. Gynecol.* 174, 1433–1440. doi: 10.1016/S0002-9378(96)70585-9

Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., et al. (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J. Neurosci.* 34, 11929–11947. doi: 10.1523/jneurosci.1860-14.2014

Zhao, C., Fancy, S. P., ffrench-Constant, C., and Franklin, R. J. (2008). Osteopontin is extensively expressed by macrophages following CNS demyelination but has a redundant role in remyelination. *Neurobiol. Dis.* 31, 209–217. doi: 10.1016/j.nbd.2008.04.007

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Boardman, Ireland, Sullivan, Pataky, Fleiss, Gressens and Miron. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 22 November 2018
doi: 10.3389/fphys.2018.01664

Supine vs. Prone Position With Turn of the Head Does Not Affect Cerebral Perfusion and Oxygenation in Stable Preterm Infants ≤32 Weeks Gestational Age

*Dietmar Spengler, Elisa Loewe and Martin F. Krause**

Department of Pediatrics, University Hospital Schleswig-Holstein, Kiel, Germany

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Max Berry,
University of Otago, New Zealand
Michael Stark,
Women's and Children's Hospital,
Australia

***Correspondence:**
Martin F. Krause
martin.krause@uksh.de

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *28 June 2018*
Accepted: *05 November 2018*
Published: *22 November 2018*

Citation:
Spengler D, Loewe E and Krause MF (2018) Supine vs. Prone Position With Turn of the Head Does Not Affect Cerebral Perfusion and Oxygenation in Stable Preterm Infants ≤32 Weeks Gestational Age. Front. Physiol. 9:1664. doi: 10.3389/fphys.2018.01664

Intraventricular hemorrhage (IVH) is a frequent major damage to the brain of premature babies ≤32 weeks gestational age, and its incidence (20–25%) has not significantly changed lately. Because of the intrinsic fragility of germinal matrix blood vessels, IVH occurs following disruption of subependymal mono-layer arteries and is generally attributed to ischemia-reperfusion alterations or venous congestion, which may be caused by turn of the head. Therefore, supine position with the head in a midline position is considered a standard position for preterm infants during their first days of life. We asked whether a change in body position (supine vs. prone) linked with a turn of the head by 90° in the prone position would change blood flow velocities and resistance indices in major cerebral arteries and veins of stable premature babies at two different time points (t0, day of life 2, vs. t1, day 9). Moreover, we assessed cerebral tissue oxygenation (cStO2) by near-infrared spectroscopy and determined correlations for changes in velocities and oxygenation. Twenty one premature infants [gestational age 30 (26–32) weeks] with sufficiently stable gas exchange and circulation were screened by ultrasonography and near-infrared spectroscopy. Peak systolic and end-diastolic blood flow velocities in the anterior cerebral arteries (29 ± 6 m/s vs. 28 ± 7 peak flow at t0, 36 ± 8 vs. 35 ± 7 at t1), the basilar artery, the right and the left internal carotid artery, and the great cerebral vein Galen (4.0 ± 0.8 m/s vs. 4.1 ± 1.0 maximum flow at t0, 4.4 ± 0.8 vs. 4.4 ± 1.0 at t1) did not show significant differences following change of body and head position. Also, there were no differences in $cStO_2$ (83 ± 7% vs. 84 ± 7 at t0, 76 ± 10 vs. 77 ± 11 at t1) and in vital signs such as heart rate and blood pressure. We conclude that change in body position with turn of the head in the prone position does not elicit significant alterations in cerebral blood flow velocities or in oxygenation of cerebral tissues. Maturational changes in arterial flow velocities and $cStO_2$ are not correlated. For this subgroup of premature infants at low risk of IVH our data do not support the concept of exclusive preterm infant care in supine position.

Keywords: cerebral blood flow velocity, regional cerebral oxygenation, body position, turn of head, near-infrared spectroscopy, intraventricular hemorrhage

INTRODUCTION

Intraventricular hemorrhage (IVH) is a frequent major damage to the brain of premature infants ≤32 weeks of gestational age (WGA)/<1500 g birthweight, and despite great advances in neonatal care its incidence (20–25%) has not significantly changed within the last two decades (Humberg et al., 2017).

Long-term neurodevelopmental outcome depends on the immaturity of the infant and the degree of parenchymal damage by IVH ranging from mild germinal matrix bleeding to widespread periventricular hemorrhagic infarction. Neurologic sequelae of IVH include all degrees of motor deficits, cognitive deficits, learning disabilities, and seizure disorders: in high-risk premature infants <1000 g birthweight Sherlock et al. (2005) found a cerebral palsy incidence of 6% in infants with grade I IVH, 24% with grade II IVH, and 100% with grade IV IVH at 8 years of age.

The risk of bleeding is inversely correlated to gestational age and may be attributed to the following three factors: vascular immaturity of the germinal matrix, deficient extravascular matrix, and cerebral blood flow disturbances (Inder et al., 2018). First, vascular immaturity bases on the observation that the germinal matrix blood vessels are composed of endothelial cells only and lack muscle tissue and collagen. This immature microvascular network contains large diameter blood vessels without arterial or venous specification, is deficient in tight junction proteins (e.g., claudin and zonula occludens protein-1), and continues to be subject to involution and remodeling until the 37th WGA (Ballabh et al., 2005; Anstrom et al., 2007). Then, the extravascular matrix is rich in fibrinolytic activity (in contrast to developmental shortfalls in platelet and coagulation factor function), but lacks direct contact with perivascular structures due to slow astrocytic development (Rao et al., 2016) making the germinal matrix a gelatinous friable tissue deficient of mesenchymal and glial elements. Finally and most important, perfusion disturbances such as fluctuating cerebral blood flow (e.g., ischemia-hyperperfusion), a pressure-passive state of the cerebral perfusion, and sudden increases in cerebral venous pressure make the premature infant subject to germinal matrix bleeding (Baburamani et al., 2012).

As the internal jugular veins are the major blood outflow tract of the brain, turn of the head associated compromise of the venous blood drainage might lead to venous congestion, increased intracranial pressure, reduced cerebral oxygenation, and ultimately germinal matrix IVH (de Bijl-Marcus et al., 2017). Therefore, the head midline position has been advocated for many years for preventing the occurrence or extension of IVH in routine neonatal care, but an updated Cochrane systematic review was unable to support this approach due to a lack of adequate studies (Romantsik et al., 2017). In routine care of the respiratory and hemodynamically stable mature infant, however, prone position is preferred for reasons of improved oxygenation (secondary to augmented functional residual capacity) (Bhat et al., 2003), saturation stability (Heimann et al., 2010), airway patency (Francois et al., 1992), drainage of oropharyngeal secretions (Pickens et al., 1989; Waisman, 2006), reduction in obstructive apnea (Heimann et al., 2010), and improved quality of sleep (Bhat et al., 2006).

Our aim was to study the impact of supine position with the head in a midline position vs. prone position with turn of the head by 90° on arterial peak and end-diastolic flow, on resistance indices in cerebral arteries, and maximum flow in cerebral veins because this practical approach to stable preterm infant nursing has not been investigated before. In addition, we assessed cerebral oxygen saturation by near-infrared spectroscopy to capture any compromise in blood supply by a supportive technique, and compared the results of two different times of examination [t0 on day 2 (median) vs. t1 on day 9].

MATERIALS AND METHODS

The detailed study protocol was approved by the institutional ethics commission of the medical faculty of the Christian-Albrechts-University, Kiel (reference number A 102/13), in accordance with the World Medical Association's Declaration of Helsinki. The parents or the legal guardians of the infants admitted to the neonatal intensive care unit of the university hospital gave their written informed consent that could be withdrawn any time. A total of 32 premature infants ≤32 weeks of gestational age with sufficiently stable oxygenation/circulation (two infants on invasive mechanical ventilation with low pressures and FiO_2 <0.3) in a calm resting state [grade 1–3 according to Prechtl (Cioni and Prechtl, 1990)] were recruited to avoid motion-related inaccuracies in data acquisition and sampling. Exclusion criteria following the initial screening were all conditions of low blood pressure [i.e., below the 95% confidence limit for systolic and diastolic blood pressure for age 2 and 9 days (Zubrow et al., 1995)] with or without the administration of catecholamines, large patent ductus arteriosus [defined by the following three criteria: LA:Ao-ratio of >1.6:1, ductal diameter > 2.0 mm, retrograde diastolic flow in descending Ao > 30% of forward flow (Skinner, 2000)], congenital heart disease, IVH ≥ grade 2 on an initial ultrasound examination immediately after birth, and all kinds of congenital malformations. Two infants of 27 WGA were diagnosed with unilateral IVH grade 1 on day 2 that was almost completely reabsorbed on day 9.

In each infant two sets of investigations were carried out on day 2 (median, range 1–3; t0) and on day 9 (8–11; t1) of life to capture any maturational changes in all measured parameters. 6/21 infants received their t0 evaluations on day 1. The cerebral tissue oxygen saturation ($cStO_2$) was assessed by the application of an infant frontal adhesive sensor to the Invos 5100C near-infrared spectroscopy device (Somanetics Corp., Troy, MI, United States). The $cStO_2$ sensor was always taped to the middle of the forehead with the connecting flexible hose running to the right side. To assess cerebral perfusion an Acuson X300 (Siemens Healthcare, Erlangen, Germany) connected to a 9 MHz sector transducer was used to examine the side up anterior cerebral artery (ACA), the basilar artery (BA), the right and the left carotid artery (RICA and LICA), and the great cerebral vein Galen (GCV, the largest vessel in the midline inferior to the

splenium of the corpus callosum) by sagittal and coronal sections performed via the great fontanelle as the acoustic window. For the Doppler measurements, the angle of insonation was kept <30° to reduce the error of velocity measurements and was manually corrected to a minimum (**Figure 1**). Color Doppler ultrasound was used to clearly identify the different blood vessels, and all recordings were cross-checked by two experienced ultrasound investigators (DS, EL). From the arterial flow profiles the peak systolic velocity (PSV) and the end-diastolic velocity (EDV) were identified, and a resistance index (RI) was calculated by PSV-EDV/PSV. Venous flow profiles of the GCV were measured in pairs using a sagittal and a coronal approach to correct for any bias in low flow venous patterns. For comparisons the maximum systolic velocities (MSV) were used to compensate for possible waveform fluctuations (Ikeda et al., 2015). The ultrasound examinations started from either the supine or the prone position without any head-up angulation, and within the examination time of 25–35 min the body position was changed (from supine with the head in a midline position to prone with a turn of the head to the right or left by 90°, or *vice versa*). The same side of head turning was used for the t0 and the t1 examinations.

We also assessed pCO_2 (mmHg) and hemoglobin concentrations (g/dl) in capillary blood prior to each ultrasound investigation.

For statistics we applied a paired t-test to compare the data obtained from different body positions and from different time windows by the use of Prism 5 software (GraphPad, La Jolla, CA, United States). Pearson correlation of PSV and NIRS as independently measured variables were calculated. A p-value <0.05 was considered statistically significant.

RESULTS

Of the 32 preterm infants, 2 were excluded after withdrawal of informed consent; the registration of the Doppler profiles were greatly disturbed by motion-associated artifacts in 1 infant; complete sets of Doppler ultrasound recording (i.e., missing t1 recordings) were not available in two infants; in six infants the acquisition of t1 parameters could not be accomplished because of back transport to the referring hospitals. The data presented here base on the examination of 21 infants with each infant serving as its own control.

Among these 21 infants 10 (48%) were girls and 11 (52%) were boys. The gestational age was 29 ± 2 weeks, and the average weight was 1211 ± 342 g. 19/21 (90%) infants had received antenatal steroids. None of the infants was diagnosed with perinatal asphyxia (defined by an umbilical artery pH <7.1, and a 5-min Apgar score <5), even though 4/21 (19%) were prenatally screened with an absent end-diastolic umbilical artery flow. The co-morbidities are listed on **Table 1**. As mentioned above only infants with sufficiently stable oxygenation ($SpO_2 \geq 92\%$, $FiO_2 \leq 0.25$) and circulation (blood pressure above lower 95% CL, capillary refill ≤2 s) were screened for inclusion to this study depending on the vote of the attending neonatologist. When starting $cStO_2$ recordings and Doppler ultrasound examinations, the saturation by pulsed oximetry was 96% ± 2 at t0 and 97% ± 1 at t1; the heart rate was 148 bpm ± 14 at t0 and 161 ± 14 at t1; the blood pressure was 53 ± 8 mmHg (systolic)/30 ± 6 (diastolic) at t0 and 59 ± 9/35 ± 7 at t1. The results of the blood gases were: pCO_2 39 ± 6 mm Hg at t0 and 40 ± 4 at t1; hemoglobin 17.2 ± 2.8 g/dl at t0 and 14.6 ± 2.5 at t1.

PSV, EDV, and RI from the four arteries investigated (ACA, BA, RICA, LICA) are shown in **Figure 2**. There were no statistically significant differences between supine and prone position at t0 and at t1 except of RI of ACA at t1 ($p < 0.05$, **Figure 2B**). In contrast PSV and EDV (and RI) differences of all 4 arteries between t0 and t1 suggest maturational changes and come along with an increase in blood pressure level.

Maximum systolic velocities of the GCV was obtained by the use of two different approaches (sagittal vs. coronal) to the anterior fontanel as the acoustic window yielding no differences between supine vs. prone position, t0 vs. t1, and sagittal vs. coronal (**Figure 3**).

Figure 4 displays the results of the $cStO_2$ recordings without differences between supine vs. prone position, however, between t0 and t1 recordings. The calculation of correlation of PSV/EDV and $cStO_2$ yielded values of $r = 0.38$ to -0.14 (not significant). Despite differences in physiologic stability in the infants with early t0 scans (i.e., day 1) the flow velocity and $cStO_2$ analyses of

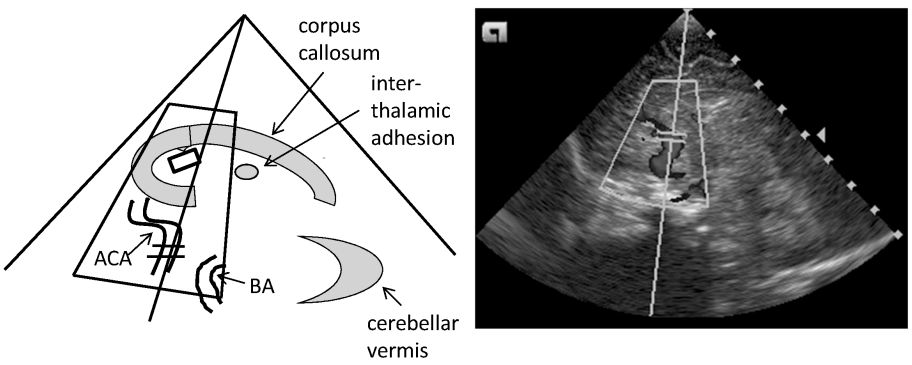


FIGURE 1 | Standard sagittal plane of the cerebral color Doppler ultrasound investigation performed through the anterior fontanel. This midline sagittal image shows the corpus callosum, the interthalamic adhesion, and the cerebellar vermis as reference structures. ACA, anterior cerebral artery; BA, basilar artery.

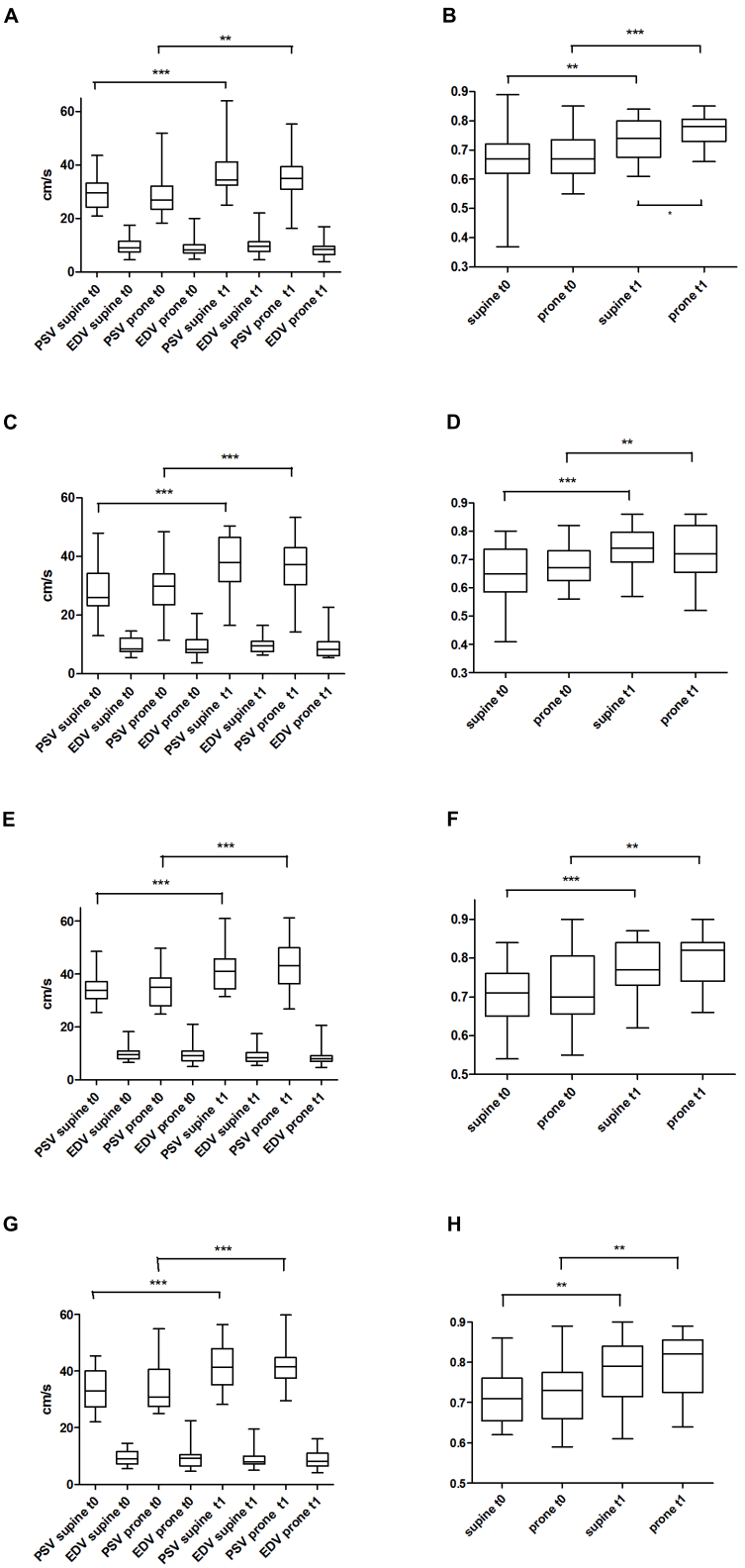


FIGURE 2 | Peak systolic velocity (PSV) and end-diastolic velocity (EDV) at two different times (t0, t1) and two different body positions (supine vs. prone) of the side up anterior cerebral artery (ACA) **(A)**; resistance indices of ACA **(B)**; PSV and EDV of the basilar artery (BA) **(C)**; resistance indices of BA **(D)**; PSV and EDV of the right internal carotid artery (RICA) **(E)**; resistance indices of RICA **(F)**; PSV and EDV of the left internal artery (LICA) **(G)**; resistance indices of LICA **(H)**. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

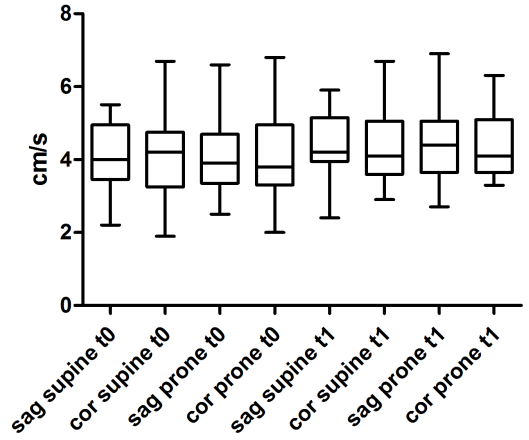


FIGURE 3 | Maximum systolic flow (MSV) of the great cerebral vein (GCV) using a sagittal (sag) and a coronal (cor) approach to the anterior fontanel as an acoustic window at two different times (t0, t1) and two different body positions (supine vs. prone).

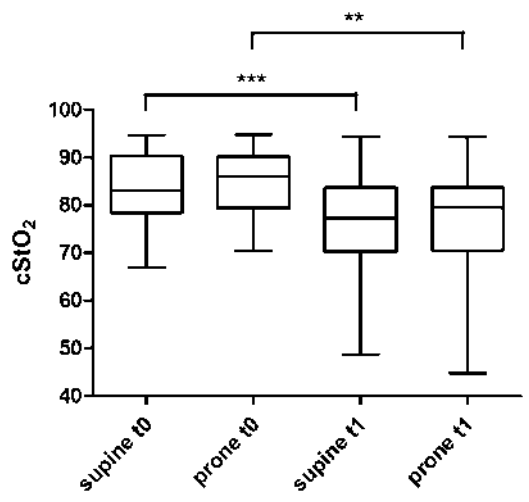


FIGURE 4 | $cStO_2$ (cerebral tissue oxygen saturation) recordings by NIRS technique from a frontal probe at two different times (t0, t1) and two different body positions (supine vs. prone). $^{**}p < 0.01$; $^{***}p < 0.001$.

this subgroup do not show significant differences when compared to the group examined on day 2 and 3 (data not shown).

DISCUSSION

Our data demonstrate that arterial and venous blood flow velocities do not depend on body position comparing supine with the head in a midline position vs. prone with a turn of the head by 90° position. In contrast we show that PSV and EDV are subject to maturational changes with increases in PSV (and RI) comparing t0 vs. t1. These changes were not observed in venous flow patterns which remained stable over time and were independent of the approach to the anterior fontanel as an acoustic window (sagittal vs. coronal). PSV and $cStO_2$ were not correlated and need to be interpreted individually.

TABLE 1 | Comorbidities of the study group.

	t0* (*n* = 21)	t1 (*n* = 21)
IVH grade I**	2 (9%)	2 (9%)
RDS***	18 (85%)	11 (52%)
Large PDA****	0	0
Apnea/bradycardia	12 (57%)	16 (76%)
Infection (antibiotic use)	15 (71%)	3 (14%)

*t0 examinations on day 2 (median, range 1–3); t1 examinations on day 9 (8–11). **IVH, intraventricular hemorrhage; infants with IVH ≥ grade II were excluded from the study. ***RDS, respiratory distress syndrome, mild to moderate; two infants received invasive mechanical ventilation at t0. ****Large PDA (defined by LA:Ao-ratio of >1.6:1, ductal diameter >2.0 mm, retrograde diastolic flow in descending Ao > 30% of forward flow).

It needs to be stressed that these findings concern a subgroup of premature infants with sufficient respiratory and hemodynamic stability and may not be generalized. Infants at greatest risk of IVH typically are <28 weeks gestational age, <1000 g body weight, in their first 3–5 days of life, on invasive mechanical ventilation, equipped with umbilical lines, diagnosed with PDA or other hemodynamic instabilities, treated with specific medication for PDA closure, and often times without antenatal corticosteroids administration. Explicitly, this kind of infants was not included into this study to avoid possible undesired side effects by changes in body and head position.

However, newer technologies such as assisted non-invasive ventilation and surfactant application by different LISA (less invasive surfactant application) maneuvers allow us to provide sufficient respiratory support in a growing high-risk subgroup of premature infants as described above not receiving invasive ventilation and not being equipped with umbilical lines. Nursing in prone position with turn of the head to stabilize respiratory function is therefore mandatory and carried out in our institution beyond 96 h of age also in this subgroup of premature infants given the fact that a Cochrane Systematic Review (Romantsik et al., 2017) could not show differences in germinal matrix IVH, severe IVH, and neonatal mortality comparing supine vs. prone position.

Our findings are in line with 6 studies (Lawson et al., 1987; Buckley et al., 2009; Ancora et al., 2010; Al-Abdi et al., 2011; Elser et al., 2012; Liao et al., 2015) investigating the effect of body position (including body/head elevation) or turn of the head on oxidative phosphorylation, cerebral blood flow velocities and $cStO_2$; however, the current study is the only study using both interventions simultaneously (i.e., change from supine to prone and from midline to turn of the head by 90° positions, or *vice versa*). The alteration of cerebral blood flow following turn of the head was studied by Lawson et al. (1987) in preterm and term infants using ^{31}phosphorus nuclear magnetic resonance spectroscopy. The phosphocreatine/inorganic phosphate ratio did not change regardless of head-turning or supine vs. prone body position in both cohorts. Buckley et al. (2009) studied body/head elevation by 12° in 4 premature infants of 26 WGA and found no significant change in PSV, EDV,

and diffuse correlation spectroscopy. They calculated a weak correlation (Pearson's r^2 = 0.13–0.58) of PSV/EDV and spectroscopy when performing body/head elevation. NIRS was used by Ancora et al. (2010) when studying 24 premature infants ≤32 WGA in six consecutive positions: supine/neutral head/bed 0°, supine/head-turning 90°/bed 0°, supine/head-turning 90°/bed + 30°, supine/neutral head/bed + 30°, prone/head minimally turned/bed + 30°, prone/head minimally turned/bed 0°. Changes in tissue hemoglobin/tissue oxygenation indices were not observed. Al-Abdi et al. (2011) assessed the incidence of IVH comparing preterm infants positioned supine with the head either in a neutral or turn of the head by 90° position and described a similar incidence (26% vs. 20%). Cerebral oxygen saturation was studied by Elser et al (Elser et al., 2012) in six different body positions involving supine/lateral/prone positions, body elevation by 15°, and turn of the head by 15–45° without any change in oxygenation (range 69–76%). Finally, Liao et al. (2015) assessed cerebral oxygen saturations in elevated supine position by 30° changing the head position several times from midline to the left/right 90° head-turning position. The range in average saturation (71–75%) was even closer as in the previous publication.

Fluctuations in the venous perfusion waveform of the GCV appear as a strong predictor of IVH in preterm infants. Ikeda et al. (2015) categorized four different GCV flow patterns in 80 VLBW infants that were repeatedly assessed over the first 6 days of life: grade 0: steady flow, constant perfusion speed; grade 1: fluctuating flow, minimum speed never less than half maximum speed; grade 2: fluctuating flow, minimum speed less than half maximum speed; grade 3: fluctuating flow, speed eventually dropping to 0 cm/s. They showed that higher grades mainly occur between days 2–4 of life which duplicates the postnatal time window of IVH occurrence; they gradually disappear beyond 4 days of life for reasons not clearly identified. Thus, the minor flow fluctuations in our recordings (grade 0 and 1 only) underline the low risk of IVH in our cohort of infants. Increased flow fluctuations in the GCV have been also described in growth retarded fetuses and are associated with an increased risk of perinatal morbidity, especially if combined with a reduction in cerebral vascular resistance and an overall increment in brain blood flow (Figueroa-Diesel et al., 2008).

The perception of head-turning related increase in intracranial pressure (ICP) and reduced cerebral perfusion by a hampered venous drainage was pursued as early as the 70th of the last century using techniques being replaced nowadays. Watson (1974) demonstrated in 1974 the complete obstruction of the ipsilateral internal jugular vein following turn of the head by 90° in anesthetized and non-anesthetized children using a contrast medium directly injected into the jugular vein. Goldberg et al. (1983) then were first to study position-related changes in ICP in preterm infants applying a Ladd monitor probe attached to the anterior fontanelle. They described a reduction [from 10.0 ± 1.2 (SEM) cm H_2O to 6.9 ± 1.2, ns] after switching the head from a right lateral to a midline position; this effect, however, was mildly augmented in a subgroup of infants starting with a higher baseline level ICP (i.e., ≥7 cm H_2O; from 11.9 ± 1.5 cm H_2O to 10.0 ± 1.6, $p < 0.01$). Using the same ICP measurement technique Emery and Peabody (1983) also observed an increase in ICP by head turning [from 13.8 ± 2.5 (SEM) cm H_2O to 17.2 ± 2.1, $p < 0.01$] along with unchanged PI (blood flow velocity parameters not provided), as was described by Urlesberger et al. (1991) in preterm infants with posthemorrhagic hydrocephalus following cancelation of head/body elevation to a horizontal position. It remains questionable whether position-related changes in ICP present a valid risk parameter for IVH as ICP depends on a manifold of factors such as cardiac output, blood pressure, cerebral perfusion, compliance of the infant skull, cerebrospinal fluid drainage, carbon dioxide level, state of vigilance, body position, stress, and gestational age (to name a few). An emerging topic is the vulnerability of the blood-brain barrier in the preterm infant following perinatal perfusion disturbances with the consequences of a delayed enwrapping of astrocytes in the germinal matrix and a reduced expression of platelet-derived growth factor beta as a key player in angiogenesis (Lai et al., 2017).

As a quality control, we compared our arterial blood flow velocity data with data from Forster et al. (2018) assessing term neonates within their first 3 days of life and found an overall consistency: ACA PSV 36.3 ± 6.6 cm/s (our data: 29.6 ± 6.2 at t0/36.8 ± 8.5 at t1); ACA EDV 12.4 ± 3.9 cm/s (9.3 ± 2.8 at t0/8.9 ± 3.3 at t1); RI 0.67 ± 0.06 (0.66 ± 0.09 at t0/0.68 ± 0.08 at t1). GCV data had to be compared to the data from Figueroa-Diesel et al. (2008) studying growth restricted fetuses: maximum flow 8.2 ± 5.5 cm/s (our data: sagittal 4.0 ± 0.8, coronal 4.1 ± 1.0 at t0). For the comparison of $cStO_2$ data Fuchs et al. (2011) demonstrated a rapid rise in regional cerebral oxygenation values in preterm infants, starting at values of 35% immediately after birth and peaking within 7–10 min at 75% (our data: 83 ± 7% at t0, 76 ± 10 at t1). The slightly lower data measured in term infants may occur due to a lower oxygen extraction in the brain, a different compartment of the arterial and the venous compartment, and the registration of more central parts of the brain in preterm infants (Almaazmi et al., 2013). In addition, different devices use different types of probes to be attached to the infant's forehead and use different algorithms for data computation. Lower $cStO_2$ values of the t1 recordings may also have occurred secondary to an improved oxygen delivery to the brain parenchyma and an increased systemic blood flow (cardiac output) typically seen in neonatal transition (Vrancken et al., 2018).

There are limitations to this study concerning the small number of study patients included, the high percentage of patients excluded because of incomplete data acquisition, and the small number of extremely low birth weight infants (i.e., birthweight <1000 g, 6/21 = 28%). Around 90% of the infants received antenatal steroids, a rate in line with international publications describing large cohorts (Stichtenoth et al., 2012; Schindler et al., 2017). Major IVH is clearly linked with low gestational age, birth asphyxia, and missed administration of prenatal steroids. Indeed, our cohort of infants does not represent this subgroup of infants at highest risk for IVH.

Taken together, our data demonstrate that the change from supine with the head in a midline position to prone with turn of the head by 90° position does not affect arterial or venous cerebral blood flow velocities or regional cerebral oxygenation in stable preterm infants ≤32 weeks gestational age on day 2 or day 9 of life. Cerebral blood flow and regional oxygenation are not correlated probably due to transitional hemodynamic changes, however, blood flow and regional oxygenation show strong maturational changes. Our data do not support the widespread clinical practice to nurse preterm infants in supine position with midline head position solely. Even though our findings must not be generalized for infants at high risk of IVH, a growing number of premature infants may profit by less invasive treatment protocols requiring changes in body and head position.

AUTHOR CONTRIBUTIONS

DS and EL performed all Doppler ultrasound examinations and supervised each other. EL collected the clinical data and performed the statistics. DS and MK designed the study. MK wrote the first draft of the manuscript. All authors approved the final manuscript.

REFERENCES

Al-Abdi, S. Y., Nojoom, M. S., Alshaalan, H. M., and Al-Aamri, M. A. (2011). Pilot-randomized study on intraventricular hemorrhage with midline versus lateral head positions. *Saudi Med. J.* 32, 420–421.

Almaazmi, M., Schmid, M. B., Havers, S., Reister, F., Lindner, W., Mayer, B., et al. (2013). Cerebral near-infrared spectroscopy during transition of healthy term newborns. *Neonatology* 103, 246–251. doi: 10.1159/000345926

Ancora, G., Maranella, E., Aceti, A., Pierantoni, L., Grandi, S., Corvaglia, L., et al. (2010). Effect of posture on brain hemodynamics in preterm newborns not mechanically ventilated. *Neonatology* 97, 212–217. doi: 10.1159/000253149

Anstrom, J. A., Thore, C. R., Moody, D. M., and Brown, W. R. (2007). Immunolocalization of tight junction proteins in blood vessels in human germinal matrix and cortex. *Histochem. Cell Biol.* 127, 205–213. doi: 10.1007/s00418-006-0232-z

Baburamani, A. A., Ek, C. J., Walker, D. W., and Castillo-Melendez, M. (2012). Vulnerability of the developing brain to hypoxic-ischemic damage: contribution of the cerebral vasculature to injury and repair? *Front. Physiol.* 3:424. doi: 10.3389/fphys.2012.00424

Ballabh, P., Hu, F., Kumarasiri, M., Braun, A., and Nedergaard, M. (2005). Development of tight junction molecules in blood vessels of germinal matrix, cerebral cortex, and white matter. *Pediatr. Res.* 58, 791–798. doi: 10.1203/01.PDR.0000180535.14093.FB

Bhat, R. Y., Hannam, S., Pressler, R., Rafferty, G. F., Peacock, J. L., and Greenough, A. (2006). Effect of prone and supine position on sleep, apneas, and arousal in preterm infants. *Pediatrics* 118, 101–107. doi: 10.1542/peds.2005-1873

Bhat, R. Y., Leipälä, J. A., Singh, N. R.-P., Rafferty, G. F., Hannam, S., and Greenough, A. (2003). Effect of posture on oxygenation, lung volume, and respiratory mechanics in premature infants studied before discharge. *Pediatrics* 112, 29–32. doi: 10.1542/peds.112.1.29

Buckley, E. M., Cook, N. M., Durduran, T., Kim, M. N., Zhou, C., Choe, R., et al. (2009). Cerebral hemodynamics in preterm infants during positional intervention measured with diffuse correlation spectroscopy and transcranial Doppler ultrasound. *Opt. Express* 17, 12571–12581. doi: 10.1364/OE.17.012571

Cioni, G., and Prechtl, H. F. (1990). Preterm and early postterm motor behaviour in low-risk premature infants. *Early Hum. Dev.* 23, 159–191. doi: 10.1016/0378-3782(90)90012-8

de Bijl-Marcus, K. A., Brouwer, A. J., de Vries, L. S., and van Wezel-Meijler, G. (2017). The effect of head positioning and head tilting on the incidence of hemorrhage in very preterm infants: a systematic review. *Neonatology* 111, 267–279. doi: 10.1159/000449240

Elser, H. E., Holditch-Davis, D., Levy, J., and Brandon, D. H. (2012). The effects of environmental noise and infant position on cerebral oxygenation. *Adv. Neonatal Care* 12, S18–S27. doi: 10.1097/ANC.0b013e31826853fe

Emery, J. R., and Peabody, J. L. (1983). Head position affects intracranial pressure in newborn infants. *J. Pediatr.* 103, 950–953. doi: 10.1016/S0022-3476(83)80728-8

Figueroa-Diesel, H., Hernandez-Andrade, E., Benavides-Serralde, A., Crispi, F., Acosta-Rojas, R., Cabero, L., et al. (2008). Cerebral venous blood flow in growth restricted fetuses with an abnormal blood flow in the umbilical artery before 32 weeks of gestation. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 140, 201–205. doi: 10.1016/j.ejogrb.2008.04.006

Forster, D. E., Koumoundouros, E., Saxton, V., Fedai, G., and Holberton, J. (2018). Cerebral blood flow velocities and cerebrovascular resistance in normal-term neonates in the first 72 hours. *J. Paediatr. Child Health* 54, 61–68. doi: 10.1111/jpc.13663

Francois, M., Elmaleh, M., Garel, C., and Narcy, P. (1992). Dimensions of the pharynx in function of the posture in neonates and infants. *Arch. Fr. Pediatr.* 49, 23–26.

Fuchs, H., Lindner, W., Buschko, A., Trischberger, T., Schmid, M., and Hummler, H. D. (2011). Cerebral oxygenation in very low birth weight infants supported with sustained lung inflations after birth. *Pediatr. Res.* 70, 176–180. doi: 10.1203/PDR.0b013e318220c1e0

Goldberg, R. N., Joshi, A., Moscoso, P., and Castillo, T. (1983). The effect of head position on intracranial pressure in the neonate. *Crit. Care Med.* 11, 428–430. doi: 10.1097/00003246-198306000-00006

Heimann, K., Vaeßen, P., Peschgens, T., Stanzel, S., Wenzl, T. G., and Orlikowsky, T. (2010). Impact of skin to skin care, prone and supine positioning on cardiorespiratory parameters and thermoregulation in premature infants. *Neonatology* 97, 311–317. doi: 10.1159/000255163

Humberg, A., Härtel, C., Paul, P., Hanke, K., Bossung, V., Hartz, A., et al. (2017). Delivery mode and intraventricular hemorrhage risk in very-low-birth-weight infants: observational data of the german neonatal network. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 212, 144–149. doi: 10.1016/j.ejogrb.2017.03.032

Ikeda, T., Amizuka, T., Ito, Y., Mikami, R., Matsuo, K., Kawamura, N., et al. (2015). Changes in the perfusion waveform of the internal cerebral vein and intraventricular hemorrhage in the acute management of extremely low-birth-weight infants. *Eur. J. Pediatr.* 174, 331–338. doi: 10.1007/s00431-014-2396-1

Inder, T. E., Perlman, J. M., and Volpe, J. J. (2018). "Preterm intraventricular hemorrhage / posthemorrhagic hydrocephalus," in *Volpe's Neurology of the Newborn*, ed. J. J. Volpe (Philadelphia, PA: Elsevier), 637–698.

Lai, J. C. Y., Rocha-Ferreira, E., Ek, C. J., Wang, X., Hagberg, H., and Mallard, C. (2017). Immune responses in perinatal brain injury. *Brain Behav. Immun.* 63, 210–223. doi: 10.1016/j.bbi.2016.10.022

Lawson, B., Anday, E., Guillet, R., Wagerle, L. C., Chance, B., and Delivoria-Papadopoulos, M. (1987). Brain oxidative phosphorylation following alteration in head position in preterm and term neonates. *Pediatr. Res.* 22, 302–305. doi: 10.1203/00006450-198709000-00013

Liao, S. M.-C., Rao, R., and Mathur, A. M. (2015). Head position change is not associated with acute changes in bilateral cerebral oxygenation in stable preterm infants during the first three days of life. *Am. J. Perinatol.* 32, 645–652. doi: 10.1055/s-0034-1390348

Pickens, D. L., Schefft, G. L., and Thach, B. T. (1989). Pharyngeal fluid clearence and aspiration preventive mechanisms in sleeping infants. *J. Appl. Physiol.* 66, 1164–1171. doi: 10.1152/jappl.1989.66.3.1164

Rao, V. T., Ludwin, S. K., Fuh, S. C., Sawaya, R., Moore, C. S., Ho, M. K., et al. (2016). MicroRNA expression patterns in human astrocytes in relation to anatomical location and age. *J. Neuropathol. Exp. Neurol.* 75, 156–166. doi: 10.1093/jnen/nlv016

Romantsik, O., Calevo, M. G., and Bruschettini, M. (2017). Head midline position for preventing the occurrence or extension of germinal matrix-intraventricular

hemorrhage in preterm infants. *Cochrane Database Syst. Rev.* 7:CD012362. doi: 10.1002/14651858.CD012362

Schindler, T., Koller-Smith, L., Lui, K., Bajuk, B., Bolisetty, S., and New South Wales and Australia Capital Territory Neonatal Intensive Care Units's Data Collection (2017). Causes of death in very preterm infants cared for in neonatal intensive care units: a population-based retrospective cohort study. *BMC Pediatr.* 17:59. doi: 10.1186/s12887-017-0810-3

Sherlock, R. L., Anderson, P. J., Doyle, L. W., and Victorian Infant Collaborative Study Group (2005). Neurodevelopmental sequelae of intraventricular haemorrhage at 8 years of age in a regional cohort of ELBW/very preterm infants. *Early Hum. Dev.* 81, 909–916. doi: 10.1016/j.earlhumdev.2005.07.007

Skinner, J. (2000). "Ductal shunting," in *Echocardiography for the Neonatologist*, eds J. Skinner, D. Alverson, and S. Hunter (London: Churchill Livingstone), 151–170.

Stichtenoth, G., Demmert, M., Bohnhorst, B., Stein, A., Ehlers, S., Heitmann, F., et al. (2012). Major contributors to hospital mortality in very-low-birth-weight infants: data of the birth year 2010 cohort of the german neonatal network. *Klin. Padiatr.* 224, 276–281. doi: 10.1055/s-0032-1306344

Urlesberger, B., Müller, W., Ritschl, E., and Reiterer, F. (1991). The influence of head position on the intracranial pressure in preterm infants with posthemorrhagic hydrocephalus. *Child's Nerv. Syst.* 7, 85–87. doi: 10.1007/BF00247862

Vrancken, S. L., van Heijst, A. F., and de Boode, W. P. (2018). Neonatal hemodynamics: from developmental physiology to comprehensive monitoring. *Front. Pediatr.* 6:87. doi: 10.3389/fped.2018.00087

Waisman, D. (2006). Non-traumatic nasopharyngeal suction in premature newborn infants with upper airway obstruction from secretions following nasal CPAP. *J. Pediatr.* 149:279. doi: 10.1016/j.jpeds.2006.02.044

Watson, G. H. (1974). Effect of head rotation on jugular vein blood flow. *Arch. Dis. Child.* 49, 237–239. doi: 10.1136/adc.49.3.237

Zubrow, A. B., Hulman, S., Kushner, H., and Falkner, B. (1995). Determinants of blood pressure in infants admitted to neonatal intensive care units: a prospective multicenter study. Philadelphia neonatal blood pressure study group. *J. Perinatol.* 15, 470–479.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Spengler, Loewe and Krause. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 07 January 2019
doi: 10.3389/fphys.2018.01858

Ketamine Reduces Inflammation Pathways in the Hypothalamus and Hippocampus Following Transient Hypoxia in the Late-Gestation Fetal Sheep

*Eileen I. Chang[1], Miguel A. Zarate[1], Thomas J. Arndt[1], Elaine M. Richards[2], Maria B. Rabaglino[3], Maureen Keller-Wood[2] and Charles E. Wood[1]**

[1] Department of Physiology and Functional Genomics, University of Florida College of Medicine, Gainesville, FL, United States, [2] Department of Pharmacodynamics, University of Florida College of Pharmacy, Gainesville, FL, United States, [3] CEPROCOR, National Scientific and Technical Research Council (CONICET), Córdoba, Argentina

OPEN ACCESS

Edited by:
Carina Mallard, University of Gothenburg, Sweden

Reviewed by:
Adam John Watkins, University of Nottingham, United Kingdom
Mhoyra Fraser, The University of Auckland, New Zealand

***Correspondence:**
Charles E. Wood
woodc@ufl.edu

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *02 August 2018*
Accepted: *11 December 2018*
Published: *07 January 2019*

Citation:
Chang EI, Zarate MA, Arndt TJ, Richards EM, Rabaglino MB, Keller-Wood M and Wood CE (2019) Ketamine Reduces Inflammation Pathways in the Hypothalamus and Hippocampus Following Transient Hypoxia in the Late-Gestation Fetal Sheep. Front. Physiol. 9:1858. doi: 10.3389/fphys.2018.01858

The physiological response to hypoxia in the fetus has been extensively studied with regard to redistribution of fetal combined ventricular output and sparing of oxygen delivery to fetal brain and heart. Previously, we have shown that the fetal brain is capable of mounting changes in gene expression that are consistent with tissue inflammation. The present study was designed to use transcriptomics and systems biology modeling to test the hypothesis that ketamine reduces or prevents the upregulation of inflammation-related pathways in hypothalamus and hippocampus after transient hypoxic hypoxia. Chronically catheterized fetal sheep (122 ± 5 days gestation) were subjected to 30 min hypoxia (relative reduction in P_aO_2∼50%) caused by infusion of nitrogen into the inspired gas of the pregnant ewe. RNA was isolated from fetal hypothalamus and hippocampus collected 24 h after hypoxia, and was analyzed for gene expression using the Agilent 15.5 k ovine microarray. Ketamine, injected 10 min prior to hypoxia, reduced the cerebral immune response activation to the hypoxia in both brain regions. Genes both upregulated by hypoxia and downregulated by ketamine after hypoxia were significantly associated with gene ontology terms and KEGG pathways that are, themselves, associated with the tissue response to exposure to bacteria. We conclude that the results are consistent with interruption of the cellular response to bacteria by ketamine.

Keywords: fetal hypoxia, ketamine, fetal brain, inflammation, transcriptomics

Abbreviations: CASP8, caspase8; CD14, CD14 molecule; CXCL10, chemokine (C-X-C motif) ligand 10; ERK, extracellular signal-regulated kinase; HC, hypoxic control; HK, hypoxia + ketamine; IL1β, interleukin 1 beta; JAK/STAT, janus kinase/signal transducers and activators of transcription; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinases; MYD88, myeloid differentiation primary response 88; NC, normoxic control; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, normoxia + ketamine; NMDA, *N*-Methyl-$_D$-aspartate; PTGS2, prostaglandin-endoperoxide synthase 2; TLR, toll-like receptor; TNFα, tumor necrosis factor-alpha.

INTRODUCTION

Transient fetal hypoxia is a common homeostatic disturbance during fetal life. Any compromise of maternal oxygen supply, for example, high altitude or maternal hypoventilation secondary to any cause, decreases partial pressure of oxygen in the fetal blood (Assali et al., 1962; Makowski et al., 1968). Physiologists commonly view the fetal response to hypoxia through the lens of homeostatic cardiovascular responses that spare brain and heart viability (Dawes et al., 1969; Cohn et al., 1974). Cardiovascular and neuroendocrine reflex responses to hypoxia redirect blood flow such that a larger proportion of the combined ventricular output flows toward the brain, heart, and adrenal glands (Cohn et al., 1974).

We have recently reported that 30 min of transient hypoxia of the mother and fetus (caused by reducing the oxygen content of the mother's inspired gas) results in increased abundance of live bacteria in the fetal brain 24 h after the hypoxic episode (Zarate et al., 2017). Whole genome sequencing of bacteria recovered from the fetal cerebral cortex revealed that the bacteria isolated from the fetal brain were identical to those isolated from the placenta (Zarate et al., 2017). This suggested a route of transfer from placenta to fetal brain with hypoxia. Concomitant with appearance of bacteria in fetal brain is upregulation of molecular pathways that subserve inflammation (Chang et al., 2016a; Zarate et al., 2017). As we have previously reported, the pattern of activation of genes within inflammation-related pathways is consistent with the response to bacterial entry into the fetal brain after hypoxia (Zarate et al., 2017).

We have previously reported that ketamine, an FDA-approved agent used in Neonatal Intensive Care Units (Anand, 2007), attenuated the activation of inflammation-related pathways in fetal cerebral cortex and kidney (Chang et al., 2016a,b). Since the discovery of hypoxia-induced bacterial invasion of the fetus, we have concluded that the increase in abundance of bacteria was causative of the increased inflammation-like response in the fetal brain (Zarate et al., 2017). The mechanism of the effect of ketamine – originally hypothesized to reduce inflammation by blockade of NMDA-mediated glutamate signaling – was therefore unclear (Powers and Wood, 2007; Knutson and Wood, 2010). In this report we test the hypothesis that the effect of ketamine will be reflected by changes in inflammation-related genes in the hypothalamus and hippocampus. Furthermore, we test the hypothesis that ketamine reduces the influx of bacteria into the fetal brain, thereby blocking the stimulus to inflammation. We have reported, from these experiments, upregulated inflammation pathways in cerebral cortex, hypothalamus and hippocampus, as well as kidney cortex in response to hypoxic hypoxia. We have also reported the transfer of bacteria into the brains of these fetuses. This report specifically addresses the effect of ketamine on the inflammation response in hypothalamus and hippocampus, and addresses the question of whether ketamine reduces or prevents the appearance of bacteria in these brain regions.

MATERIALS AND METHODS

Ethics Statement

All experiments were approved by the University of Florida Animal Care and Use Committee and were performed in accordance with the Guiding Principles for Use of Animals of the American Physiological Society.

Fetal Ovine Surgery

The surgical procedures for fetal and maternal chronic femoral arterial and venous catheterizations were described previously (Chang and Wood, 2015; Chang et al., 2016b). Briefly, ewes were fasted 24 h before surgery, and received 750 mg ampicillin (Polyflex®, Boehringer Ingelheim VetMedica, Inc., St. Joseph, MO, United States) before the induction of anesthesia with 0.5–2% isoflurane with oxygen and intubation. The fetal hindlimbs were catheterized with a set of femoral arterial, venous, and amniotic catheters. Ampicillin (500 mg) was injected into the amniotic fluid before the uterus was sutured closed. The ewe also received a set of femoral arterial and venous vascular catheters. In addition, a non-occlusive catheter was implanted in the maternal trachea for the infusion of nitrogen gas as previously described

TABLE 1 | Primers and probes used for qPCR analysis (all sequences 5′–3′).

Gene	Forward primer	Reverse primer	Probe
ACTB	TTCCTTCCTGGG CATGGA	GACGTCACACTT CATGATGGAATT	TCCTGCGGCA TTCACGAAACT ACCTT
CASP8	TGGCTGCCCTCA AGTTCCT	GGAATAGCATCA AGGCATCCTT	SYBR Green
CD14	CCTAAAGGAC TGCCGACCAA	GCGGCTCCCTG CTTAGCT	SYBR Green
CRH	TCCCATTTC CCTGGATCTCA	GAGCTTGCTGCG CTAACTGA	TTCCACCTCCT CCGAGAAGTCTT GGAAAT
CXCL10	TTGAACTGATTC CTGCAAGTCA	TTCCTTTTCATT GTGGCAATAATCT	SYBR Green
IL1B	CGTGGCCAT GGAGAAGCT	GGTCATCATCACG GAAGACATGT	SYBR Green
MYD88	GCCTGAGTATTT TGATGCCTTCA	GCTGCCGGATC ATCTCATG	SYBR Green
NFKB1	TCCCACAGATGT TCACAAACAGT	GACGCTCAATCTT CATCTTGTGAT	SYBR Green
NFKBIA	CTACACCTTGCC TGTGAGCA	AGACACGTGTGG CCATTGTA	SYBR Green
OAS1	GAGGAAAGAGG GCGAGTTCT	GGATGAGGCTCT TCAGCTTG	SYBR Green
PTGS2	GCACAAATCTGA TGTTTGCATTCT	CTGGTCCTCGTT CATATCTGCTT	TGCCCAGCACTT CACCCATCAATTTT
PTX3	GCACCTGGGATT CAAAGAAA	TGTTTCATCAAA GCCACCAA	SYBR Green
TLR2	GATTCTGCTGGA GCCCATTG	TCATGATCTTCCG CAGCTTACA	SYBR Green
TLR4	ACTCGCTCCGG ATCCTAGACT	CCTTGGCAAATT CCGTAGTTCT	SYBR Green
TNF	CCCTTCCACCCC CTTGTT	ATGTTGACCTTG GTCTGGTAGGA	SYBR Green

(Chang and Wood, 2015). Post-operative care was provided to the ewe for a minimum of 5 days, including prophylactic administration of ampicillin (15–20 mg/kg, IM, bid), wound care, and monitoring of rectal temperature, food consumption and signs of infection or distress.

In vivo Transient Hypoxia

Studies were performed on chronically catheterized singleton (n = 1) and twin (n = 15) ovine fetuses at gestational age 122 ± 5 days (full term = 145–147 days), while the ewes were conscious and freestanding in their pens with access to food. Each pregnancy was randomly assigned to the experimental protocol of normoxia or hypoxia, therefore producing the four groups: normoxia control (NC), normoxia+ketamine (NK), HC, and hypoxia+ketamine (HK). For groups treated with ketamine, the fetuses received 3 mg/kg of ketamine intravenously through the femoral venous catheter 10 min prior to normoxic or hypoxic stimuli (30 min). Transient hypoxia was produced by infusing nitrogen gas into the maternal tracheostomy tube, resulting in a 50% decrease in arterial partial pressures of oxygen (P_aO_2) for both the ewe and the fetus. Changes in blood gas compositions (ABL80 Radiometer, Copenhagen, Denmark) were closely monitored by anaerobically drawing arterial blood (1 mL) at regular intervals. Fetal physiological responses (hemodynamics, blood gasses, and neuroendocrine values) to hypoxia, ketamine, and the combination of hypoxia and ketamine have been reported previously (Chang and Wood, 2015). Fetuses were euthanized 24 h post initial stimulation of normoxia or

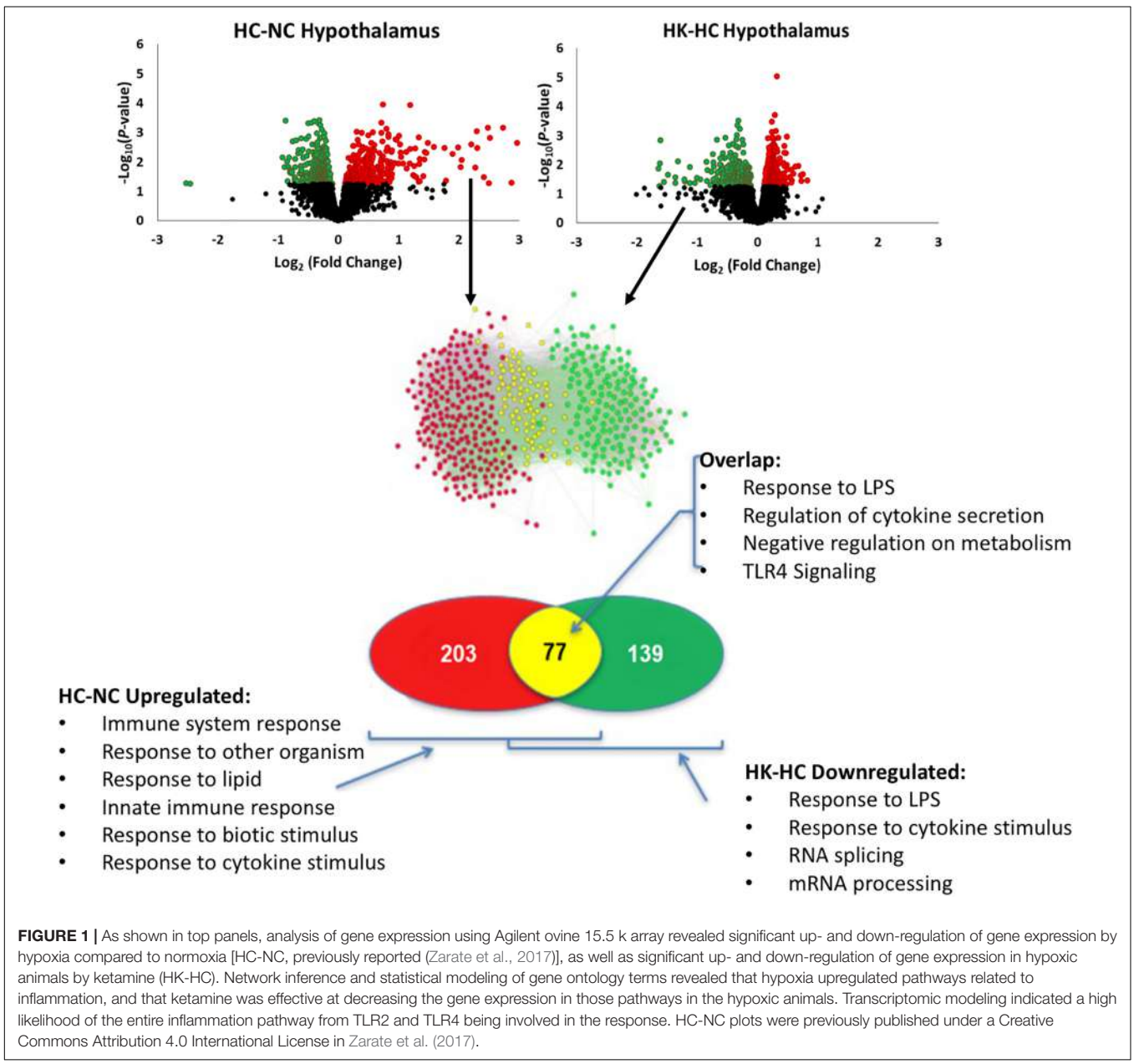


FIGURE 1 | As shown in top panels, analysis of gene expression using Agilent ovine 15.5 k array revealed significant up- and down-regulation of gene expression by hypoxia compared to normoxia [HC-NC, previously reported (Zarate et al., 2017)], as well as significant up- and down-regulation of gene expression in hypoxic animals by ketamine (HK-HC). Network inference and statistical modeling of gene ontology terms revealed that hypoxia upregulated pathways related to inflammation, and that ketamine was effective at decreasing the gene expression in those pathways in the hypoxic animals. Transcriptomic modeling indicated a high likelihood of the entire inflammation pathway from TLR2 and TLR4 being involved in the response. HC-NC plots were previously published under a Creative Commons Attribution 4.0 International License in Zarate et al. (2017).

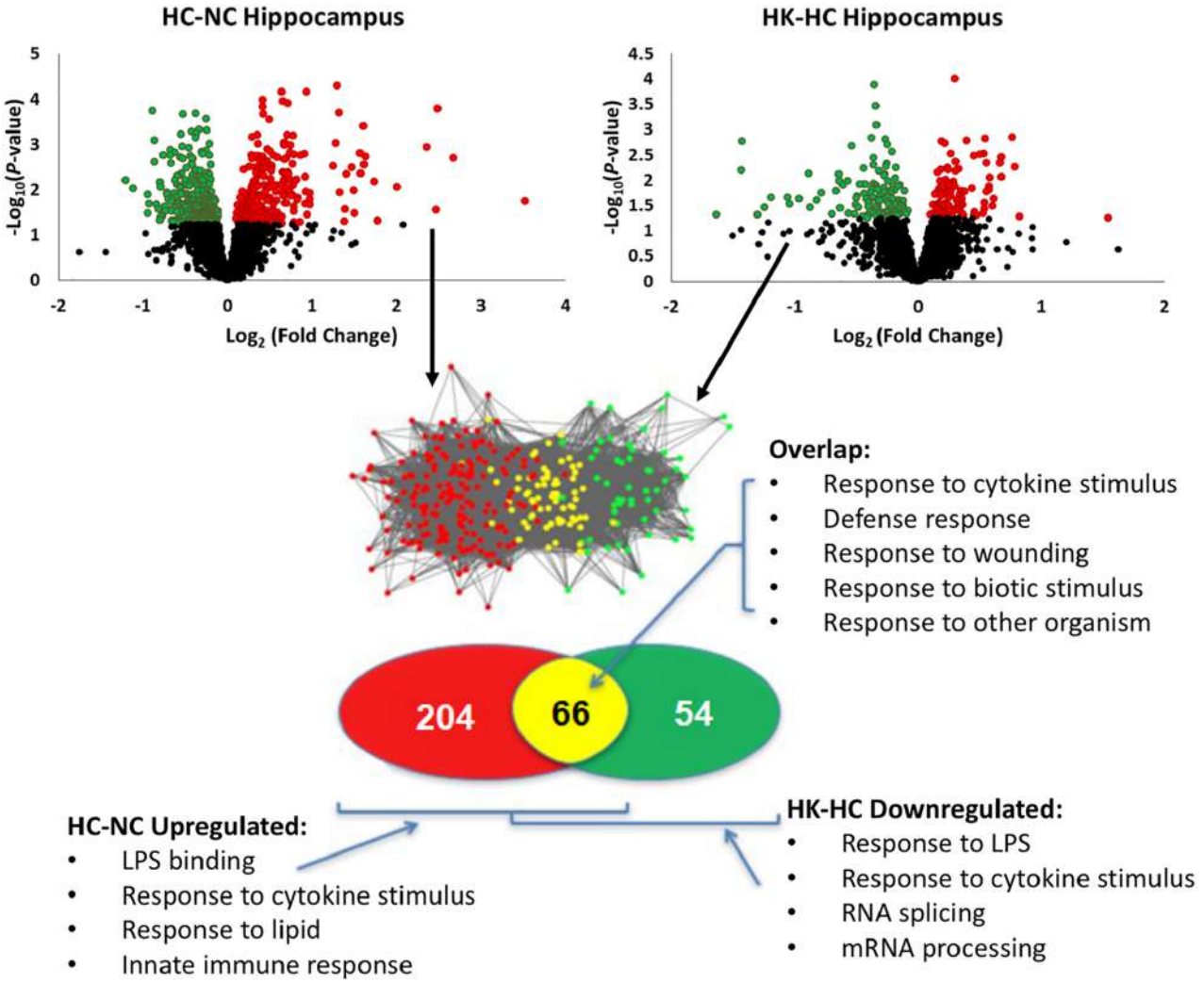


FIGURE 2 | Top panels, significant up- and down-regulation of gene expression in hippocampus by hypoxia compared to normoxia [HC-NC, previously reported (Zarate et al., 2017)], as well as significant up- and down-regulation of gene expression in hypoxic animals by ketamine (HK-HC). Network inference and statistical modeling of gene ontology terms were as described in legend to **Figure 1**. HC-NC plots were previously published under a Creative Commons Attribution 4.0 International License in Zarate et al. (2017).

transient hypoxia. The fetal brain was hemisected, with one half reserved for fixation and histological analysis and with the other half dissected for molecular analysis. Hypothalamus was removed as a single block of tissue, bounded on the rostral edge by the rostral edge of the optic chiasm, on the caudal side by the caudal edge of the median eminence, and on the side by the edges of the median eminence (Gersting et al., 2009). Hippocampus was dissected as the entire hippocampus (Gersting et al., 2009). Medulla oblongata was defined as the area between the obex and the caudal medulla-rostral spinal cord border (Gersting et al., 2009).

Microarray Procedures

Total mRNA was extracted from snap frozen fetal hypothalamus tissues using Trizol extraction followed by RNeasy Plus Mini Kit (Qiagen, Valencia, CA, United States), with on-column DNase digestion. RNA integrity number (RIN) values ranged between 8.0 and 9.0, RNA was labeled with cyanine 3 CTP with the Quick Amp Labeling Kit (Cat# 5190-0442, Agilent Technologies, Santa Clara, CA, United States), according to the manufacturer's protocol. The specific activities of the labeled cRNAs ranged from 11.6 to 15.2 pmol Cy3/μg RNA and yielded 8.9 to 10.96 μg. The cRNA samples were hybridized and processed for one-channel Sheep Gene Expression Microarray (8 × 15 K slide) – 8 arrays with 15208 oligomers each (Cat# G4813A-019921, Agilent Technologies, Santa Clara, CA, United States) as described (Rabaglino et al., 2014). The slides were scanned with Microarray Scanner System (G2505-90021, Agilent) and the measured fluorescence was detected and converted using Agilent Feature Extraction 9.1 software at the Genomics Division of the University of Florida's Interdisciplinary Center for Biotechnology Research. All microarray data have been uploaded to the Gene

TABLE 2 | Top 10 gene ontology biological processes and enriched KEGG pathways associated with genes that were significantly up regulated in the hypothalamus during acute hypoxic stress, but were down regulated with ketamine.

Analysis	Pathways, processes, functions, components	# Genes involved	Adjusted *P*-values
Biological Process	Defense response	30	1.93E-11
	Response to stress	45	2.09E-10
	Immune response	26	7.72E-09
	Immune system process	33	7.72E-09
	Regulation of defense response	16	1.67E-07
	Response to lipopolysaccharide	12	2.05E-07
	Response to other organism	18	2.05E-07
	Response to wounding	24	2.30E-07
	Innate immune response	17	2.85E-07
	Response to molecule of bacterial origin	12	2.85E-07
KEGG Pathways	MAPK signaling pathway	9	1.05E-07
	Osteoclast differentiation	7	1.17E-07
	Cytokine-cytokine receptor interaction	8	6.45E-07
	Hematopoietic cell lineage	5	8.36E-06
	Toll-like receptor signaling pathway	5	1.28E-05
	Chemokine signaling pathway	6	1.28E-05
	Pathways in cancer	7	1.94E-05
	Toxoplasmosis	5	2.96E-05
	Hepatitis C	5	3.96E-05
	Adipokine signaling pathway	4	4.60E-05

TABLE 3 | Top 10 gene ontology biological processes and enriched KEGG pathways associated with genes that were significantly up regulated in the hippocampus during acute hypoxic stress, but were down regulated with ketamine.

Analysis	Pathways, processes, functions, components	# Genes involved	Adjusted *P*-values
Biological Process	Response to lipid	18	4.49E-08
	Response to cytokine stimulus	15	1.49E-06
	Response to organic substance	27	1.49E-06
	Response to biotic stimulus	16	1.53E-06
	Response to chemical stimulus	33	1.53E-06
	Response to other organism	15	4.53E-06
	Response to lipopolysaccharide	10	4.53E-06
	Response to molecule of bacterial origin	10	6.95E-06
	Response to external stimulus	21	1.54E-05
	Response to stress	32	1.61E-05
KEGG Pathways	Cytokine-cytokine receptor interaction	7	8.15E-06
	Malaria	4	2.32E-05
	Toll-like receptor signaling pathway	4	2.00E-04
	MAPK signaling pathway	5	6.00E-04
	Adipocytokine signaling pathway	3	1.20E-03
	Chemokine signaling pathway	4	1.50E-03
	Amoebiasis	3	3.10E-03
	Osteoclast differentiation	3	4.60E-03
	African trypanosomiasis	2	5.20E-03
	Jak-STAT signaling pathway	3	6.50E-03

Expression Omnibus of the National Center for Biotechnology Information (accession numbers GSE82016 and GSE97916). We performed array analyses in the hippocampus and the hypothalamus for the following groups: NC (n = 3), NK (n = 4), HC (n = 5), and HK (n = 4).

Network Inference and Analysis, and Functional Annotation of Gene Ontology

Gene networks were inferred using the GeneMania (Montojo et al., 2010) plugin of Cytoscape (Shannon et al., 2003). The functional annotation of gene ontology for groups or networks of genes was analyzed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) (Zhang et al., 2005; Wang et al., 2013).

Immunohistochemistry

Fetal brain tissues (n = 4 per group) were fixed in 4% buffered paraformaldehyde overnight, and stored in 70% reagent alcohol until embedded in paraffin wax. All tissue samples were sectioned with a microtome (5 μm) and counterstained with methyl green. To identify the presence of microglia or macrophages, the hypothalamus, hippocampus, and medulla were stained for Iba-1 (ionized calcium binding adaptor protein-1) antibody (Cat.# 019-19741, Waco Pure Chemical Industries, Richmond, VA, United States; 1:500 dilution) and peroxidase-PAP sandwich technique (Vectastain, Vector Labs, Burlingame, CA, United States). For each animal, five (hypothalamus and medulla) and ten (hippocampus) images (40× magnification) were randomly selected for quantification. Vascular integrity was assessed using immunostaining of ovine albumin (anti-Sheep Serum Albumin Polyclonal Antibody MBS715170, MyBiosource Inc., San Diego, CA, United States, dilution 1:200) with peroxidase-PAP sandwich visualization. The number of broken and leaky blood vessels were counted using seven (hypothalamus), ten (cerebral cortex), and five (medulla) random images (10× magnification) per animal. All microscopic quantification was done using a blinded protocol.

Real-Time PCR (qPCR)

The same set of mRNA used for the microarray was also used for qPCR validations. The primers were designed based on the known *Ovis aries* and *Bos taurus* genomes (**Table 1**) for SYBR green or TaqMan chemistry. Chemistries used for this analysis were Applied Biosystems Fast SYBR Green master mix, and Taqman Fast Advanced master mix (Cat. # 4385612, 4444558, respectively; Thermo-Fisher Scientific, Waltham, MA, United States). The ovine β-actin primers and probe were used as the house keeping gene control, as described previously (Chang et al., 2016b). Bacterial 16S rRNA was measured using universal 16S primers as described by Nadkarni and coworkers (Nadkarni et al., 2002). For each sample, the relative mRNA

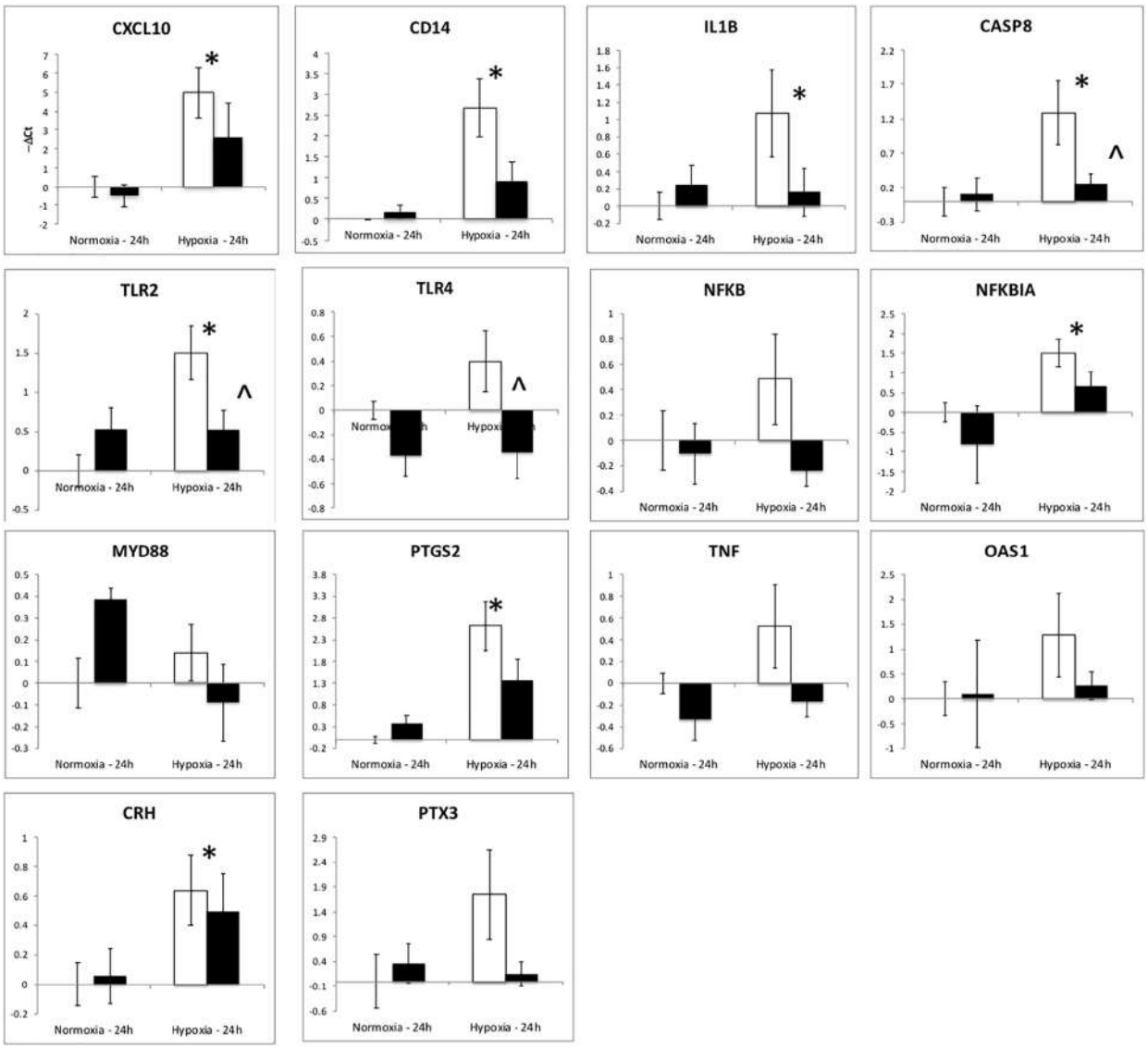


FIGURE 3 | Gene expression (mRNA abundance) in fetal hypothalamus measured by real-time qPCR for genes in the toll-like receptor inflammation pathway. Values are represented as negative delta cycle threshold ($-\Delta C_t$) compared to the NC group. Open bars represent experiments in which ketamine was not administered. Filled bars represent experiments in which ketamine was administered before normoxia or hypoxia. The criterion for statistical significance was $P < 0.05$ (Student's *t*-test). Data are presented as means ± SEM, and the y-axis scale varies between plots. *Statistically significant difference of hypoxia group compared to normoxia control. ∧Statistically significant difference of hypoxia control group compared to hypoxia + ketamine group.

expression was calculated by the difference in threshold cycle (ΔC_t) between the triplicate mean C_t for each gene and for β-actin.

Statistics

As previously described, statistical analysis for Agilent 15.5 k array was performed with Bioconductor's Limma package for R software v.2.15.1, which utilized moderated *t*-test and empirical Bayes method for small sample size per group ($p < 0.05$) (Chang et al., 2016b). The qPCR data were analyzed by Student's *t*-test (Zar, 1984). Two-way nested ANOVA design was applied for immunohistochemistry data analysis, using the Genmod Procedure of SAS/STAT® 9.3 (SAS Institute Inc., Cary, NC, United States). Data are presented as mean values ± standard error of the mean (SEM), and the criterion for statistical significance was $p < 0.05$.

RESULTS

The blood gas and cardiovascular responses to hypoxia and normoxia, and the effect of ketamine on these variables in a larger cohort of experiments have been previously reported (Chang and Wood, 2015).

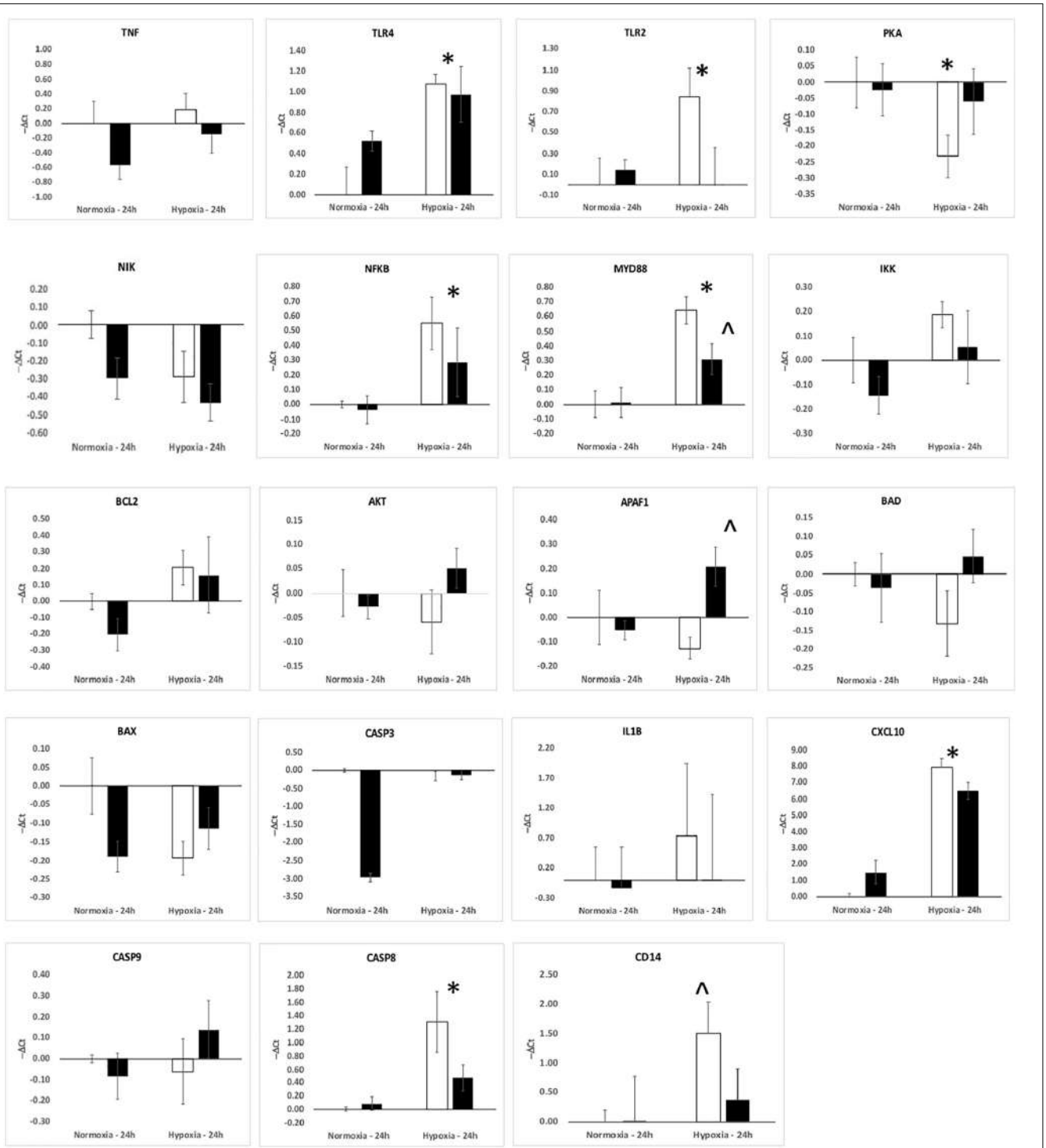


FIGURE 4 | Gene expression (mRNA abundance) in fetal hippocampus measured by real-time qPCR for genes in the toll-like receptor inflammation pathway. Values are represented as negative delta cycle threshold ($-\Delta C_t$) compared to the NC group. Open bars represent experiments in which ketamine was not administered. Filled bars represent experiments in which ketamine was administered before normoxia or hypoxia. The criterion for statistical significance was $P < 0.05$ (Student's *t*-test). Data are presented as means ± SEM, and the y-axis scale varies between plots. *Statistically significant difference of hypoxia group compared to normoxia control. ∧Statistically significant difference of hypoxia control group compared to hypoxia + ketamine group.

Twenty-four hours after the onset of a 30 min period of hypoxia, 280 genes were upregulated and 357 genes were downregulated in hypothalamus compared to fetuses subjected to normoxia alone. In hippocampus, 270 genes were upregulated

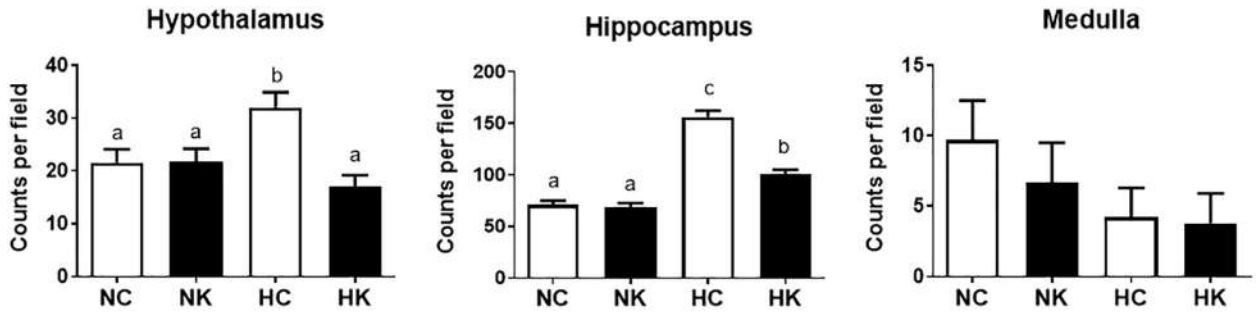


FIGURE 5 | Ketamine reduces the number of microglia and macrophages in the hypothalamus and hippocampus 24 h after transient hypoxia. For each brain region, the Iba-1 positive cells counted per 40X field (average of 7 fields analyzed for each animal) were averaged from 4 animals per group. Data from hippocampus are average measurements from regions CA1, CA2, CA3, CA4, and Dentate Gyrus. Data are expressed as mean ± SEM. Different letters indicate statistically significant difference ($P < 0.05$). NC, normoxia control; NK, normoxia + ketamine; HC, hypoxia control; HK, hypoxia + ketamine.

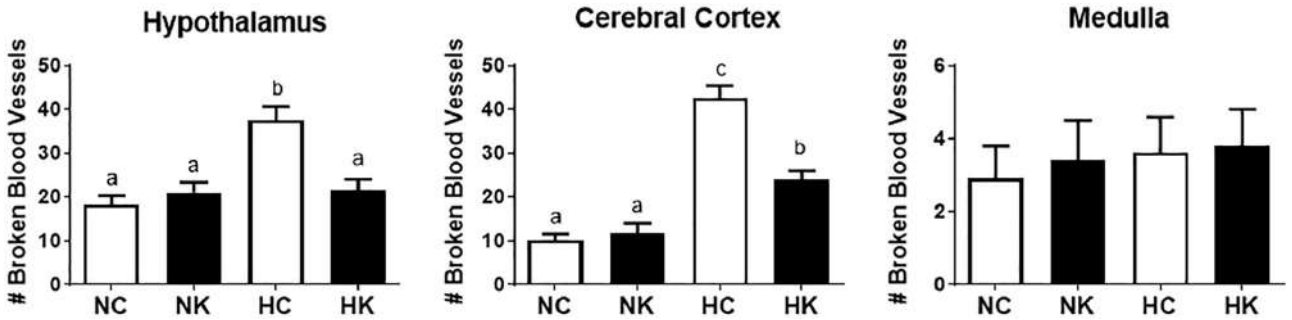


FIGURE 6 | Ketamine reduces the number of broken or leaky blood vessels in the fetal hypothalamus and cerebral cortex 24 h after transient hypoxia. Number of broken blood vessels were counted in 10X field images and averaged for each animal per brain region: hypothalamus, cerebral cortex, and medulla. Data are expressed as means ± SEM ($n = 4$/group). Different letters indicate statistically significant difference ($P < 0.05$). NC, normoxia control; NK, normoxia + ketamine; HC, hypoxia control; HK, hypoxia + ketamine.

and 240 were downregulated. We have previously reported these changes in gene expression as well as statistically significant over-represented gene ontology terms and KEGG pathways activation for these differentially regulated genes elsewhere (Zarate et al., 2017). Inflammation- and infection-related pathways were significantly upregulated in both brain regions (Zarate et al., 2017).

Ketamine alone (in normoxic fetuses) upregulated 15 genes and downregulated 21 genes in hypothalamus and upregulated 22 and downregulated 29 genes in hippocampus. Gene ontology analysis of the upregulated genes revealed significant association with extracellular matrix binding and collagen binding in hypothalamus, but the downregulated genes did not reveal any significant biological processes in hypothalamus. In the hippocampus, there was no significant biological processes revealed by gene ontology analysis that were associated with either the upregulated or the downregulated genes.

Ketamine had a substantial effect on the transcriptomic response to hypoxia. In hypothalamus, ketamine decreased the expression of 293 genes in and increased the expression of 238 genes in the fetuses subjected to hypoxia (**Figure 1**). In hippocampus, ketamine upregulated 125 and downregulated 130 genes when compared to hypoxia alone (**Figure 2**).

Comparison of the genes upregulated by hypoxia but downregulated by ketamine after hypoxia, allows identification of those pathways that are activated by hypoxia but whose activation is reversed by ketamine pretreatment. This analysis in hypothalamus revealed 77 genes with this pattern (**Figure 1**). Gene ontology analysis revealed that these genes were significantly associated with immune response, cellular response to lipopolysaccharide, response to other organism, innate immune response, and response to molecule of bacterial origin (**Table 2**). KEGG pathways significantly associated with these genes include MAPK signaling pathway, cytokine-cytokine receptor interaction, hematopoietic cell lineage, chemokine signaling, and TLR signaling pathway (**Table 2**).

The same analysis strategy in hippocampus revealed 66 genes that were upregulated by hypoxia but downregulated by ketamine (**Figure 2**). Significantly associated biological process terms of these genes included response to cytokine stimulus, response to lipopolysaccharide, response to molecule of bacterial origin, response to external stimulus, and response to stress (**Table 3**). KEGG pathways significantly overrepresented in this gene set were cytokine-cytokine receptor interaction, TLR signaling pathway, MAPK signaling pathway, adipocytokine

signaling pathway, chemokine signaling pathway, osteoclast differentiation, and JAK/STAT signaling pathway (**Table 3**).

There were 57 genes in hypothalamus and 31 in hippocampus that were downregulated by hypoxia but increased in the fetuses subjected to hypoxia and ketamine. In both brain regions, these genes were not significantly associated with gene ontology terms. KEGG analysis of the hypothalamic genes revealed a significant association with metabolic pathways, vitamin digestion and absorption, fructose/mannose metabolism, endocytosis, and spliceosome, while a similar analysis in hippocampus did not yield any significantly associated KEGG pathways.

The upregulation of inflammation-related genes (MYD88, NFKB, etc.) and the subsequent modeling of the transcriptome led us to further explore the relationship between hypoxia and inflammation, and the modification of this relationship by ketamine at both the RNA and histological levels. We chose to probe, at the mRNA expression level, the TLR pathway and the apoptosis pathway. Hypoxia increased the abundance of mRNA for multiple inflammation-related genes in hypothalamus (**Figure 3**) and hippocampus (**Figure 4**), including TLR2, CD14, NFKBIA, IL1B, CXCL10, PTGS2, and CASP8 (previously reported in Zarate et al., 2017). Ketamine reduced the CD14, TLR2, TLR4, MYD88, NFKB, and CASP8 mRNA abundance in one or both of these brain regions compared to fetuses that were subjected to hypoxia but not to ketamine treatment (**Figures 3**, **4**).

The gene ontology analysis and the qPCR analysis indicated that a major component of the response 24 h after hypoxia is inflammation, and more specifically genes related to the toll-like receptor (TLR2 and TLR4) inflammation pathway. We have reported, previously, that hypoxia increases microglia/macrophage abundance in cerebral cortex (Chang et al., 2016a), renal cortex (Chang et al., 2016b), and in hypothalamus and hippocampus (Zarate et al., 2017). We have also previously reported that anti-inflammatory actions of ketamine pretreatment were accompanied by reductions in microglia/macrophages in both fetal cerebral (Chang et al., 2016a) and renal (Chang et al., 2016b) cortex. In this report, we extend this effect of ketamine to hypothalamus and hippocampus (**Figure 5**).

We have previously demonstrated that hypoxia resulted in an increase in the abundance of bacterial 16S rRNA gene expression and vascular permeability in fetal cerebral cortex, hypothalamus, and hippocampus (Zarate et al., 2017). Ketamine significantly reduced the effect of hypoxia on vascular integrity in hypothalamus and cerebral cortex (**Figure 6**) and decreased the macrophage/microglia infiltration of hypothalamus and hippocampus (**Figure 5**). Likewise, ventilatory hypoxic hypoxia produced an increase in bacterial load though an upregulation of 16S gene; however, ketamine did not significantly reduce or block the hypoxia-induced increase in qPCR-detectable 16S bacterial rRNA (**Figure 7**). While not specifically the topic of this analysis, medulla was analyzed for Iba-1 and albumin immunostaining: neither hypoxia nor ketamine had any effect on either of these measured variables in medulla (**Figures 5**, **6**).

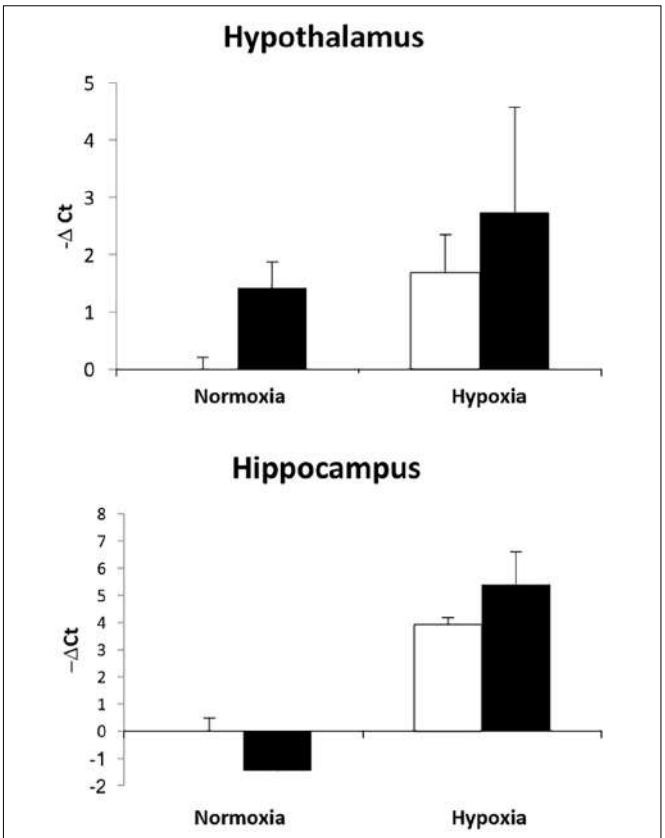


FIGURE 7 | Effect of ketamine on the expression of 16S bacterial rRNA in hypothalamus and hippocampus 24 h after transient hypoxia. Data are presented as $-\Delta C_t$ values relative to the NC group for each brain region ($n = 4$ animals per group). Data are expressed as mean ± SEM.

DISCUSSION

We hypothesized that ketamine would reduce hypothalamic inflammation secondary to its action to block NMDA receptor activity, thereby reducing the damaging effects of intense glutamate neurotransmission. Our original concept was that glutamate neurotransmission, followed by calcium influx into neurons, would increase apoptosis and cell death, ultimately initiating inflammation. Calcium is also released from macrophages into the extracellular space. It is possible that cell death and the associated release of cellular contents into the extracellular space could cause or contribute to the inflammation and increase in macrophage/microglia in hypothalamus and hippocampus. While the results of the present study do not reveal the ultimate cause of the inflammation, they do demonstrate that the entire inflammation cascade at a gene transcript level is activated, similar to the response to infection. Ketamine attenuates the activation of genes in inflammation-related pathways.

Although, we designed this experiment on the assumption that the mechanism of action of ketamine was the blockade of NMDA receptors, we propose that the anti-inflammatory action of the drug in the context of the present experiments is likely

to involve other mechanisms. For example, the results could be explained by an action of ketamine to block macrophage activation or translocation. Shimaoka and colleagues reported that ketamine, but not other NMDA receptor antagonists, reduced nitrate production and TNFα production by J774 cells (a murine macrophage-like cell line) (Shimaoka et al., 1996). Ketamine reduces NFkB activation in A172 human glioblastoma cells exposed to LPS (Sakai et al., 2000) and reduces TNFα and IL6 production by LPS-stimulated RAW 267.4 (transformed mouse macrophage) cells (Wu et al., 2008). The mechanism of action may involve a reduction in ERK1/2 phosphorylation (Chang et al., 2010) and may, in cases of gram negative bacterial infection, interfere with LPS binding protein binding to LPS (Chen et al., 2009). The majority of bacteria appearing in fetal brain in the present experiments are gram positive (*Staphylococcus*), as reported previously (Zarate et al., 2017). Ketamine is known to reduce TLR2-mediated activation of ERK1/2 and NFKB by lipoteichoic acid, the principal component of cell wall in gram positive bacteria (Chang et al., 2010). However, in this study, ketamine did not produce any significant effects on bacterial load in the fetal hippocampus or hypothalamus. We believe that ketamine failed to have any affects on bacterial 16S gene expression because it has neither antibiotic properties nor any obstructive effects on the bacterial movement from maternal to fetal circulation. Histological data also indicates that hypoxia negatively affects the integrity of the blood vessels, and ketamine administration reduced the number of broken vessels. We have previously reported (Chang and Wood, 2015) that ventilatory hypoxia produces hemodynamic modifications in the fetus, and this response was attenuated by ketamine. Alterations in hemodynamics during acute episodes of hypoxia have been reported to affect the microvascular system, especially in the brain (Mark and Davis, 2002; Luo et al., 2012; Baburamani et al., 2013). These previous findings strongly supports our data and together they provide new insights on the therapeutical effects of ketamine in episodes of hypoxia-induced cerebral edema (Shu et al., 2012). The exact mehanisms on how blood vessels integrity are affected by hypoxia are not fully elucidated.

It is perhaps tempting to assume that inflammation in the fetal hypothalamus is damaging, or that inflammation might "program" an increased propensity for adult disease. The results of the present study do not, however, address the question of whether inflammation and the increase in tissue macrophages/microglia is damaging or whether the changes that we observe are reparative. Indeed, it is not clear from the present experiments that the inflammation reflects damage or cell death in any of the brain regions. Microglia and macrophages are important participants in tissue development and maturation. For example, microglia appear to be important in the turnover of neuronal progenitor cells in the developing brain. Arno and colleagues have proposed that microglia are a critical part of maintaining the balance between cell division and cell death in the developing brain (Arno et al., 2014). Loss of microglial function might be causative with regard to known conditions involving cellular overgrowth (Arno et al., 2014). Unknown variables important to fetal brain inflammation may have great relevance to the ultimate outcome of the offspring. Knowing, for example, that fetal brain response to transient hypoxic hypoxia involves several heretofore unrecognized variables – including bacteria after, for example, maternal antibiotic treatment or prophylactic indomethacin treatment during pregnancy or labor/delivery, or the use of ketamine for analgesia in the Neonatal Intensive Care Unit.

We conclude that the action of ketamine in the fetal brain (or, perhaps in the premature neonatal brain when ketamine is used in the Neonatal Intensive Care Unit) reflects a reduction in the inflammatory response to bacteria or to other pathogen-associated molecular patterns to which the brain is exposed. Future work should perhaps be focused on the programming effects of maternal ventilatory hypoxia as well as elucidating possible sex effects differences induced by hypoxic episodes in late gestation.

AUTHOR CONTRIBUTIONS

EC and CW conceived and designed the experiments, collected, analyzed, and interpreted the data, drafted the article, and critically revised the article for important intellectual content. MZ, TA, and MR collected, analyzed and interpreted the data, and critically revised the article for important intellectual content. ER and MK-W interpreted the data and critically revised article for important intellectual content. All authors approved the final version submitted for publication.

FUNDING

This research was supported by the National Institutes of Health (NIH) grant HD33053 (CW), and by the NIH Training in Endocrine, Metabolic, and Prenatal Basis of Chronic Kidney Disease Predoctoral Fellowship T32DK076541 (EC and CW).

ACKNOWLEDGMENTS

We thank Ms. Xiaoying (Lisa) Fang, Ms. Kristina Steinfeldt, and Ms. Heidi Straub for their expert technical assistance. We also thank the Genomics Division of the University of Florida's Interdisciplinary Center for Biotech Research for the use of the Agilent Bioanalyzer and Agilent scanner.

REFERENCES

Anand, K. J. (2007). Pharmacological approaches to the management of pain in the neonatal intensive care unit. *J. Perinatol.* 27(Suppl. 1), S4–S11. doi: 10.1038/sj.jp.7211712

Arno, B., Grassivaro, F., Rossi, C., Bergamaschi, A., Castiglioni, V., Furlan, R., et al. (2014). Neural progenitor cells orchestrate microglia migration and positioning into the developing cortex. *Nat. Commun.* 5:5611. doi: 10.1038/ncomms6611

Assali, N. S., Holm, L. W., and Sehgal, N. (1962). Hemodynamic changes in fetal lamb in uteroin response to asphyxia, hypoxia,

and hypercapnia. *Circ. Res.* 11, 423–430. doi: 10.1161/01.RES.11.3.423

Baburamani, A. A., Castillo-Melendez, M., and Walker, D. W. (2013). VEGF expression and microvascular responses to severe transient hypoxia in the fetal sheep brain. *Pediatr. Res.* 73, 310–316. doi: 10.1038/pr.2012.191

Chang, E. I., and Wood, C. E. (2015). Ketamine attenuates the ACTH response to hypoxia in late-gestation ovine fetus. *Neonatology* 107, 249–255. doi: 10.1159/000369374

Chang, E. I., Zarate, M. A., Rabaglino, M. B., Richards, E. M., Arndt, T. J., Keller-Wood, M., et al. (2016a). Ketamine decreases inflammatory and immune pathways after transient hypoxia in late gestation fetal cerebral cortex. *Physiol. Rep.* 4:e12741. doi: 10.14814/phy2.12741

Chang, E. I., Zarate, M. A., Rabaglino, M. B., Richards, E. M., Keller-Wood, M., and Wood, C. E. (2016b). Ketamine suppresses hypoxia-induced inflammatory responses in the late-gestation ovine fetal kidney cortex. *J. Physiol.* 594, 1295–1310. doi: 10.1113/JP271066

Chang, H. C., Lin, K. H., Tai, Y. T., Chen, J. T., and Chen, R. M. (2010). Lipoteichoic acid-induced TNF-alpha and IL-6 gene expressions and oxidative stress production in macrophages are suppressed by ketamine through downregulating Toll-like receptor 2-mediated activation oF ERK1/2 and NFkappaB. *Shock* 33, 485–492. doi: 10.1097/SHK.0b013e3181c3cea5

Chen, T. L., Chang, C. C., Lin, Y. L., Ueng, Y. F., and Chen, R. M. (2009). Signal-transducing mechanisms of ketamine-caused inhibition of interleukin-1 beta gene expression in lipopolysaccharide-stimulated murine macrophage-like Raw 264.7 cells. *Toxicol. Appl. Pharmacol.* 240, 15–25. doi: 10.1016/j.taap.2009.06.013

Cohn, H. E., Sacks, E. J., Heymann, M. A., and Rudolph, A. M. (1974). Cardiovascular responses to hypoxemia and acidemia in fetal lambs. *Am. J. Obstet. Gynecol.* 120, 817–824. doi: 10.1016/0002-9378(74)90587-0

Dawes, G. S., Duncan, S. L., Lewis, B. V., Merlet, C. L., Owen-Thomas, J. B., and Reeves, J. T. (1969). Hypoxaemia and aortic chemoreceptor function in foetal lambs. *J. Physiol.* 201, 105–116. doi: 10.1113/jphysiol.1969.sp008745

Gersting, J. A., Schaub, C. E., and Wood, C. E. (2009). Development of prostaglandin endoperoxide synthase expression in the ovine fetal central nervous system and pituitary. *Gene Expr. Patterns* 9, 603–611. doi: 10.1016/j.gep.2009.08.003

Knutson, N., and Wood, C. E. (2010). Interaction of PGHS-2 and glutamatergic mechanisms controlling the ovine fetal hypothalamus-pituitary-adrenal axis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 299, R365–R370. doi: 10.1152/ajpregu.00163.2010

Luo, J., Martinez, J., Yin, X., Sanchez, A., Tripathy, D., and Grammas, P. (2012). Hypoxia induces angiogenic factors in brain microvascular endothelial cells. *Microvasc. Res.* 83, 138–145. doi: 10.1016/j.mvr.2011.11.004

Makowski, E. L., Battaglia, F. C., Meschia, G., Behrman, R. E., Schruefer, J., Seeds, A. E., et al. (1968). Effect of maternal exposure to high altitude upon fetal oxygenation. *Am. J. Obstet. Gynecol.* 100, 852–861. doi: 10.1016/S0002-9378(15)33590-0

Mark, K. S., and Davis, T. P. (2002). Cerebral microvascular changes in permeability and tight junctions induced by hypoxia-reoxygenation. *Am. J. Physiol. Heart Circ. Physiol.* 282, H1485–H1494. doi: 10.1152/ajpheart.00645.2001

Montojo, J., Zuberi, K., Rodriguez, H., Kazi, F., Wright, G., Donaldson, S. L., et al. (2010). GeneMANIA Cytoscape plugin: fast gene function predictions on the desktop. *Bioinformatics* 26, 2927–2928. doi: 10.1093/bioinformatics/btq562

Nadkarni, M. A., Martin, F. E., Jacques, N. A., and Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 148, 257–266. doi: 10.1099/00221287-148-1-257

Powers, M. J., and Wood, C. E. (2007). Ketamine inhibits fetal ACTH responses to cerebral hypoperfusion. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292, R1542–R1549. doi: 10.1152/ajpregu.00300.2006

Rabaglino, M. B., Keller-Wood, M., and Wood, C. E. (2014). Transcriptomics of the late gestation ovine fetal brain: modeling the co-expression of immune marker genes. *BMC Genomics* 15:1001. doi: 10.1186/1471-2164-15-1001

Sakai, T., Ichiyama, T., Whitten, C. W., Giesecke, A. H., and Lipton, J. M. (2000). Ketamine suppresses endotoxin-induced NF-kappaB expression. *Can. J. Anaesth.* 47, 1019–1024. doi: 10.1007/BF03024876

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Shimaoka, M., Iida, T., Ohara, A., Taenaka, N., Mashimo, T., Honda, T., et al. (1996). Ketamine inhibits nitric oxide production in mouse-activated macrophage-like cells. *Br. J. Anaesth.* 77, 238–242. doi: 10.1093/bja/77.2.238

Shu, L., Li, T., Han, S., Ji, F., Pan, C., Zhang, B., et al. (2012). Inhibition of neuron-specific CREB dephosphorylation is involved in propofol and ketamine-induced neuroprotection against cerebral ischemic injuries of mice. *Neurochem. Res.* 37, 49–58. doi: 10.1007/s11064-011-0582-3

Wang, J., Duncan, D., Shi, Z., and Zhang, B. (2013). WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res.* 41, W77–W83. doi: 10.1093/nar/gkt439

Wu, G. J., Chen, T. L., Ueng, Y. F., and Chen, R. M. (2008). Ketamine inhibits tumor necrosis factor-alpha and interleukin-6 gene expressions in lipopolysaccharide-stimulated macrophages through suppression of toll-like receptor 4-mediated c-Jun N-terminal kinase phosphorylation and activator protein-1 activation. *Toxicol. Appl. Pharmacol.* 228, 105–113. doi: 10.1016/j.taap.2007.11.027

Zar, J. H. (1984). *Biostatistical Analysis*, Vol. 2. Englewood Cliffs, NJ: Prentice-Hall.

Zarate, M. A., Rodriguez, M. D., Chang, E. I., Russell, J. T., Arndt, T. J., Richards, E. M., et al. (2017). Post-hypoxia Invasion of the fetal brain by multidrug resistant Staphylococcus. *Sci. Rep.* 7:6458. doi: 10.1038/s41598-017-06789-6

Zhang, B., Kirov, S., and Snoddy, J. (2005). WebGestalt: an integrated system for exploring gene sets in various biological contexts. *Nucleic Acids Res.* 33, W741–W748. doi: 10.1093/nar/gki475

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Chang, Zarate, Arndt, Richards, Rabaglino, Keller-Wood and Wood. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 28 January 2019
doi: 10.3389/fphys.2019.00015

Emergent Prophylactic, Reparative and Restorative Brain Interventions for Infants Born Preterm With Cerebral Palsy

Megan Finch-Edmondson[1,2], *Catherine Morgan*[1,2], *Rod W. Hunt*[3,4,5,6] *and Iona Novak*[1,2]*

[1] The Discipline of Child and Adolescent Health, The Children's Hospital at Westmead Clinical School, The University of Sydney Medical School, Sydney, NSW, Australia, [2] Cerebral Palsy Alliance Research Institute, The University of Sydney, Sydney, NSW, Australia, [3] Department of Neonatal Medicine, The Royal Children's Hospital, Melbourne, VIC, Australia, [4] Department of Paediatrics, University of Melbourne, Melbourne, VIC, Australia, [5] Neonatal Research, Murdoch Children's Research Institute, Melbourne, VIC, Australia, [6] Department of Obstetrics and Gynaecology, Monash University, Melbourne, VIC, Australia

Worldwide, an estimated 15 million babies are born preterm (<37 weeks' gestation) every year. Despite significant improvements in survival rates, preterm infants often face a lifetime of neurodevelopmental disability including cognitive, behavioral, and motor impairments. Indeed, prematurity remains the largest risk factor for the development of cerebral palsy. The developing brain of the preterm infant is particularly fragile; preterm babies exhibit varying severities of cerebral palsy arising from reductions in both cerebral white and gray matter volumes, as well as altered brain microstructure and connectivity. Current intensive care therapies aim to optimize cardiovascular and respiratory function to protect the brain from injury by preserving oxygenation and blood flow. If a brain injury does occur, definitive diagnosis of cerebral palsy in the first few hours and weeks of life is difficult, especially when the lesions are subtle and not apparent on cranial ultrasound. However, early diagnosis of mildly affected infants is critical, because these are the patients most likely to respond to emergent treatments inducing neuroplasticity via high-intensity motor training programs and regenerative therapies involving stem cells. A current controversy is whether to test universal treatment in all infants at risk of brain injury, accepting that some patients never required treatment, because the perceived potential benefits outweigh the risk of harm. Versus, waiting for a diagnosis before commencing targeted treatment for infants with a brain injury, and potentially missing the therapeutic window. In this review, we discuss the emerging prophylactic, reparative, and restorative brain interventions for infants born preterm, who are at high risk of developing cerebral palsy. We examine the current evidence, considering the timing of the intervention with relation to the proposed mechanism/s of action. Finally, we consider the development of novel markers of preterm brain injury, which will undoubtedly lead to improved diagnostic and prognostic capability, and more accurate instruments to assess the efficacy of emerging interventions for this most vulnerable group of infants.

Keywords: preterm, brain injury, cerebral palsy, neuroplasticity, neuroprotection, neuro-regeneration, neuro-repair

OPEN ACCESS

Edited by:
Carina Mallard,
University of Gothenburg, Sweden

Reviewed by:
Emily Camm,
University of Cambridge, United Kingdom
Angela Leigh Cumberland,
RMIT University, Australia

***Correspondence:**
Iona Novak
inovak@cerebralpalsy.org.au

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *15 October 2018*
Accepted: *08 January 2019*
Published: *28 January 2019*

Citation:
Finch-Edmondson M, Morgan C, Hunt RW and Novak I (2019) Emergent Prophylactic, Reparative and Restorative Brain Interventions for Infants Born Preterm With Cerebral Palsy. Front. Physiol. 10:15. doi: 10.3389/fphys.2019.00015

PREMATURITY

Prematurity remains the leading cause of morbidity and mortality in childhood within the developed world. Global rates of prematurity range from 5 to 18% of all births (Blencowe et al., 2012). In 2015, 9% of all Australian births occurred <37 weeks' gestational age, giving a burden of prematurity of around 27,000 babies (AIHW, 2017). The incidence of babies born extremely prematurely (22–27^{+6} weeks' gestation) is lower, however over 3,000 Australian babies were born very preterm (<30 weeks' gestation) during the same period (AIHW, 2017). Advances in neonatal intensive care ensure that 85% of these very preterm infants survive, but 55% survive with neurobehavioral impairments, 22% with intellectual disability and 7% with cerebral palsy (Aarnoudse-Moens et al., 2009). In the long term it is estimated that their lifetime cost to the health economy will exceed $200 million (Access Economics, 2008).

Current Usual Care for Preterm Infants

Usual care of the preterm infant in the Neonatal Intensive Care Unit (NICU) environment is generally highly protocolized and whilst some variation in practice exists between units, the basic "recipe" is the same (Malcolm, 2014). Almost without exception preterm infants with surfactant deficiency receive exogenous surfactant, with the expectation that reduced severity of lung disease will minimize hypoxia and fluctuations in blood pressure (Polin and Carlo, 2014). Oxygen saturations are targeted to around 90–95%, with a recent increase in our understanding that both hypoxia and hyperoxia increase the risk of cerebral and other end organ injury (Askie et al., 2017). Mean blood pressure is targeted to the gestational age at birth in weeks, and fluctuations in blood pressure are avoided where possible, through judicious use of fluid following assessment of fluid status by clinical indicators such as urine output, perfusion and in some centers, ultrasound measures (Kenner et al., 1998). Where there is any risk of infection/inflammation, there is generally a low threshold for the use of broad spectrum antibiotics to cover against the common bacteria that pose a risk to the preterm infant, most notably Group B streptococcus and *Escherichia coli* (Simonsen et al., 2014). The most premature and smallest infants are at most risk of organ damage, however a recent study has demonstrated that even those infants born in the late premature phase are at risk of neurodevelopmental impairments (Cheong et al., 2017). The goal of all of these therapies collectively is to maintain adequate and steady perfusion and oxygenation of the brain to avoid inflammation and brain injury, but none specifically target either neuroprotection or neural repair.

Brain Injury in Preterm Infants

Brain Injury Diagnostics

Historically, cerebral injury during the perinatal period until term corrected age was detected with cranial ultrasound (cUS). However, the sensitivity and specificity of this imaging modality is variable between operators. cUS remains in common use because of its clinical utility with imaging possible at the bedside, and lower costs compared to magnetic resonance imaging (MRI). With cUS, it is possible to detect common patterns of severe preterm brain injury, such as intraventricular hemorrhage (IVH) and periventricular leukomalacia (PVL), however subtle lesions can be indiscernible (Hintz et al., 2007). Serial cUS is therefore widely used for screening, however approximately one third of preterm infants diagnosed with cerebral palsy may not have lesions identified by cUS (Beaino et al., 2010). The accuracy of cUS to predict long term disability is enhanced when scans are taken sequentially until term-equivalent age, and are then combined with MRI to detect and classify the presence of white matter injury (Martinez-Biarge et al., 2016). MRI provides better anatomical detail and has the sensitivity to detect subtle white matter injury that may not be discernible on ultrasound. However, in experienced hands cUS can still be reliable in the detection of cerebral injury, and whilst MRI provides modest benefit to injury detection (Leijser et al., 2008; Edwards et al., 2018), its cost and availability preclude its routine use in some centers.

White Matter Injury

With increasing use of MRI, a more insidious cerebral insult—*diffuse white matter injury* has been recognized. Damage to the white matter is the most common type of preterm brain injury with severity ranging from mild to severe (Inder et al., 2003). In white matter injury, the preoligodendrocytes are "arrested" in their development, preventing full maturation and thus myelination (Ferriero, 2016). In addition, recurrent infection and long term ventilation exacerbate neuronal and axonal damage (Khwaja and Volpe, 2008), and results in a higher likelihood of white matter injury (Smilga et al., 2018).

Cystic PVL is a condition in which cystic cavitations develop in the periventricular white matter, and their extent can be graded from grade 1 to 3 (classification by De Vries et al., 1992). PVL has been associated with spastic cerebral palsy, with severity of impairment influenced by the location and extent of the lesions (De Vries et al., 2011). With increasing use of MRI and detection of more subtle white matter injury, the term PVL has been applied to non-cystic diffuse white matter injury (Khwaja and Volpe, 2008). Non-cystic PVL is characterized by microscopic focal necroses which generally evolve to form glial scars (Khwaja and Volpe, 2008). The prevalence of cystic PVL has been declining for some time (Van Haastert et al., 2011), and non-cystic PVL now accounts for the majority of cerebral white matter injury observed in premature infants (Khwaja and Volpe, 2008).

Inder et al. showed that cerebral injury in premature infants typically manifests as diffuse white matter atrophy, ventriculomegaly, immature gyral development, and enlarged subarachnoid space (Inder et al., 2003). In addition, this group (Inder et al., 2005) have shown that white matter injury results in reductions in volumes of deep nuclear gray matter and cortical gray matter as well as disturbed white matter microstructure (Cheong et al., 2009).

In addition to these abnormalities, children born prematurely experience interruptions to their third trimester brain development, growth and gyration, and arrested development of the oligodendrocyte cell lineage which disrupts myelination and brain connectivity development (Back et al., 2001). Recent

research has demonstrated that preterm infants are at greater risk of: (a) neurodevelopmental difficulties at 2 years (Woodward et al., 2006); (b) motor deficit at 4 years (Spittle et al., 2011); and (c) a range of functional outcomes at 7 years (Anderson et al., 2017). Moreover, preterm birth is a risk factor for lower IQ and poorer educational performance (Cheong et al., 2013). Doyle et al. have shown that biological influences on cerebral structure in early postnatal life have a greater influence on long term outcome than subsequent early childhood environmental factors (Doyle et al., 2015).

Intraventricular Hemorrhage

IVH is bleeding inside the lateral ventricles and occurs in up to 45% of extremely preterm-, and 30% of very preterm- infants (Mukerji et al., 2015). IVH is graded from mild (grade 1) to severe (grade 4), according to a grading system first described by Papile in 1978, and is based on the amount of bleeding into the ventricle, the presence of ventricular dilatation and involvement of the surrounding white matter (Papile et al., 1978). The presence of IVH poses a risk of subsequent post hemorrhagic ventricular dilatation, the risk of which increases from 5% with grade 1 IVH to over 80% with grade 4 (Murphy et al., 2002).

Risks of neurodisability and mortality increase with increasing grades of IVH in a similar fashion. Most studies report a higher likelihood of cerebral palsy with increasing severity of IVH (Mukerji et al., 2015). Beaino et al. reported significantly increased odds ratios of cerebral palsy at 5-years when comparing grade 4 to no IVH (Beaino et al., 2010). Contrastingly, cerebral palsy following grade 3 IVH is variably reported, but the likelihood is higher when post hemorrhagic ventricular dilatation occurs. Although bilateral IVH is common, parenchymal lesions are typically restricted to one side, and thus often lead to unilateral spastic cerebral palsy (De Vries et al., 2011).

CEREBRAL PALSY

Brain injury detected in the newborn period is predictive of cerebral palsy (Linsell et al., 2016). White matter injury, chiefly PVL, and IVH are commonly reported in preterm infants later diagnosed with cerebral palsy (Kidokoro et al., 2014).

Cerebral palsy is an overarching term used to describe a group of disorders of movement and posture that occur due to damage to the developing brain during pregnancy, around the time of birth, or in the first 28 days of life. In many countries post-neonatal brain injury up until 2 years of age (such as stroke or near-drowning) is also included in the definition of cerebral palsy. Registry data in many parts of the world provides useful information about the cerebral palsy prevalence, type and topography related to gestational age. In Australia about 43% of people with cerebral palsy were born preterm, however this proportion is likely to be higher in the United States where a greater proportion of babies are born preterm (7% in Australia vs. 10% in the US) (Martin et al., 2018). Recently published register data shows that the incidence of cerebral palsy in preterm and term populations is starting to fall in high-income countries (1.4–2.1/1,000 live births) (Galea et al., 2018). Moreover, the percentage of children with moderate to severe disability is also declining (Galea et al., 2018).

It has been well established that the rate of cerebral palsy in preterm survivors is inversely related to gestational age (Himpens et al., 2008). A more recent review by Linsell et al. found that gestational age surprisingly did not predict cerebral palsy although included studies were 32 weeks or below (Linsell et al., 2016). It appears that after 27 weeks, rates of cerebral palsy drop off quite quickly. All subtypes of cerebral palsy can result from preterm brain injury although milder subtypes are most common.

Types of Cerebral Palsy

Cerebral palsy can be classified by the type of movement disorder or the parts of the body that are affected. The most common type is spastic cerebral palsy accounting for about 85% of all cerebral palsy (ACPR Group, 2018). Dyskinetic cerebral palsy accounts for 7%, with ataxic and hypotonic cerebral palsy accounting for a combined further 7% (ACPR Group, 2018). Infants with cerebral palsy who are born preterm (<37 weeks') are most likely to have bilateral cerebral palsy, with a diplegic pattern more common than quadriplegia (44 vs. 19%), while unilateral cerebral palsy is diagnosed in about 28% of preterm babies with cerebral palsy (Towsley et al., 2011; Reid et al., 2014; ACPR Group, 2018). Where PVL is cystic and widespread, spastic quadriplegia is the most common outcome (Novak et al., 2017). In these more severe cases it is not just the white matter that is involved but also the other cerebral structures e.g., deep nuclear gray matter, leading to the description "encephalopathy of prematurity" (Volpe, 2009).

Comorbidities

The risk for comorbidities with cerebral palsy is very high, and for the most part correlates with severity of the brain injury and physical disability. Common and disabling comorbidities and co-occurring functional impairments with cerebral palsy include: chronic pain (3 in 4); intellectual disability (1 in 2); hip displacement (1 in 3); epilepsy (1 in 4); behavior disorders (1 in 4); nonverbal (1 in 4); sleep disorders (1 in 5); vision impairment (1 in 10); and hearing impairment (1 in 25) (Novak et al., 2012).

Early Detection of Cerebral Palsy

Although preterm infants are at increased risk of cerebral palsy (Spittle et al., 2018), a diagnosis of cerebral palsy has traditionally not been made until 12–24 months, unless a severe disability was clearly evident in the neonatal intensive care period (ACPR Group, 2018). An international clinical guideline, based upon systematic review evidence, indicates cerebral palsy can now be accurately identified in children as young as 12-weeks corrected age (Novak et al., 2017). Since no biomarkers yet exist for cerebral palsy, the guideline recommends using a combination of standardized assessment tools. The results of these tests should triangulate, with all the tests pointing in the direction of high-risk for cerebral palsy. If the test results are incongruent, the child may have another disability that is not cerebral palsy. The tools with the best psychometric properties for accurately predicting cerebral palsy by 3-months corrected age include: (1) Prechtl's General Movements Assessment (a video-based

observation of the infant's spontaneous movement, scored for quality of movement) (98% sensitivity); (2) term-age equivalent MRI indicating damage to the motor areas of the brain (86–89% sensitivity); and (3) the Hammersmith Infant Neurological Examination (a scored neurological physical examination, with risk cut points determined from large sample studies) (90% sensitivity) (Novak et al., 2017). The results of these assessments should be carefully and compassionately communicated to the child's parents. Early diagnosis enables referral to early intervention to improve the child's outcomes, prevent secondary complications and protect parental mental health (Novak et al., 2017).

EMERGENT INTERVENTIONS

There is a wide range of therapeutic interventions that have been proposed and are currently being tested to promote neurological repair in both infants and adults. Wherever possible, in this review, we present data generated from preclinical and clinical studies specifically utilizing preterm models and/or targeting premature populations. Where there is little published evidence, we have cast our net wider to include studies focusing on related neurological conditions such as adult stroke, traumatic brain injury and neurodegenerative conditions. Caution is warranted when interpreting the utility of interventions that are effective in adults back to the preterm infant, as the preterm baby is not simply a small adult, and their physiology often times contradicts the adult literature. Nevertheless, the results of some of these studies may be found to be relevant for preterm brain injury, and thus we see value in reviewing these studies, when there is little preterm-specific evidence available. In **Table 1** we provide a summary of the interventions covered in this review, their mechanism of action, the current phase of research translation, route and timing of administration, plus, potential advantages and controversies to overcome through research. In addition, the timing of administration for known effective and emergent therapies for preterm infants is further illustrated in **Figure 1**.

Biological and Pharmacological Therapies

Cell-Based Therapy

Cell therapies offer great promise for treating neurological diseases and are emerging as a new paradigm in human medicine (Trounson and Dewitt, 2016). The mechanisms by which candidate cell therapies might work for brain injury include (1) anti-inflammatory mechanisms: attenuation of the inflammatory immune response to brain injury via a reduction in the release of excitotoxins, cytotoxins, and reactive oxygen species; (2) trophic mechanisms: to promote cell survival via release of neurotrophic factors to induce endogenous cell migration, proliferation and differentiation and/or promote angiogenesis; and (3) regenerative mechanisms: replacement of damaged brain tissue by engraftment, proliferation and differentiation of transplanted cells (reviewed in Novak et al., 2016). Different types of cells have been proposed, including: amnion epithelial cells (AECs); mesenchymal stromal/stem cells (MSCs); umbilical cord blood (UCB), and neural progenitor/neural stem cells (NSCs) (Novak et al., 2016), each with different advantages and disadvantages that must be considered when contemplating therapeutic application. Substantial preclinical evidence now exists to support the conduct of human trials evaluating efficacy of various cell therapies for perinatal brain injury (reviewed by Fleiss et al., 2014).

There are ethical complexities and controversies to be considered when conducting clinical trials of stem cells in newborns with perinatal brain injury. First, these children have a lot to gain but also a lot to lose from an adverse event. Thus, human ethics committees often favor "first in human" studies to be conducted in adult populations where the patient can consent, rather than consent being obtained by proxy from a parent. Hence there have been no clinical trials in preterm infants where the cells have been delivered in the acute newborn period. Second, not all preterm babies have a disability. Accurate determination of which patients will have a normal outcome and which patients will have a disability can take months, and even years for subtle literacy problems in reading and writing to be apparent. Therefore, the dilemma exists about timing of stem cell treatment for preterm infants. Should universal treatments be applied to all preterm patients in the newborn period given that the risks of disability are high, and the newborn period is the optimal therapeutic window in terms of an effect in the acute phase? Or should we wait and provide targeted therapies once the children with definite long-term disability can be identified, all the while lowering the potential treatment effect size from late intervention in the chronic phase of brain injury? Third, the mechanism of action should match the clinical indication. Cells that have anti-inflammatory and trophic properties (i.e., AECs, MSCs, and UCB) will theoretically will be of most benefit during the acute period of neuroinflammation, if neuro-repair is sought. If administered in the chronic phase, smaller gains may still be possible via trophic effects and the emerging evidence that inflammation persists into the tertiary phase of injury (Fleiss and Gressens, 2012). Contrastingly, cells that have regenerative capacity (i.e., NSCs) are a better therapeutic target for chronic stage injury. Fourth the origin of the cells can be controversial for some. AEC, MSC, and UCB cells can be obtained using morally uncomplicated methods: AECs are obtained from placental tissue under maternal consent, UBC from cords with maternal consent, and MSCs are available in off-the-shelf commercial formats or can be obtained autologously from bone marrow harvesting with patient consent. NSCs cannot be safely obtained from adult donors and therefore need to be obtained with consent from embryonic or fetal sources, or grown autologously from induced pluripotent cells.

Given the challenges of designing stem cell trials for preterm infants, there are limited published studies. However, Rudnicki et al. assessed the safety and feasibility of autologous UCB for extremely preterm (<32 weeks') neonates who developed anemia due to prematurity (Rudnicki et al., 2015). In this trial, infants received an intravenous transfusion of 15 mL/kg of body weight of either autologous UCB ($n = 5$) or allogeneic red blood cells ($n = 9$; control group), administered on average 3.2 and 7.8 days after birth for UCB and red blood cells, respectively. Results from this small study group indicated that autologous UCB transplantation in preterm newborns was generally safe and well

TABLE 1 | Potential therapeutic agents and research progress.

Agent	Mechanism of action	Sub-population of patients that might be treatment responsive	Research phase for preterm brain injury				Route of administration	Timing of administration	Advantages	Disadvantages/ controversies
			Preclinical	Phase 1	Phase 2	Phase 3				
Cell therapy|amnion epithelial cells (AEC) Cells obtained from the placenta	Anti-inflammatory and trophic TARGET = Motor, cognitive, behavioral and respiratory gains	• BPD	✓	✓	✗	✗	IV	If 28-weeks GA and still requiring O2 at 36-weeks GA	• Proven safe for newborns in BPD Phase 1 trial (Lim et al., 2018) • Easily obtained from placenta • Morally uncomplicated source of cells • Low immunogenicity • Can be stored for later use • Can be isolated and expanded to treat multiple patients • Crosses the BBB	• Unknown if it is better to administer AECs as a universal treatment for all preterm births at risk of disability, in the very acute phase OR whether to wait until a disability can be diagnosed and then offer a targeted AEC treatment later after injury
		• All preterm births as a universal neuro-protectant	✓	✗	✓	✗	IV	3 doses in the first week of life		
Cell therapy|mesenchymal stem cells (MSC) Multipotent adult stem cells, found in bone marrow and umbilical cord tissue and blood	Anti-inflammatory and trophic TARGET = Motor and cognitive gains	• CP >6-months of age, of all sub-types and causal pathways	✓	✓	✓	✗	IV and intrathecal from autologous source, bone marrow derived	Tested late in the tertiary phase of injury. Autologous MSC doses of 1–2 $\times 10^7$	• Proven safe in humans • Can be obtained autologously from bone marrow or umbilical cord • Available in off-the-shelf commercial formats for emergency and high/multi-dose administration • Low immunogenicity • Can be stored for later use • Can be isolated and expanded • Short survival following transfusion, lowering the risk of adverse events • Some MSCs may cross the BBB	• Unknown if the cells might be more efficacious when transplanted stereotactically to increase the concentration of cells crossing the BBB • Unknown if the cells might be more efficacious when transplanted in combination with other stem cell types with longer duration effects
		• IVH	✓	✓	✗	✗	Intra-cerebroventricular injection	Within 7 days of IVH		
		• Perinatal stroke	✓	✗	✗	✗	Intranasal and intra-arterial	On day 1 of injury		

(Continued)

TABLE 1 | Continued

Agent	Mechanism of action	Sub-population of patients that might be treatment responsive	Research phase for preterm brain injury				Route of administration	Timing of administration	Advantages	Disadvantages/ controversies
			Preclinical	Phase 1	Phase 2	Phase 3				
									• Bone marrow derived MSCs shown to be more effective than bone marrow mononuclear cells in a clinical trial for CP (Liu et al., 2017)	
Cell therapy|neural stem cells (NSC) Stem cells that make the 3 types of brain cells	Regenerative, anti-inflammatory and trophic TARGET = Cure or Motor and cognitive gains in tertiary phase injury	• CP 1 year old and up, of all sub-types and causal pathways	✓	✓	✓	✗	Intracranial, stereotactically placed neurosurgically	Unknown, may be effective in the tertiary phase of injury as a cure	• Regenerative capabilities • Shown to be safe in human trials when transplanted with immunosuppression • Can be stored for later use • One donor's cells can be expanded to treat multiple patients	• Must be obtained from embryos, fetal or iPSC sources, as it is unsafe for adults to donate • Neurosurgical administration required
Cell therapy|umbilical cord blood (UCB) Umbilical cord blood rich in an array cell types including stem cell populations	Anti-inflammatory and trophic TARGET = Motor and cognitive gains in late tertiary phase treatment; disability-free survival in primary and secondary phase treatment	• CP 1 year old and up, of all sub-types and causal pathways	✓	✓	✓	✗	IV (Autologous and allogeneic)	Tested late in the tertiary phase of injury. Autologous UCB doses >2 × 10^7/kg produced better results (Sun et al., 2017)	• Proven safe in humans • Easily obtained from umbilical cords • Morally uncomplicated source of cells • Source can be autologous or allogeneic and can be HLA matched • Can be stored for later use • Theoretically can be expanded	• Difficult to collect and reinfuse autologous cords in an emergency birth with HI injury • Unknown risks of autologous reinfusion for genetic and/or infection causal pathways to CP, i.e., could the injury be worsened? • Preterm infants have small low volume cords with a different cell make-up to term cords, and therefore autologous infusions may have less therapeutic value
		• HI injury	✓	✓	✓	✗	IV (autologous)	After 6 h of injury and initiation of hypothermia. Multiple doses in the first week of life		
		• All preterm births as a universal neuro-protectant	✓	✗	✗	✗	IV (allogeneic)	Unknown		

(Continued)

TABLE 1 | Continued

Agent	Mechanism of action	Sub-population of patients that might be treatment responsive	Research phase for preterm brain injury				Route of administration	Timing of administration	Advantages	Disadvantages/ controversies
			Preclinical	Phase 1	Phase 2	Phase 3				
										• Efficacy appears higher with higher HLA matching, meaning large, diverse cord banks are needed • Need for off-the-shelf expanded products for emergency and/or high-dose/repeat treatments • Unknown GVHD risk from combining allogeneic cords to deliver high/multi-dose treatments • Unknown effect of delayed cord clamping on cord volumes and efficacy
Extracellular vesicles (EVs) Membrane fragments released from cells (exosomes and microvesicles) with neuroprotective effects	Anti- inflammatory, restoring myelination and cell microstructure TARGET = Cognitive gains	• Preterm stroke • HI injury	✓	✗	✗	✗	IV	Unknown, but presumably in the primary and secondary phases of injury	• Safer alternative to stem cells • Can be stored for later use • Collected in large volumes • Administered off-the-shelf • Low immunogenicity • Can transport cargo • Crosses the BBB	• Autologous preterm derived EVs may lack sufficient aerobic potential to be therapeutic and therefore allogeneic term equivalent sources may be required
Erythropoietin (EPO) Natural red blood cell hormone with neuroprotective effects. Also manufactured synthetically enabling dose titration	Anti-inflammatory, anti-excitotoxic, anti-oxidant, trophic, plus enhanced neurogenesis and angiogenesis	• IVH • Hemorrhagic parenchymal infarction • HI injury	✓	✓	✓	✓	IV	3 doses of 1,000 U/kg of EPO in the first week of life, during the secondary and tertiary phases of injury (Wu et al., 2012)	• Proven safe in neonates • Administered off-the-shelf • Can be administered in the tertiary phase of injury	• Cost • Refrigeration requirements • Lack of standardization between international products

(Continued)

TABLE 1 | Continued

Agent	Mechanism of action	Sub-population of patients that might be treatment responsive	Research phase for preterm brain injury				Route of administration	Timing of administration	Advantages	Disadvantages/ controversies
			Preclinical	Phase 1	Phase 2	Phase 3				
	TARGET = Cognitive (and motor) gains									• Some infants will be discharged from NICU before all 3-doses can be administered
Melatonin Natural hormone that regulates circadian rhythm. Also manufactured synthetically enabling dose titration	Anti-inflammatory and anti-apoptotic TARGET = Motor and cognitive gains	• IUGR	✓	✓	✓	✗	Oral (Maternal)	Antenatal 3x daily 10 mg, from baseline until birth	• Administered off-the-shelf • Low cost • Proven safe in humans	• Large sample sizes required to run Phase 3 public health trials, powered to detect a protective benefit
		• HI injury	✓	✓	✗	✗	Enteral	0.5–3-5 mg/kg dose escalation in first 6 h of life		
Creatine Natural diet compound that builds muscle, with neuroprotective effects	Anti-excitotoxic and anti-apoptotic TARGET = Disability-free survival from HI injury	• Mothers with pre-eclampsia, cervical incompetence, placental abruption, placental previa, IUGR	✓	✗	✗	✗	Oral diet supplementation (Maternal)	Antenatal, especially during the 3rd trimester of pregnancy	• Proven safe in humans • Straightforward bioavailability • Administered off-the-shelf • Low cost • Simple and feasible intervention for patients to adhere to • Manufactured synthetically enabling dose titration • May have the added benefit of reducing labor pains	• Large sample sizes required to run Phase 3 public health trials, powered to detect a protective benefit
		• TBI as proof of concept for HI injury	✓	✓	✓	✗	Oral	0.4 g/kg daily for 6-months duration		
Granulocyte-colony stimulating factor (G-CSF) hematopoietic growth factor	Stimulate neural stem and progenitor cell production TARGET = Motor gains	• CP 2-10 years old, all sub-types and causal pathways	✓	✓	✓	✗	IV	10 μg/kg for 5 days. Should be used >60 h after injury	• Appears safe	• Unclear if there is a clinical benefit

(Continued)

TABLE 1 | Continued

Agent	Mechanism of action	Sub-population of patients that might be treatment responsive	Research phase for preterm brain injury				Route of administration	Timing of administration	Advantages	Disadvantages/ controversies
			Preclinical	Phase 1	Phase 2	Phase 3				
Thyroxine Thyroid hormone	Normalization of thyroid levels as a neuroprotectant TARGET = Disability free survival	• Hypothyroxinemia • IVH	✓	✓	✓	✓	IV	Birth to 42-days	• Proposed reduced mortality • Proposed improved brain structure	• Clinical trials indicated improved long-term neurodevelopmental outcomes but these results are not supported in a meta-analysis
Minocycline Broad-spectrum antibiotic	Inhibition of microglial activation TARGET = Motor and cognitive gains	• HI injury	✓	✗	✗	✗	IV	Before and after injury	• Promising preclinical data indicating a reduction in white matter damage	• Conflicting results in human studies of other adult degenerative neurological disorders, with some studies showing harm • Timing of administration affects the result
Epidermal growth factor (EGF) Growth factor	Regulation of NSC migration, proliferation and oligodendrocyte differentiation to increase myelination TARGET = Motor gains	• Focal demyelination • HI injury • IVH	✓	✗	✗	✗	Overexpression of EGF receptor in oligodendrocytes Intranasal Intra-cerebroventricular injection	Before and after injury Immediately after injury 3 doses at 24, 72 and 120 h post-IVH	• Preliminary preclinical data indicating increased myelination and functional recovery	• Cost • Can induce neurotoxicity • Might promote tumor growth and progression
Diazoxide Vasodilator	Prevention of hypoglycemia TARGET = Motor and cognitive gains	• HI injury	✓	✗	✗	✗	Intraperitoneal	Daily during induction of HI injury	• Well established safety profile (from other indications) • Rapid acting • Inhibits the secretion of insulin	• Does not cross the BBB but affects circulation • May cause hypotension • Fluid retention
Nanoparticles Microscopic particles including dendrimers (branched molecules that can be tailored to store and transport materials)	Drug or gene transporter TARGET = Motor gains; reduction in rates of preterm birth	• HI injury • Infection and PVL	✓	✗	✗	✗	IV	Unknown, but presumably in the primary, secondary and tertiary phases of injury when inflammation is present	• Can transport cargo, including anti-inflammatory agents • Crosses the BBB • Targets abnormal microglia and astrocyte activity • Low immunogenicity	• High manufacturing costs • Potential toxicity

(Continued)

TABLE 1 | Continued

Agent	Mechanism of action	Sub-population of patients that might be treatment responsive	Research phase for preterm brain injury				Route of administration	Timing of administration	Advantages	Disadvantages/ controversies
			Preclinical	Phase 1	Phase 2	Phase 3				
Gene Therapy Delivery of DNA as a drug	Anti-excitotoxic to excessive glutamate via gene delivery of BDNF TARGET = Motor gains via protection of white matter volume loss	• PVL	✓	✗	✗	✗	Intracerebellar	Research indicates in the primary and secondary phases of injury (Gressens et al., 1997)	• Unknown	• Prolonged administration required because precise timing of the insult is usually unknown • Neurosurgical administration required
Parent attachment training Teaching parents to read and respond sensitively to their infant's cues	Experience dependent plasticity	• IVH • PVL	✓	✓	✓	✗	Parent Education Manual guidance	1 h weekly	• Lasting benefits to cognition	• Heterogeneous, under powered samples • Limited effect on motor skills (Nelson et al., 2001; Ohgi et al., 2004)
NIDCAP A low stress and low stimuli NICU environment that mimics the uterine environment	Experience dependent plasticity	• IVH • PVL • Stroke • HIE	✓	✓	✓	✓	Adapted environment e.g., low lighting and noise	24/7	• Possible short term protection of brain structure	• No long term benefits conferred (Ohlsson and Jacobs, 2013)
SPEEDI Parent coaching and environmental enrichment	Experience dependent plasticity Parent Education	• IVH • WMI • HIE • Hydrocephalus	✓	✓	✓	✗	Adapted environment e.g., promoting child active learning	21 days + 12 weeks of daily practice	• Improved problem solving (Dusing et al., 2018)	• Heterogeneous, under powered samples
GAME Task-specific motor training, parent coaching and environmental enrichment	Experience dependent plasticity Parent education	• IVH • PVL • Stroke • HIE	✓	✓	✓	✓	Adapted environment Task practice	Daily 45 min of practice by the child (Morgan et al., 2016b)	• Improved motor skills • Improved cognitive skills	• Co-occurring vision impairment may lower the rate of gains

✓ = Yes; ✗ = No; AEC, Amnion epithelial cells; BBB, Blood brain barrier; BDNF, Brain-Derived Neurotrophic Factor; BPD, Bronchopulmonary dysplasia; CP, Cerebral palsy; CIMT, Constraint induced movement therapy; DNA, Deoxyribonucleic acid; EGF, Epidermal growth factor; EPO, Erythropoietin; EVs, Extracellular vesicles; g, grams; GA, Gestational age; GAME, Goals Activity Motor Enrichment; GVHD, Graft vs. host disease; HI, Hypoxic ischemic; HIE, Hypoxic ischemic encephalopathy; HLA, Human Leukocyte Antigens; iPSC, Induced pluripotent stem cell; IUGR, Intrauterine growth restriction; IV, Intravenous; IVH, Intraventricular hemorrhage; kg, kilograms; µg, micrograms; mg, milligrams; MSC, Mesenchymal stem cell; MRI, Magnetic resonance imaging; NICU, Neonatal Intensive Care Unit; NIDCAP, Newborn Individualized Care and Assessment Program; NSC, Neural stem cell; O2, Oxygen; PVL, Periventricular leukomalacia; SPEEDI, Supporting Play Exploration and Early Developmental Intervention; TBI, Traumatic brain injury; U, Units; UCB, Umbilical cord blood; WMI, White Matter Injury.

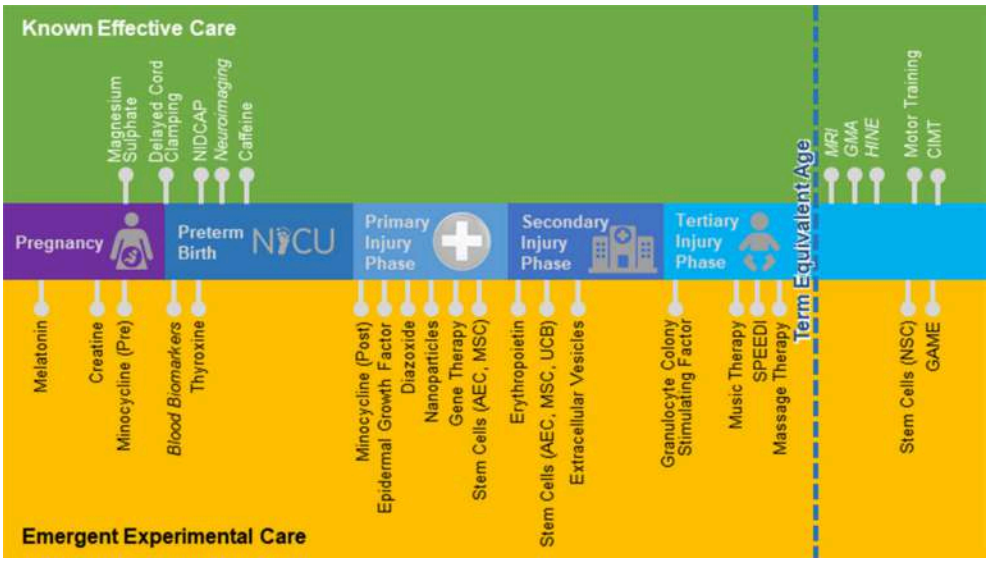


FIGURE 1 | Known effective and emergent treatments and assessments. AEC, Amnion epithelial cells; CIMT, Constraint induced movement therapy; GAME, Goals Activity Motor Enrichment; GMA, General Movements Assessment; HINE, Hammersmith Infant Neurological Examination; MSC, Mesenchymal stem cell; MRI, Magnetic resonance imaging; NICU, Neonatal Intensive Care Unit; NIDCAP, Newborn Individualized Care and Assessment Program; NSC, Neural stem cell; SPEEDI, Supporting Play Exploration and Early Developmental Intervention; UCB, Umbilical cord blood.

tolerated. Recently published, Won Soon Park's team at Samsung Medical Center in South Korea conducted a Phase 1 dose-escalation study of Pneumostem® transplantation in preterm infants with severe IVH (grade 3–4) (NCT02274428) (Ahn et al., 2018). Pneumostem® is a human allogeneic UCB-derived MSC product. Eligible infants were born at 23–34 weeks' gestation, and received cells (5×10^6 cells/kg or 1×10^7 cells/kg) within 7 days of IVH diagnosis. Study results indicated that Pneumostem® transplantation by intracerebroventricular injection was safe; cell transplantation at both doses was not associated with any serious adverse events or dose-limiting toxicity, and there were no cases of mortality in their cohort of 9 infants (Ahn et al., 2018). This is being followed up by a currently-recruiting Phase 2a study to evaluate the efficacy and safety of intraventricular administration of Pneumostem® for treatment of IVH (grade 3–4) in 22 high-risk premature infants (NCT02890953). Participants will be randomized to receive either Pneumostem® or saline control within 28 postnatal days. Outcomes include death, ventricular shunt operation, and ventricular dilatation.

There are now multiple clinical trials underway examining the safety and efficacy of cell therapies (chiefly MSCs and AECs) for other morbidities associated with prematurity, in particular bronchopulmonary dysplasia. It is feasible that these therapies may provide non-specific neuro-protective benefit through reduction of systemic inflammation. These trials should therefore be designed (e.g., length of follow up), and sufficiently powered, to capture any potential neurological benefits of these therapies. As an example, the long-term safety and efficacy follow-up study of Pneumostem® (NCT02023788) in patients who completed the previously mentioned Phase I study (NCT02274428) includes neurological development test outcomes at 5 years (corrected age) comprising the K-ASQ (Korean Ages and Stages Questionnaires) and Bayley Scales of Infant and Toddler Development (Bayley).

Although not specifically targeting preterm infants, the following clinical trials warrant mention. First is the open label trial of 23 term-born neonates (≥35 weeks') with hypoxic ischemic encephalopathy (HIE) who received non-cryopreserved autologous volume- and red blood cell-reduced UCB intravenously (Cotten et al., 2014). Infants received up to four infusions of $1–5 \times 10^7$ cells/kg, with the first dose as soon as possible after birth, and at 24, 48, and 72 postnatal hours. The authors found that whilst autologous UCB for infants with HIE is feasible, UCB collection at delivery for all "obstetric emergencies" was a challenge that initially resulted in extremely low recruitment rates; they originally intended to recruit 52 HIE infants, however only enrolled 23. They go on to describe how this challenge was overcome by multidisciplinary collaboration. Nevertheless, timely collection of autologous UCB may be a potential barrier for this therapy in certain settings. There are now multiple currently-recruiting Phase 1 and 2 studies of autologous UCB for HIE listed on clinical trial registries. These include the follow-on study (NCT02612155) at Duke University, as well as other studies in the United States (NCT02434965), France (NCT02881970), and China (NCT02551003, NCT03352310). Finally, for a different indication, there is the phase 1/2 open-label study of intranasally administered bone marrow-derived allogeneic MSCs (5×10^7 cells) in term neonates (≥36 weeks') with perinatal arterial stroke (PAIS) (NCT03356821). Referred to as the PASSIoN trial, this study aims to recruit 10 participants for cell administration as soon as possible after confirmation of PAIS (maximum within the

first week). PAIS is an important perinatal cause of long-lasting neurodevelopmental problems.

Due to the limited number of published clinical trials of stem cells for perinatal/preterm brain injury, it is not surprising that no systematic reviews have yet been published. However, three reviews with meta-analyses have been conducted analyzing stem cell clinical trials for other indications including two for adult stroke and one for children with cerebral palsy. Interestingly, meta-analysis of the effects of MSCs for ischemic stroke, which included seven studies, indicated no significant difference between stem cell and cell-free treatments (Wang et al., 2016). This was in contrast to the analysis of Chen et al., which revealed stem cell transplantation for patients with ischemic stroke can significantly improve neurological deficits, motor function, daily life quality and functional independence (Chen et al., 2016). This analysis included 18 studies, the majority of which used MSCs ($n = 10$) however neural stem cells ($n = 4$), bone marrow mononuclear cells ($n = 2$), peripheral blood cells ($n = 1$), and umbilical cord MSCs ($n = 1$) were also used (Chen et al., 2016). It is therefore possible that cell types other than MSCs contributed to the larger effect size observed. For children with cerebral palsy, stem cells appeared to induce short-term improvements in gross motor skills, despite the acknowledged limitation of heterogeneous data e.g., cell type, participant age range and cerebral palsy sub-type (Novak et al., 2016). Collectively, these papers conclude that further research, in particular randomized controlled trials (RCTs), using rigorous methodologies, is warranted to determine the optimal stem cell treatments for neurological conditions.

Extracellular Vesicles (EVs)

MSCs, and likely other types of cells, exert their neuroprotective/reparative effects via secretion of extracellular vesicles (EVs), namely exosomes and microvesicles (reviewed by Willis et al., 2017). EVs are small membrane-bound particles with a size of 70–1,000 nm that contain an array of bioactive "cargo" which includes DNA, messenger RNA, microRNA, proteins, and lipids (Willis et al., 2017). EVs offer an attractive option as a novel, cell-free alternative therapy for the treatment of perinatal brain injury, since their use eliminates some of the largest perceived risks of cell therapy: tumorigenic potential of administered live cells, as well as immunologic reactions and/or rejection of transplanted cells. Another benefit of EVs is that they can be easily collected in large quantities and stored for future use (Willis et al., 2017), providing "off-the-shelf" capability for clinical emergencies.

The efficacy of EVs for perinatal brain injury is still being explored in preclinical studies, including a rodent model of inflammation-induced preterm birth with resultant brain damage (Drommelschmidt et al., 2017), and a preterm sheep model of hypoxic-ischemic (HI) brain injury (Ophelders et al., 2016). In rats, MSC-EVs ameliorated inflammation-induced cellular damage, restoring myelination deficits and abnormal microstructure, indicated by reduced fractional anisotropy measured using diffusion tensor MRI, resulting in improved long-term cognitive function assessed using the spatial probe test as an indicator for adaptive memory function (Drommelschmidt et al., 2017). Similarly in sheep, MSC-EVs partially rescued brain function following HI-induced injury, and appeared to mildly protect against hypomyelination (Ophelders et al., 2016). Additionally, there are numerous published studies demonstrating the utility of EVs for adult brain injury, in particular traumatic brain injury (TBI) (Zhang et al., 2015, 2017b; Kim et al., 2016; Li et al., 2017; Williams et al., 2019), and stroke (Otero-Ortega et al., 2018). Together, these studies highlight the potential of EVs for the treatment of brain injury, and more research into their use for neurological conditions is merited. Lessons learned from EV studies using these models may be beneficial for preterm indications.

An important consideration for EV therapy is the source of the EVs and the timing of retrieval. EVs can be derived from different cell types. It seems logical to favor autologous products because, as for cell-based therapies, they are considered safer due to the eliminated risk of immune rejection of the cell product or development of graft-versus-host disease, or transmission of infectious diseases from donor to recipient. Intriguingly, a 2016 study presents evidence that autologous EVs for preterm infants might not be best choice (Panfoli et al., 2016). In this study, exosomes derived from umbilical cord-MSCs of preterm babies (28–30 weeks' gestation) had reduced aerobic potential compared to those derived from term babies (≥37 weeks') (Panfoli et al., 2016). Reduced aerobic potential might affect their ability to rescue the bioenergetics of damaged tissues (i.e., by restoring ATP and NADH levels). Thus, preterm exosomes may have a diminished capacity to provide therapeutic benefit, which may have design implications for EV-therapies in preterm infants.

Interestingly, EVs may also be utilized to deliver gene therapy to target cells. In a series of *in vitro* experiments, Lee et al., used MSCs genetically modified to express fluorescently labeled microRNA-124 to demonstrate that MSCs can deliver microRNA to neural cells via the secretion of exosomes (Lee et al., 2014). MicroRNA-124 was selected because it plays a role in neurogenesis, indeed miRNA-124 is the most abundant miRNA in the brain, which suppresses cell proliferation, promotes neuronal differentiation and neurite outgrowth, and represses astrocytic differentiation (reviewed in Sun et al., 2015). Consistent with this, delivery of microRNA-124 induced neuronal differentiation in targeted neural progenitor cells. This concept has now progressed to clinical trial: a Phase 1 trial will examine the safety and efficacy of allogeneic MSC-derived exosomes "enriched" with microRNA-124 in five adults (40–80 years) with acute ischemic stroke (NCT03384433). MicroRNA-124 enrichment will be achieved via transfection of MSCs. Outcome measures include the incidence of treatment-emergent adverse events and the Modified Rankin Scale to measure change in disability or dependence in daily activities. Other developments in the EV clinical trial space include published studies of chronic kidney disease (Nassar et al., 2016) and graft-vs.-host disease (Kordelas et al., 2014), as well as the currently recruiting study for healing of large and refractory macular holes using MSC-derived exosomes (NCT03437759).

Given the potential advantages of EVs (Hall et al., 2016), this raises an ethical controversy of whether researchers should be progressing predominantly EV- rather than cell-based- therapies.

Studies such as that published by Doeppner et al., which compared the efficacy of human bone marrow-derived MSCs with EVs obtained from the same cells in a rodent model of stroke (Doeppner et al., 2015), are highly valuable. This study showed that EVs were equally effective as cells in promoting neurological recovery and brain remodeling following transient focal cerebral ischemia. More such head-to-head studies are warranted to confirm the utility of EVs for a range of indications. The efficacy of EVs for neuro-regeneration/repair is expected to be limited to situations that involve anti-inflammatory and/or trophic signaling mechanisms; it is unlikely that EVs would be useful when cell engraftment is desired, for example in neural stem cell replacement therapy.

Erythropoietin (EPO)

Originally identified for its role in erythropoiesis, erythropoietin (EPO) was later shown to be neuroprotective in a variety of animal models of neurological conditions including stroke, TBI and multiple sclerosis (MS) (Siren et al., 2009). Unsurprisingly, EPO garnered significant interest as a potential neuroprotectant for preterm infants, and numerous preclinical- and clinical-studies have since been completed. Three recent systematic reviews with meta-analyses of EPO for reducing neurodevelopmental disability in preterm infants have been published. These studies comprised: four RCTs with 297 infants (Zhang et al., 2014), two RCTs and three quasi-RCTs with 233 infants (Wang et al., 2015), and four RCTs with 1,133 infants (Fischer et al., 2017), in the meta-analyses, respectively. Analyses were conducted on mostly non-overlapping trials: seven original studies in total, and all three concluded that EPO treatment improved cognitive outcome in preterm infants. Important to note is that no significant effect was observed for other neurodevelopmental outcomes including cerebral palsy, visual impairment, severe hearing deficit, and necrotizing enterocolitis.

New clinical trials of EPO for preterm infants continue to be launched. A currently recruiting, multicenter, randomized, placebo-controlled study plans to recruit 312 infants (<32 weeks' gestation, ≤1,500 grams) for repeated administration of high-dose EPO within 48 h of birth (NCT02550054). The primary outcomes are neurodevelopmental function at 18 months measured using the Bayley and GMFM-88. Similarly, "EpoRepair" (NCT02076373) is an RCT of repetitive high-dose EPO in 120 preterm infants (23–31 weeks'). Contrastingly however, recruited infants will be those with a diagnosed brain injury (IVH and/or hemorrhagic parenchymal infarction), and neurodevelopmental follow-up using a composite intelligence quotient will be conducted at the longer time-point of 5 years of age (Ruegger et al., 2015). Moreover, the active, though not recruiting Phase 3 Preterm Erythropoietin Neuroprotection "PENUT" Trial (NCT01378273) has enrolled 941 preterm infants specifically in the lower age range of 24–27 weeks' gestation. Infants in this study will likewise receive repeated high-dose EPO initiated soon after birth. The primary endpoint is 24–26 months corrected age, at which neurodevelopmental outcome will be measured using the Bayley (Juul et al., 2015). The selection of extremely low gestational age neonates for the PENUT Trial is significant since in a subgroup analysis reported by Fischer et al., no significant benefit of EPO was seen on cognitive outcome in infants <28 weeks' (Fischer et al., 2017). Whilst the authors acknowledged that the small sample size used for the analysis was a limitation, the results of the PENUT Trial should definitively address the efficacy of EPO for neuroprotection in extremely preterm infants. Of particular note, whilst the EPO regimens of the above listed trials differ, the effect of EPO on cognitive outcome appears to be robust enough to withstand variations in both timing and dose (Fischer et al., 2017). Thus, hopefully the results of these studies will provide conclusive evidence to support change in the clinical practice guidelines for the care of preterm infants.

Melatonin

Melatonin (N-acetyl-5-methoxytryptamin) is a hormone produced by the pineal gland that is responsible for regulating circadian rhythms via activation of specific melatonin receptors. Melatonin also acts as an antioxidant and has demonstrated anti-inflammatory and anti-apoptotic effects (Welin et al., 2007). It is these properties that make melatonin a promising candidate for the treatment of perinatal brain injury in preterm neonates. Accordingly, there is now substantial evidence from animal studies supporting a neuroprotective role of melatonin administered either antenatally, or postnatally after the injurious event (reviewed in Biran et al., 2014; Wilkinson et al., 2016). For example, in a murine model of excitotoxic periventricular white matter injury, which mimics human PVL, melatonin given immediately following intracerebral injection of the glutamatergic analog ibotenate promoted secondary lesion repair, effectively reducing the size of white matter cysts (Husson et al., 2002). Similarly, in a rat model of acute neonatal hemorrhagic brain injury commonly observed in very low birth weight preterm infants, systemic administration of melatonin 1 h after injury normalized brain atrophy and led to improved cognitive and sensorimotor function (Lekic et al., 2011). Moreover, Watanabe et al., demonstrated that antenatal administration of melatonin reduced *in utero* ischemia/reperfusion oxidative brain injury in neonatal rats. Rat pups born to mothers who received melatonin exhibited reduced markers of oxidative stress, had numbers of intact mitochondria comparable to control animals, and had reduced brain damage quantified by protection of hippocampal pyramidal neurons from ischemia/reperfusion-induced degeneration (Watanabe et al., 2012). Likewise, maternal antenatal melatonin reduced brain injury in an ovine model of fetal growth restriction (Miller et al., 2014). In this model, fetal growth restriction was induced surgically by single umbilical artery ligation at 0.7 gestation after which melatonin was administered for the remainder of the pregnancy. Antenatal melatonin rescued oxidative stress, white matter hypomyelination and axonal damage leading to significant functional improvements in neonatal lamb behaviors, in particular suckling (Miller et al., 2014). Collectively, these studies demonstrate the broad utility of melatonin in improving outcomes following perinatal injury from a variety of etiologies. In addition, there is encouraging data on the neuroprotective efficacy of melatonin in a range of related adult conditions including stroke (Watson et al., 2016), TBI (Barlow et al.,

2018), and neurodegenerative diseases (Wongprayoon and Govitrapong, 2017).

The results of these pivotal animal studies have led to pilot clinical trials of either antenatal or postnatal melatonin for neuroprotection of preterm or growth restricted infants. Though few trials have been published to date, a single-arm, open-label study of antenatal oral melatonin administration to 16 women with a growth restricted fetus (diagnosed <34 weeks gestation) was completed in November 2014 (NCT01695070) (Alers et al., 2013). This study is being followed by the 2017-registered "Protect Me Trial" which aims to recruit 292 mothers with a growth restricted fetus for a Phase 2 RCT of daily antenatal melatonin supplementation for improving early childhood neurodevelopmental outcomes at 2-years (ACTRN12617001515381). Trial participants will be stratified according to gestational age (<28 weeks' and 28 to <32 weeks'), and recruitment is anticipated to start in late-2018. For postnatally administered melatonin, a pharmacokinetic study was conducted in which 18 preterm infants (<31 weeks') were administered melatonin intravenously at varying concentrations for up to 6 h (NCT00649961). The objective was to determine the dose required to achieve melatonin blood levels in the preterm infant similar to that of the mother, to guide potential future therapeutic trials. It was found that peak adult melatonin concentration could be achieved by a relatively short (2 h) infusion of low dose (0.1 μg/kg/h) melatonin in preterm infants (Merchant et al., 2013). Using this dosing regime, a Phase 2 RCT (The Mint Study) was conducted to evaluate the neuroprotective effect of melatonin in 58 preterm infants (30 received melatonin and 28 received placebo) (Merchant et al., 2014). No between group difference was shown for the primary outcome, which was a 5% difference in the fractional anisotropy in the white matter measured using MRI at term equivalent age. Similar to the Mint Study, "PREMELIP" aimed to assess the neuroprotective effect of melatonin administered in the immediate prepartum period in very preterm infants (<28 weeks') (NCT02395783). Also designed to measure white matter injury at term equivalent age using MRI as the primary outcome, this study was terminated in 2018 after recruiting 14 participants. No reason was provided by study investigators. Melatonin is also being studied in *term*-born neonatal populations (Aly et al., 2015), however this is beyond the scope of this review.

Creatine

Creatine is a natural compound that is synthesized in the liver, kidney and pancreas, and is also obtained through diet. Creatine's main role is in cellular energy homeostasis; creatine participates in a reversible reaction with creatine kinase to continuously and efficiently replenish ATP (the energy currency of the cell) from ADP, to meet energy demands (Wallimann et al., 1992). There is evidence to suggest that creatine is neuroprotective for the developing perinatal brain (Dickinson et al., 2014b). Specifically, offspring born to pregnant spiny mice fed a creatine-supplemented diet had a significantly increased capacity to survive a hypoxic birth event, and these pups showed improved postnatal weight gain (Ireland et al., 2008). In a follow-up mechanistic study, maternal creatine supplementation protected pups from hypoxia-induced brain lipid peroxidation and apoptosis, hypothesized to be mediated via preservation of mitochondrial function (Ireland et al., 2011). However, no clinical trials of antenatal creatine supplementation to protect against perinatal brain injury have yet been conducted (Dickinson et al., 2014a).

Complexities exist around the practicalities of antenatal creatine administration for neuroprotection of preterm infants. For example, should creatine be administered as a prophylactic to all pregnant women on the off chance that their babies experience birth asphyxia and/or prematurity? Alternatively, the utility of postnatal creatine for neuroprotection of preterm infants who do not experience birth asphyxia is unclear. Creatine has been pilot tested in clinical trial for children with TBI, with daily administration from the day of injury to 6 months post-injury. The results suggested improved memory and functional skills, with reduced intensive care stay (Sakellaris et al., 2006). Thus, creatine could potentially be given postnatally to newborns with encephalopathic brain injury at high-risk of cerebral palsy. Notably, creatinine (the breakdown product of creatine) is excreted by the kidneys, which are known to have reduced function in premature infants (Stritzke et al., 2017). Thus, the effects of administering potentially high therapeutic doses of creatine to preterm infants with reduced kidney function warrants careful consideration and thorough investigation. Creatine is also being studied in adult neurodegenerative diseases (Pastula et al., 2012; Xiao et al., 2014; Bender and Klopstock, 2016), however these are beyond the scope of this review.

Granulocyte-Colony Stimulating Factor (G-CSF)

Granulocyte-colony stimulating factor (G-CSF) is a hematopoietic growth factor that is used clinically in cancer patients diagnosed with neutropenia following chemotherapy, due to its ability to stimulate hematopoietic stem cell mobilization and neutrophil differentiation. G-CSF also exhibits non-hematopoietic neurotrophic functions, with evidence from experimental studies indicating that G-CSF is neuroprotective in stroke, TBI, and neurodegenerative diseases (reviewed by Solaroglu et al., 2007). Despite this, results of studies examining the effect of G-CSF in perinatal brain injury were initially controversial. While two studies of HI injury in neonatal rats showed that G-CSF inhibited neuronal apoptosis to attenuate brain damage (Yata et al., 2007; Kim et al., 2008), Keller et al., found that G-CSF actually increased cortical and white matter lesions in a newborn mouse model of excitotoxic brain injury (Keller et al., 2006). Similarly, in a follow-up study, acute administration of G-CSF led to increased hippocampal brain damage in a murine model of neonatal HI (Schlager et al., 2011). This disparity was explained 5 years later, when it was revealed that the neuroprotective effects of G-CSF are contingent on the timing of administration following the insult (Neubauer et al., 2016). Delayed administration of G-CSF (in combination with stem cell factor or flt3-ligand) at 60 h post-injury, compared to 1 h used previously, conveyed significant neuroprotection against excitotoxic insult (Neubauer et al., 2016). This was consistent with other studies showing G-CSF reduced white matter injury in a sheep model of preterm brain injury (Jellema et al., 2013),

and attenuated neuroinflammation and stabilized the blood brain barrier (BBB) following HI (Li et al., 2015). However, it remains unclear why Schlager et al., failed to find any long-term neuroprotective effects of G-CSF after neonatal HI (Schlager et al., 2011).

Thus far, the only clinical trials assessing the neuroreparative potential of G-CSF in pediatric patients has been in children with cerebral palsy (NCT02983708, NCT02866331). In the first study (NCT02983708), G-CSF was given to 57 children with non-severe cerebral palsy for 5 days to mobilize peripheral blood mononuclear cells. These were then collected and re-infused at either 1- or 7-months post-cell collection, in a double-blind, cross-over study (Rah et al., 2017). Neurodevelopmental improvement, above what would normally be expected, was seen in 42% of patients in response to G-CSF treatment. Interestingly, larger improvements in test scores were observed in patients who received delayed cell re-infusion at 7 months, although this was in contrast to the results of the MRI analysis. The authors concluded that further studies are needed to delineate the effect of repeated G-CSF either alone, or in combination with reinfusion of G-CSF-stimulated peripheral blood cells. The second study, a subsequent trial from the same lead investigator, is currently recruiting (NCT02866331). In this study, the efficacy of autologous UCB mononuclear cells and/or repeated G-CSF administration for children with cerebral palsy will be assessed. In addition to these limited pediatric studies, numerous clinical trials of G-CSF for adult stroke have been completed. Meta-analyses of clinical trial data however paint a less-than-convincing picture, with conflicting conclusions (Fan et al., 2015; England et al., 2016; Huang et al., 2017). There has also been substantial interest in G-CSF for ALS, however early phase clinical trials have failed to show efficacy (Cashman et al., 2008; Nefussy et al., 2010; Chio et al., 2011; Duning et al., 2011). This serves as a timely reminder that compelling preclinical results, like creatine for neurodegenerative conditions, does not always translate to clinical benefit.

Thyroid Hormone (Thyroxine)

The thyroid hormones (thyroxine and triiodothyronine) are essential for brain development during fetal and postnatal life (reviewed by Williams, 2008). Preterm infants often exhibit transient low levels of thyroid hormones (hypothyroxinemia) (Rooman et al., 1996), which is thought to be due to immaturity of the hypothalamic-pituitary-thyroid axis. Long-term follow up of very preterm and/or very low birth weight infants revealed that neurologic dysfunction at age 5, and school failure at age nine, significantly correlated with lower neonatal thyroxine levels (Den Ouden et al., 1996). This result has been challenged by another cohort study in which thyroxine levels in the first 6 weeks of life were correlated with cognitive outcome at 7 years of age, and in this study higher thyroxine exposure was associated with poorer cognitive outcome (Scratch et al., 2014). Consequently, thyroxine substitution therapy was proposed to improve neurodevelopmental outcomes in this vulnerable population. Multiple clinical trials have been conducted, however a systematic review failed to find any significant difference in neonatal mortality, morbidity or neurodevelopmental outcomes in preterm infants who received thyroid hormones compared to controls (Osborn and Hunt, 2007). Since then, additional studies have been completed (Suzumura et al., 2011; Ng et al., 2014; Van Wassenaer-Leemhuis et al., 2014), however only Suzumura et al., found any neurodevelopmental benefit of thyroxine supplementation in preterm infants.

The failure of thyroxine substitution therapy to provide neurological benefit at clinical trial may be related to a lack of monocarboxylate transporter 8 (MCT8, also known as SLC16A2) which is required for transport of thyroid hormones across the cell membrane for its action and metabolism (Friesema et al., 2003). MCT8 expression has been shown to decrease following lipopolysaccharide (LPS)-induced systemic inflammation (Wittmann et al., 2015) and severe intrauterine growth restriction (Chan et al., 2014). It is feasible that preterm infants may similarly exhibit a lack of MCT8, thus may be unresponsive to thyroxine substitution. Future preclinical studies should examine whether up-regulation of MCT8 could improve response to thyroxine, and/or investigate use of a thyroxine hormone analog that does not require MCT8 to cross the plasma membrane.

Nevertheless, two new clinical trials of thyroxine for preterm infants have been registered on clinicaltrials.gov, both yet to start recruiting. The first is a Phase 3 study of 1,224 preterm infants (<28 weeks') randomized to receive either continuous infusion of thyroxine plus oral potassium iodide, or placebo, for 42 days (NCT02103998). The primary outcome for this trial is a composite endpoint of cerebral palsy or Bayley score <85 at 36 months' corrected age. The second is a Phase 2 trial which aims to recruit 100 preterm infants (<28 weeks') with a grade 3–4 IVH to determine whether thyroxine treatment can improve brain structure measured using MRI (NCT03390530). Secondary outcomes include infant mortality and neurodevelopmental impairment at 2 years of age.

Minocycline

Minocycline is a semi-synthetic broad-spectrum antibiotic that has been in commercial use for nearly 50 years. It is lipid soluble and can cross the BBB, making it an attractive drug for neurological application. Due to its ability to inhibit microglial activation, minocycline has demonstrated efficacy in various models of adult neurological conditions such as stroke, MS, Parkinson's disease and ALS (Yong et al., 2004; see reviews by Garrido-Mesa et al., 2013). Studies have also shown that minocycline can improve outcomes following perinatal brain injury. For example, minocycline administered 12 h before, and then periodically after LPS exposure, protected against white matter injury, oligodendrocyte loss and abnormal neurobehavioral performance in the neonatal rat (Fan et al., 2005). Similarly, in a preterm rodent model, post-HI treatment with minocycline abolished neuroinflammation and white matter injury (Carty et al., 2008). Notably however, other studies have shown that minocycline actually worsened brain injury in neonatal models (Tsuji et al., 2004; Strahan et al., 2017), although this effect was alleviated when minocycline was administered at a lower dose or later time-point (Strahan et al., 2017). Clearly,

dose and timing is very important, and the use of minocycline in pediatric patients warrants careful consideration.

There have been numerous clinical trials conducted of minocycline for various neurological conditions with mixed results. Whilst a 2010 futility study concluded that further investigation of minocycline for Huntington's disease was not warranted (Huntington Study Group Domino Investigators, 2010), data from a recent systematic review indicated efficacy of minocycline in acute stroke patients (Malhotra et al., 2018). Similarly, in a Phase 2 trial for Parkinson's disease, minocycline could not be rejected as futile based on the threshold of a 30% reduction in UPDRS progression (Ninds Net-Pd Investigators., 2006), though no Phase 3 clinical trial has yet been launched. Results are also pending from a dose-escalation study of minocycline for TBI (NCT01058395). Worryingly however, is the finding from a multicenter, randomized Phase 3 trial, that minocycline had a harmful effect on patients with ALS (Gordon et al., 2007). Thus, trials of minocycline, and indeed other neuroprotective agents, should proceed with caution.

Epidermal Growth Factor (EGF)

Epidermal growth factor (EGF), together with its receptor EGFR, is a key mediator of neural progenitor cell proliferation, migration and differentiation (reviewed by Galvez-Contreras et al., 2013). In preclinical studies, overexpression of EGFR shifted neural progenitor cells from a non-migratory- to a migratory-phenotype (Aguirre et al., 2005), and EGF stimulated *in vitro* neural progenitor cell oligodendrogenesis (Aguirre and Gallo, 2007). Importantly, EGFR overexpression enhanced proliferation of neural progenitors and their migration to the lesion following lysolecithin-induced focal demyelination in the mouse brain (Aguirre et al., 2007). This led to accelerated and more extensive remyelination, with more rapid functional recovery (Aguirre et al., 2007). Similarly, in a mouse model of preterm hypoxic brain injury, EGFR overexpression or intranasal administration of heparin-binding EGF decreased oligodendrocyte death, promoted oligodendrocyte-progenitor maturation, and led to improved behavioral recovery of mice assessed using various white matter-dependent sensori-motor tests (Scafidi et al., 2014). Moreover, in rabbits with IVH, intracerebroventricular injection of recombinant human EGF significantly increased myelination, promoted proliferation and maturation of oligodendrocyte-progenitors, and enhanced neurobehavioral recovery (Vinukonda et al., 2016). Thus, EGF is an exciting potential neuroprotective therapeutic.

Diazoxide

Diazoxide is a potassium channel activator that is used as a vasodilator in the treatment of acute hypertension, and to counter hypoglycemia caused by congenital hyperinsulinism in infants. The neuroprotective potential of diazoxide following ischemia/reperfusion injury was first investigated nearly 20 years ago (Domoki et al., 1999). Despite positive findings, the utilization of diazoxide for reducing neuronal injury in the newborn has been slow to progress. Nevertheless, in a series of *in vitro* and *in vivo* experiments, Fogal et al., demonstrated that diazoxide stimulated proliferation of cultured oligodendrocyte-progenitor cells, enhanced myelination of cerebellar-slice fibers, and prevented hypoxia-induced ventriculomegaly and hypomyelination (Fogal et al., 2010). In a follow-up mechanistic study, the authors' showed that diazoxide actually enhanced maturation of oligodendrocytes, rather than increasing oligodendrocyte proliferation, as their previous *in vitro* studies suggested (Zhu et al., 2014). Given the well-established safety profile of diazoxide, and accumulating efficacy data in stroke, further preclinical studies, in other models of perinatal brain injury, are warranted.

Nanoparticle-Targeted Drug Delivery

Nanoparticles for targeted drug delivery are emerging as a promising strategy for the treatment of a range of disorders. Nanoparticle delivery can improve drug bioavailability and targeting, particularly across the BBB. Dendrimers are nanoparticles that have a branching, tree-like structure with a high density of tailorable surface functional groups. Systemically administered hydroxyl-terminated polyamidoamine dendrimers crossed the BBB to localize in activated microglia and astrocytes in the brain of newborn rabbits with cerebral palsy, but not healthy controls (Kannan et al., 2012). Moreover, administration of dendrimers conjugated with the anti-oxidant and anti-inflammatory agent N-acetyl-L-cysteine (NAC; D-NAC), suppressed neuroinflammation and led to a dramatic improvement in motor function. NAC has long been used clinically for acetaminophen poisoning, and has been shown to be an effective treatment for a multitude of psychiatric and neurological conditions including autism, Alzheimer's disease, addiction, schizophrenia, and progressive myoclonic epilepsy (reviewed by Deepmala et al., 2015).

In a mouse model of ischemia-induced neonatal white matter injury, administration of D-NAC suppressed the "detrimental" pro-inflammatory response and increased myelination in regions of white matter injury, indicative of functional recovery (Nance et al., 2015). Furthermore, in a mouse model of perinatal HIE, systemically delivered D-NAC targeted all three types of neural cells involved in brain injury after HI (microglia, neurons, and astrocytes), but only in injured tissue (Nemeth et al., 2017). Importantly, uptake of D-NAC was not significantly altered by exposure to therapeutic hypothermia, suggesting that D-NAC can be used as a combinatorial therapy for the clinical management of HIE (Nemeth et al., 2017). Interestingly, using a mouse model of intrauterine inflammation Lei et al., demonstrated that a single dose of D-NAC, maternally delivered, significantly reduced the rate of preterm birth in pregnant dams exposed to LPS (Lei et al., 2017). Maternal D-NAC treatment altered the placental immune profile, decreased placental T-cell infiltration, and reduced microglial activation. Moreover, D-NAC significantly improved the neuromotor outcomes of LPS-exposed pups (Lei et al., 2017). Whilst the concept of NAC for the prevention of preterm birth is not in itself novel, the high effective dose required for free NAC can induce side-effects of nausea, vomiting, stomatitis, and fever. This publication demonstrates for the first time that low dose NAC delivered via dendrimer-conjugation is effective in reducing preterm birth. Thus, it is

feasible that maternally administered D-NAC could be a well-tolerated prophylactic therapy for preterm birth.

Similarly, Chinese researchers have developed a novel catalase-containing nanoparticle that improved outcomes in a rodent stroke model (Zhang et al., 2017a). Catalase is an anti-oxidant and promising neuroprotectant, however its clinical application is hindered by its rapid degradation, immunogenicity and inability to cross the BBB. By modifying catalase containing nanoparticles by (a) cross-linking the polymer to increase drug stability, and (b) adding a peptide tag that is recognized and taken up by infiltrating neutrophils, catalase could effectively piggy-back across the BBB to the target site in the brain (Zhang et al., 2017a). Accordingly, nanoparticle delivery of catalase significantly reduced the infarct volume, mediated by reduced apoptosis, in a mouse model of ischemia/reperfusion injury (Zhang et al., 2017a). It is hypothesized that this technology could be beneficial in any neurological condition characterized by neuroinflammation, including preterm brain injury.

Gene Therapy

Gene therapy is the delivery of DNA as a drug to treat disease; the idea being that the transferred DNA would be stably overexpressed as a protein to provide therapeutic benefit. Gene therapy is being actively studied for a range of adult neurological conditions including Alzheimer's disease, ALS and stroke (reviewed in Choong et al., 2016). In contrast, few studies have examined gene therapy for perinatal brain injury. One example however is the overexpression of brain-derived neurotrophic factor (BDNF) to protect the newborn mouse brain from excitotoxic insult. BDNF has been shown to protect the newborn mouse brain from periventricular white matter lesions (Husson et al., 2005). However, since these lesions progress over several days to weeks, the most effective BDNF therapy should involve prolonged/continual administration. Using the same mouse model of neonatal excitotoxic challenge, Bemelmans et al., demonstrated that lentiviral-mediated gene transfer of BDNF to the newborn mouse brain is feasible and affords significant neuroprotection against excitotoxic insult (Bemelmans et al., 2006). Despite these encouraging findings, no published follow-up study was found during the literature search for this review.

Emergent Combinatorial Therapies

With the discovery of new, efficacious therapies for neuroprotection and repair of the injured preterm brain, we will undoubtedly see the emergence of combinatorial therapies in an attempt to maximize neurodevelopmental outcome. Some emergent combinatorial therapies are already being tested for various neurological conditions in the clinic, e.g., administration of allogeneic UCB plus EPO in three adults with TBI (Min et al., 2013). Moreover, besides the previously mentioned trial for children with cerebral palsy (NCT02866331), UCB plus G-CSF has been used for stroke patients (Shin and Cho, 2016), and adults with either a brain injury, cerebral palsy, Parkinson's disease or ALS (NCT02236065). Interestingly, EPO together with G-CSF has been proposed for adults with neurological diseases (NCT02018406), though the status of this trial is unknown. Unsurprisingly, various other combination therapies are being investigated at the preclinical stage. Recently, genetically engineered UCB mononuclear cells were used to deliver triple gene therapy in a rodent model of stroke (Sokolov et al., 2018). Treatment with minocycline followed by NAC is showing encouraging results for TBI (Sangobowale et al., 2018), and Jantzie et al., is exploring postnatal administration of EPO and melatonin in a rat model of perinatal brain injury caused by extreme preterm birth (Jantzie et al., 2018).

Neuroplasticity-Inducing Rehabilitation

Neuroplasticity

The brain's capacity to recover after injury is what makes early intervention scientifically possible. Neuroplasticity encompasses the ability of the brain to organize/reorganize its circuitry and produce new cells (neurogenesis) and connections (synaptogenesis) (Johnston, 2009). Environmental stimulation (early intervention) protects neurons and promotes the secretion of growth factors in the brain (Vaccarino and Ment, 2004). Neuroplasticity occurs at all ages however the developing brain has greater capacity for change. Both genetic and environmental factors play important roles in neuroplasticity during development.

Critical Periods and Timing

The preclinical work of Kolb and others show that critical periods exist during development in which the brain's capacity to reorganize in response to injury is enhanced while at other times neuroplastic mechanisms can be detrimental (Kolb et al., 2011). A suite of experiments involving various drug therapies and neurotrophic agents has demonstrated the ability of the developing brain to develop new neural networks when treatments were strategically introduced during known critical periods.

Kolb et al. describe three types of neuroplasticity: experience independent (largely genetically determined but modified by both internal and external events), experience dependent plasticity which lasts a lifetime, and experience expectant plasticity (Kolb et al., 2017). This latter type is characterized by a critical period that is moderated by a balance of inhibitory and excitatory inputs that may be chemically modifiable, thus enabling a widening of the critical period.

Environmental enrichment is the most commonly used method of inducing neuroplasticity in animal studies. The harnessing of activity-dependent mechanisms by enriching the housing and social environments of experimental animals has demonstrated improved outcomes after injury in both adult and infant models (Nithianantharajah and Hannan, 2006). The use of enriched environments for animals with perinatal brain injury produces structural brain changes including increased brain volumes as well as improved cognitive, motor and behavioral skill acquisition. Tactile stimulation, or sensory enrichment has been shown in numerous studies to improve cognitive, motor and visual function (Sale et al., 2009; Kolb et al., 2017).

Although activity-dependent plasticity is a lifelong mechanism, the timing of experience and training post injury seems important. A cat model of cerebral palsy showed that early stimulation of the corticospinal tract using forelimb training

resulted in functional skill acquisition as well as connectivity, whereas animals who received delayed training did not develop functional use of the affected limb (Friel et al., 2012). Studies in adult stroke patients have also indicated that training close to the time of the infarct leads to better functional skills and structural brain changes (Kleim and Jones, 2008).

Principles of Experience Dependent Neuroplasticity

Kleim and Jones describe activity-dependent neuroplasticity principles that should be applied to rehabilitation programs in humans with brain damage, stressing the importance of active engagement of neural circuits to avoid degradation and enhance function (Kleim and Jones, 2008). Although primarily focused on adult and animal studies these principles (task specificity, repetition, salience, timing, and intensity) also apply to infants with brain damage who need to learn for the first time, rather than re-learn, how to move, think, and communicate. Thus the principles of neuroplasticity along with environmental enrichment strategies should be applied in infant early intervention, with reference to the child's context—most importantly the parent-child relationship. Attachment to a primary caregiver is paramount for early brain development as studies of institutionalized children attest (Fox et al., 2011). Early intervention programs for infants with brain injuries should support not only the parent (Benzies et al., 2013), who are at higher risk of poor mental health (Whittingham et al., 2014) but also provide support to the child-parent relationship in order to maximize outcomes.

Measuring neuroplasticity using advanced imaging techniques is possible in adults post-stroke (Cramer et al., 2011), and increasingly advocated for children with cerebral palsy (Reid et al., 2017). Imaging of cortical thickness and gray matter can identify neuroplastic change (Reid et al., 2015) and has been conducted in very young children with unilateral cerebral palsy post constraint induced movement therapy (Sterling et al., 2013). Transcranial magnetic stimulation can also be used to measure cortical reorganization and is currently being trialed in infants post perinatal stroke (Chen et al., 2017). However, ethical dilemmas exist for this age group since conducting MRI for research purposes require anesthetics to guarantee useable images.

Neuroplasticity-Inducing Rehabilitation Trials

A multitude of early intervention trials have been conducted in cohorts of preterm infants. These interventions may be delivered during the NICU stay (Ustad et al., 2016) or commence post discharge (Spittle et al., 2010). Some intervention studies start during NICU admission and continue for a short period after discharge (Dusing et al., 2015, 2018). Since it is clear that prematurity significantly impacts all developmental domains, in most instances study inclusion is based on gestational age or birthweight. A meta-analysis of controlled trials (random and quasi randomized) demonstrates that cognitive outcomes are significantly improved through early intervention, with effects lasting until school-age (Spittle et al., 2015). Motor outcomes however were only marginally improved in these studies and the clinical significance of the improvement is not clear. This systematic review included any early developmental intervention programs that aimed to improve cognitive or motor outcomes initiated within 12 months post-term age. Types of interventions included physiotherapy, occupational therapy, neurodevelopmental therapy, parent-infant relationship enhancement, infant stimulation, and early education intervention. Thus the included interventions are heterogeneous in terms of content, dose and outcome measurement (Spittle et al., 2015), making it difficult to draw out the key ingredients for beneficial effects. Most approaches focus on training parents to recognize and respond to their infant's cues as well as providing information and guidance to support the infant to meet developmental milestones. Some early intervention approaches are more child focused and delivered by a physiotherapist or occupational therapist (Spittle et al., 2015).

Very few studies target preterm infants with brain injuries and in fact many studies specifically exclude preterm infants with imaging abnormalities. The two small RCTs that specifically targeted preterm infants with cerebral injuries did not find statistically significant between group differences in either motor or cognitive outcomes (Nelson et al., 2001; Ohgi et al., 2004), although short term neurobehavioral benefits were found in one study (Ohgi et al., 2004).

Environmental enrichment

Whilst the impact of prematurity on cerebral brain development has been extensively studied, the impact of the NICU admission itself on the brain is less certain. Painful or stressful stimuli and deprivation can have profound negative effects on brain development. It is difficult to disentangle prematurity and its associated co-morbidities from the life-saving therapies, but also the various "threats" that admission into an intensive care environment provides. The magnitude of these threats is being more accurately quantified, and we now know that the premature infant goes into an environment of light for most of the 24 h cycle (Rodriguez and Pattini, 2016), a relatively noisy environment (Shoemark et al., 2016), and a situation in which painful procedures e.g., heel lances, are common (Brummelte et al., 2012). Reproduction of the *in utero* environment, with low-level sensory input and safety, is not currently possible, however clinician scientists are now beginning to investigate the potential benefit of "normalizing" the environment for the preterm infants, for example by promoting appropriate circadian sleep-wake rhythm cycles and protecting the infant from sensory overload. Length of stay in the NICU is rarely controlled for as a confounder in RCT's, but it is easily quantified and is often used as a short term outcome in trials of neonatal interventions—with reduced length of stay considered as a significant positive outcome, both for the baby, the family, and the health service economist.

Enriching the newborn care environment via the Newborn Individualized Care and Assessment Program (NIDCAP) is an intervention that has been widely adopted (reviewed in Moody et al., 2017). NIDCAP aims to provide an environment with minimal stress that adapts to the needs of the infant through identification and response to infant cues. Though some early trials showed improved brain function and structure in

low-risk preterm infants (Als et al., 2004), subsequent studies have generally not reproduced these findings, and meta-analyses do not show significant gains from NIDCAP in the short or long term (Ohlsson and Jacobs, 2013). To date NIDCAP does not appear to prevent or ameliorate brain injury in preterm infants, nor does it do harm. Studies of infant massage (sensory enrichment) have shown positive results in a small sample of preterm infants (Guzzetta et al., 2009), as has music therapy (Lubetzky et al., 2010).

Although early intervention aims to harness naturally occurring neuroplastic mechanisms such as experience-dependent plasticity, a number of interventions that have been studied have involved the infant largely in a passive capacity in a clinic-based environment (Weindling et al., 1996).

Systematic review evidence indicates that a small effect on motor outcomes is gained for infants 0–2 years with brain injuries, when interventions are applied that enrich at least one aspect of the environment (social, motor, sensory, or cognitive) (Morgan et al., 2013). Since this review, two further studies of preterm infants (with and without brain damage) (Dusing et al., 2015, 2018) have shown early promising results in early problem solving skills. "SPEEDI" (Supporting Play Exploration and Early Developmental Intervention) is an intervention where parents are coached and supported to promote daily movement opportunities and environmental enrichment strategies with their infant. Larger trials are underway to explore the importance of timing of the intervention (NCT03518736).

Training motor skills

Traditional physiotherapy for cerebral palsy has been largely a passive experience for the child, however systematic review evidence favors approaches that are goal directed and involve repetitive practice of specific and functional tasks (Novak et al., 2013). Recent systematic reviews of early intervention (from birth to 24 months) for infants with cerebral palsy have not only revealed the lack of rigorous trial data available but also that traditional early intervention is largely ineffective for infants with cerebral palsy (Morgan et al., 2016a). The majority of studies identified in this review did not demonstrate any motor gains for infants with cerebral palsy. However, only half of the included trials began early intervention prior to 6 months of age. Delayed definitive diagnosis of cerebral palsy, after 12 months, has typically meant that a mixed group of "at risk" infants are recruited, leading to underpowered trials. Alternatively, waiting for a confirmed diagnosis prior to recruitment means that infants with cerebral palsy don't receive intervention until the second year of life.

An important study from 1988 randomized 48 infants with spastic diplegia, most of whom were preterm, to receive either a home-based active enrichment intervention ("Learning Games") or Neurodevelopmental Therapy, the most commonly applied physiotherapy intervention (Palmer et al., 1988). Infants randomized to the enrichment intervention were significantly more advanced in motor and cognitive skills after 6 months of intervention as measured using the Bayley.

Evidence for early interventions that involve active motor skill training has been growing slowly over the last 4 years. Two RCTs of GAME (Goals Activity Motor Enrichment; Morgan et al., 2014) intervention in infants aged 3–6 months with or at high risk of cerebral palsy demonstrated improved motor (Morgan et al., 2015, 2016b) and cognitive skills (Morgan et al., 2016b) after a minimum of 3 months intervention, when compared to standard care. GAME is a motor learning intervention that coaches and supports parents to train motor and cognitive skills through play, in an enriched environment. GAME is always delivered in the family home with practice carried out by parents throughout the week between face to face therapy sessions. A large multicenter single blind randomized controlled trial ($n = 300$) of GAME vs. standard care is currently being undertaken (ACTRN12617000006347).

About 20–30% of preterm infants with cerebral palsy will be diagnosed with unilateral cerebral palsy (Himpens et al., 2008; ACPR Group, 2018). High quality evidence exists for the use of constraint induced movement therapy (CIMT) in children with unilateral cerebral palsy (Novak et al., 2013) and there is early evidence to support the use of a modified version of CIMT for infants. A retrospective study of 72 infants with unilateral cerebral palsy confirmed that children who had received CIMT as an infant (baby-CIMT) were significantly more likely to have superior hand function at age two than those who did not, even when controlling for the type of lesion (Nordstrand et al., 2015). A recently published RCT ($n = 37$) confirmed that infants with unilateral cerebral palsy had better function of their affected hand after 36 h of baby-CIMT than infants who had received the same dose of infant massage (Eliasson et al., 2018). Bimanual therapy, a training intervention that targets using both hands together, is equally effective as CIMT at a matched dose in older children with hemiplegia however trials in infants are not yet published (Sakzewski et al., 2014). A randomized trial comparing baby CIMT with bimanual training is currently underway (Boyd et al., 2017).

While the evidence for early habilitation interventions in infants with cerebral palsy is small it is steadily growing evidenced by the large number of registered clinical trials underway and an increasing focus on earlier detection (Byrne et al., 2017; Novak et al., 2017). Further research is required regarding interventions that can be applied very early (during the NICU) in preterm infants at high risk of cerebral palsy since motor and sensory pathways are still developing at this point. Ideally, combining pharmacological and/or cell based therapies with habilitation interventions to drive experience-dependent plasticity, appears to be the approach that should be targeted by those working with preterm infants with cerebral palsy.

POTENTIAL FUTURE ADVANCES: BIOMARKERS OF PRETERM BRAIN INJURY

An early diagnosis of cerebral palsy at 12-weeks of age corrected is a major step forward, but still falls short of the need to accurately diagnose brain injury and predict the likelihood of disability within hours of injury, so as to instigate novel and conventional protective treatments, such as therapeutic hypothermia in the

case of HIE. The race is now on to identify reliable biomarkers of brain injury.

Blood biomarkers are under intense investigation, because the BBB "opens" after injury (Anblagan et al., 2016). This opening is mediated by a number of factors, including disruption of the tight-junction complexes connecting brain endothelial cells, increased production of inflammatory mediators resulting in infiltration of immune cells into the injured site. A permeabilized BBB enables by-products of the injury cascade to be released into the circulatory system (Graham et al., 2018). Several candidate biomarkers have been identified and have been thoroughly appraised in a systematic review (Graham et al., 2018). Candidate biomarkers include: Glial Fibrillary Acidic Protein (GFAP); Neuron-Specific Enolase (NSE); S100B; Ubiquitin Carboxy-Terminal Hydrolase L1 Protein (UCHL1); Cleaved Tau (C-TAU); Microtubule-Associated Protein 2 (MAP2); Myelin Basic Protein (MBP); Spectrin Breakdown Products (SBDP); Brain-Derived Neurotrophic Factor (BDNF); Activin A; Matrix Metalloproteinase-9 (MMP-9); Vascular Endothelial Growth Factor (VEGF); Platelet Derived Growth Factor Receptor b (PDGFRb); Thrombospondin-1 (TSP-1); Tumor Necrosis Factor Alpha (TNF-a); Granulocyte Colony Stimulating Factor (G-CSF); MicroRNA (miRNA); and Exosomes (Graham et al., 2018). These proteins, neurotrophins, factors, inflammatory and metabolic markers are only released peripherally after injury, and are therefore being studied as indicators of the presence of brain injury. Importantly, it is likely that a panel/s of biomarkers will be most useful for identifying infants with neurological injury, and a combination of markers, in addition to existing tools, would provide the greatest diagnostic sensitivity (Yokobori et al., 2013).

In addition to blood biomarkers, neuroimaging techniques including ultrasonography, MRI and electroencephalography (EEG) are crucial tools in the diagnosis and prediction of prognosis following preterm brain injury (Jin et al., 2015). Though current techniques have distinct advantages and disadvantages (e.g., cost, accessibility, sensitivity, need for general anesthetic during MRI), development of advanced techniques such as volumetric MRI, diffusion tensor imaging, metabolic imaging (MR spectroscopy), functional ultrasound (Deffieux et al., 2018) and amplitude-integrated EEG, are transforming the neuroimaging field. Advanced MRI techniques as well as the "Baby Connectome Project" (Howell et al., 2019) are likely to result in the development of improved diagnostic and prognostic markers in addition to novel outcome measures for quantifying neuroplasticity change from intervention.

Importantly, it has been proposed that a combination or panel of biomarkers may provide clues about the type of injury, allowing identification of new treatment paradigms that address the underlying cellular damage mechanisms. In addition, biomarkers may enable clinical triage of combinatory treatments and therefore they offer great promise to the field (Graham et al., 2018).

CONCLUSIONS

Preterm birth is a significant cause of morbidity and mortality for infants, and remains a significant risk factor for cerebral palsy. Encouragingly, there are numerous emerging biological, pharmacological, and rehabilitation interventions for the prevention, minimization, and even reversal of preterm brain injury. Many of these therapies are showing promising signs of efficacy in early-phase clinical trials and preclinical studies. However, as new interventions emerge, so arise various controversies surrounding their implementation, which need to be solved before they can gain acceptance and be widely implemented in the clinic. Finally, the development of reliable biomarkers of preterm brain injury, will enable better detection, diagnosis and prediction of long-term outcomes, which will undoubtedly lead to more efficacious, better-targeted therapies, with improved outcomes for infants born preterm.

AUTHOR CONTRIBUTIONS

MF-E, CM, and IN: conceived the project. MF-E, CM, RH, and IN: drafted the article. All authors were involved in editing and approval of the final manuscript.

REFERENCES

Aarnoudse-Moens, C. S., Weisglas-Kuperus, N., Van Goudoever, J. B., and Oosterlaan, J. (2009). Meta-analysis of neurobehavioral outcomes in very preterm and/or very low birth weight children. *Pediatrics* 124, 717–728. doi: 10.1542/peds.2008-2816

Access Economics (2008). *The Economic Impact of Cerebral Palsy in Australia in 2007.* Report by Access Economics Pty Limited for Cerebral Palsy Australia. Canberra, ACT: Access Economics. Available online at: https://cpaustralia.com.au/media/20379/access_economics_report.pdf

ACPR Group (2018). *Report of the Australian Cerebral Palsy Register, Birth Years 1995–2012.* The Australian Cerebral Palsy Register Group, Australia.

Aguirre, A., Dupree, J. L., Mangin, J. M., and Gallo, V. (2007). A functional role for EGFR signaling in myelination and remyelination. *Nat. Neurosci.* 10, 990–1002. doi: 10.1038/nn1938

Aguirre, A., and Gallo, V. (2007). Reduced EGFR signaling in progenitor cells of the adult subventricular zone attenuates oligodendrogenesis after demyelination. *Neuron Glia Biol.* 3, 209–220. doi: 10.1017/S1740925X08000082

Aguirre, A., Rizvi, T. A., Ratner, N., and Gallo, V. (2005). Overexpression of the epidermal growth factor receptor confers migratory properties to nonmigratory postnatal neural progenitors. *J. Neurosci.* 25, 11092–11106. doi: 10.1523/JNEUROSCI.2981-05.2005

Ahn, S. Y., Chang, Y. S., Sung, S. I., and Park, W. S. (2018). Mesenchymal stem cells for severe intraventricular hemorrhage in preterm infants: phase i dose-escalation clinical trial. *Stem Cells Transl Med.* 7, 847–856. doi: 10.1002/sctm.17-0219

AIHW (2017). *Australia's Mothers and Babies 2015—in Brief. Perinatal Statistics Series No. 33. Cat no. PER 91.* Canberra, ACT: Australian Institute of Health and Welfare.

Alers, N. O., Jenkin, G., Miller, S. L., and Wallace, E. M. (2013). Antenatal melatonin as an antioxidant in human pregnancies complicated by fetal growth restriction–a phase I pilot clinical trial: study protocol. *BMJ Open* 3:e004141. doi: 10.1136/bmjopen-2013-004141

Als, H., Duffy, F. H., Mcanulty, G. B., Rivkin, M. J., Vajapeyam, S., Mulkern, R. V., et al. (2004). Early experience alters brain function and structure. *Pediatrics* 113, 846–857. doi: 10.1542/peds.113.4.846

Aly, H., Elmahdy, H., El-Dib, M., Rowisha, M., Awny, M., El-Gohary, T., et al. (2015). Melatonin use for neuroprotection in perinatal asphyxia: a randomized controlled pilot study. *J. Perinatol.* 35, 186–191. doi: 10.1038/jp.2014.186

Anblagan, D., Pataky, R., Evans, M. J., Telford, E. J., Serag, A., Sparrow, S., et al. (2016). Association between preterm brain injury and

exposure to chorioamnionitis during fetal life. *Sci. Rep.* 6, 37932–37932. doi: 10.1038/srep37932

Anderson, P. J., Treyvaud, K., Neil, J. J., Cheong, J. L. Y., Hunt, R. W., Thompson, D. K., et al. (2017). Associations of newborn brain magnetic resonance imaging with long-term neurodevelopmental impairments in very preterm children. *J. Pediatr.* 187, 58.e1–65.e1. doi: 10.1016/j.jpeds.2017.04.059

Askie, L. M., Darlow, B. A., Davis, P. G., Finer, N., Stenson, B., Vento, M., et al. (2017). Effects of targeting lower versus higher arterial oxygen saturations on death or disability in preterm infants. *Cochr. Database Syst. Rev.* 4:Cd011190. doi: 10.1002/14651858.CD011190.pub2

Back, S. A., Luo, N. L., Borenstein, N. S., Levine, J. M., Volpe, J. J., and Kinney, H. C. (2001). Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J. Neurosci.* 21, 1302–1312. doi: 10.1523/JNEUROSCI.21-04-01302.2001

Barlow, K. M., Esser, M. J., Veidt, M., and Boyd, R. (2018). Melatonin as a treatment after traumatic brain injury: a systematic review and meta-analysis of the pre-clinical and clinical literature. *J. Neurotrauma* doi: 10.1089/neu.2018.5752. [Epub ahead of print].

Beaino, G., Khoshnood, B., Kaminski, M., Pierrat, V., Marret, S., Matis, J., et al. (2010). Predictors of cerebral palsy in very preterm infants: the EPIPAGE prospective population-based cohort study. *Dev. Med. Child Neurol.* 52, e119–e125. doi: 10.1111/j.1469-8749.2010.03612.x

Bemelmans, A. P., Husson, I., Jaquet, M., Mallet, J., Kosofsky, B. E., and Gressens, P. (2006). Lentiviral-mediated gene transfer of brain-derived neurotrophic factor is neuroprotective in a mouse model of neonatal excitotoxic challenge. *J. Neurosci. Res.* 83, 50–60. doi: 10.1002/jnr.20704

Bender, A., and Klopstock, T. (2016). Creatine for neuroprotection in neurodegenerative disease: end of story? *Amino Acids* 48, 1929–1940. doi: 10.1007/s00726-015-2165-0

Benzies, K. M., Magill-Evans, J. E., Hayden, K. A., and Ballantyne, M. (2013). Key components of early intervention programs for preterm infants and their parents: a systematic review and meta-analysis. *BMC Pregnancy Childbirth* 13:S10. doi: 10.1186/1471-2393-13-S1-S10

Biran, V., Phan Duy, A., Decobert, F., Bednarek, N., Alberti, C., and Baud, O. (2014). Is melatonin ready to be used in preterm infants as a neuroprotectant? *Dev Med Child Neurol.* 56, 717–723. doi: 10.1111/dmcn.12415

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., et al. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 379, 2162–2172. doi: 10.1016/S0140-6736(12)60820-4

Boyd, R. N., Ziviani, J., Sakzewski, L., Novak, I., Badawi, N., Pannek, K., et al. (2017). REACH: study protocol of a randomised trial of rehabilitation very early in congenital hemiplegia. *BMJ Open* 7, 385–396. doi: 10.1136/bmjopen-2017-017204

Brummelte, S., Grunau, R. E., Chau, V., Poskitt, K. J., Brant, R., Vinall, J., et al. (2012). Procedural pain and brain development in premature newborns. *Ann. Neurol.* 71, 385–396. doi: 10.1002/ana.22267

Byrne, R., Noritz, G., and Maitre, N. L. (2017). Implementation of early diagnosis and intervention guidelines for cerebral palsy in a high-risk infant follow-up clinic. *Pediatr Neurol.* 76, 66–71. doi: 10.1016/j.pediatrneurol.2017.08.002

Carty, M. L., Wixey, J. A., Colditz, P. B., and Buller, K. M. (2008). Post-insult minocycline treatment attenuates hypoxia-ischemia-induced neuroinflammation and white matter injury in the neonatal rat: a comparison of two different dose regimens. *Int. J. Dev. Neurosci.* 26, 477–485. doi: 10.1016/j.ijdevneu.2008.02.005

Cashman, N., Tan, L. Y., Krieger, C., Madler, B., Mackay, A., Mackenzie, I., et al. (2008). Pilot study of granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood stem cells in amyotrophic lateral sclerosis (ALS). *Muscle Nerve* 37, 620–625. doi: 10.1002/mus.20951

Chan, S. Y., Hancox, L. A., Martín-Santos, A., Loubière, L. S., Walter, M. N. M., González, A.-M., et al. (2014). MCT8 expression in human fetal cerebral cortex is reduced in severe intrauterine growth restriction. *J Endocrinol.* 220, 85–95. doi: 10.1530/JOE-13-0400

Chen, C. Y., Georgieff, M., Elison, J., Chen, M., Stinear, J., Mueller, B., et al. (2017). Understanding brain reorganization in infants with perinatal stroke through neuroexcitability and neuroimaging. *Pediatr. Phys. Ther.* 29, 173–178. doi: 10.1097/PEP.0000000000000365

Chen, L., Zhang, G., Khan, A. A., Guo, X., and Gu, Y. (2016). Clinical efficacy and meta-analysis of stem cell therapies for patients with brain ischemia. *Stem Cells Int.* 2016:6129579. doi: 10.1155/2016/6129579

Cheong, J. L., Anderson, P. J., Roberts, G., Burnett, A. C., Lee, K. J., Thompson, D. K., et al. (2013). Contribution of brain size to IQ and educational underperformance in extremely preterm adolescents. *PLoS ONE* 8:e77475. doi: 10.1371/journal.pone.0077475

Cheong, J. L., Doyle, L. W., Burnett, A. C., Lee, K. J., Walsh, J. M., Potter, C. R., et al. (2017). Association between moderate and late preterm birth and neurodevelopment and social-emotional development at age 2 years. *JAMA Pediatr.* 171:e164805. doi: 10.1001/jamapediatrics.2016.4805

Cheong, J. L., Thompson, D. K., Wang, H. X., Hunt, R. W., Anderson, P. J., Inder, T. E., et al. (2009). Abnormal white matter signal on MR imaging is related to abnormal tissue microstructure. *Am. J. Neuroradiol.* 30, 623–628. doi: 10.3174/ajnr.A1399

Chio, A., Mora, G., La Bella, V., Caponnetto, C., Mancardi, G., Sabatelli, M., et al. (2011). Repeated courses of granulocyte colony-stimulating factor in amyotrophic lateral sclerosis: clinical and biological results from a prospective multicenter study. *Muscle Nerve.* 43, 189–195. doi: 10.1002/mus.21851

Choong, C.-J., Baba, K., and Mochizuki, H. (2016). Gene therapy for neurological disorders. *Expert Opin. Biol. Ther.* 16, 143–159. doi: 10.1517/14712598.2016.1114096

Cotten, C. M., Murtha, A. P., Goldberg, R. N., Grotegut, C. A., Smith, P. B., Goldstein, R. F., et al. (2014). Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *J. Pediatr.* 164, 973–979.e1. doi: 10.1016/j.jpeds.2013.11.036

Cramer, S. C., Sur, M., Dobkin, B. H., O'brien, C., Sanger, T. D., Trojanowski, J. Q., et al. (2011). Harnessing neuroplasticity for clinical applications. *Brain* 134, 1591–1609. doi: 10.1093/brain/awr039

De Vries, L. S., Eken, P., and Dubowitz, L. M. (1992). The spectrum of leukomalacia using cranial ultrasound. *Behav. Brain Res.* 49, 1–6. doi: 10.1016/S0166-4328(05)80189-5

De Vries, L. S., Van Haastert, I. C., Benders, M. J., and Groenendaal, F. (2011). Myth: cerebral palsy cannot be predicted by neonatal brain imaging. *Semin. Fetal Neonatal Med.* 16, 279–287. doi: 10.1016/j.siny.2011.04.004

Deepmala, Slattery, J., Kumar, N., Delhey, L., Berk, M., Dean, O, et al. (2015). Clinical trials of N-acetylcysteine in psychiatry and neurology: a systematic review. *Neurosci. Biobehav. Rev.* 55, 294–321. doi: 10.1016/j.neubiorev.2015.04.015

Deffieux, T., Demene, C., Pernot, M., and Tanter, M. (2018). Functional ultrasound neuroimaging: a review of the preclinical and clinical state of the art. *Curr. Opin. Neurobiol.* 50, 128–135. doi: 10.1016/j.conb.2018.02.001

Den Ouden, A. L., Kok, J. H., Verkerk, P. H., Brand, R., and Verloove-Vanhorick, S. P. (1996). The relation between neonatal thyroxine levels and neurodevelopmental outcome at age 5 and 9 years in a national cohort of very preterm and/or very low birth weight infants. *Pediatr. Res.* 39, 142–145. doi: 10.1203/00006450-199601000-00021

Dickinson, H., Bain, E., Wilkinson, D., Middleton, P., Crowther, C. A., and Walker, D. W. (2014a). Creatine for women in pregnancy for neuroprotection of the fetus. *Cochr Datab Syst Rev.* 12:Cd010846. doi: 10.1002/14651858.CD010846.pub2

Dickinson, H., Ellery, S., Ireland, Z., Larosa, D., Snow, R., and Walker, D. W. (2014b). Creatine supplementation during pregnancy: summary of experimental studies suggesting a treatment to improve fetal and neonatal morbidity and reduce mortality in high-risk human pregnancy. *BMC Pregnancy Childbirth* 14:150. doi: 10.1186/1471-2393-14-150

Doeppner, T. R., Herz, J., Gorgens, A., Schlechter, J., Ludwig, A. K., Radtke, S., et al. (2015). Extracellular vesicles improve post-stroke neuroregeneration and prevent postischemic immunosuppression. *Stem Cells Transl. Med.* 4, 1131–1143. doi: 10.5966/sctm.2015-0078

Domoki, F., Perciaccante, J. V., Veltkamp, R., Bari, F., and Busija, D. W. (1999). Mitochondrial potassium channel opener diazoxide preserves neuronal-vascular function after cerebral ischemia in newborn pigs. *Stroke* 30, 2713–8; discussion, 2718–9. doi: 10.1161/01.STR.30.12.2713

Doyle, L. W., Cheong, J. L., Burnett, A., Roberts, G., Lee, K. J., and Anderson, P. J. (2015). Biological and social influences on outcomes of extreme-preterm/low-birth weight adolescents. *Pediatrics* 136, e1513–e1520. doi: 10.1542/peds.2015-2006

Drommelschmidt, K., Serdar, M., Bendix, I., Herz, J., Bertling, F., Prager, S., et al. (2017). Mesenchymal stem cell-derived extracellular vesicles ameliorate inflammation-induced preterm brain injury. *Brain Behav. Immun.* 60, 220–232. doi: 10.1016/j.bbi.2016.11.011

Duning, T., Schiffbauer, H., Warnecke, T., Mohammadi, S., Floel, A., Kolpatzik, K., et al. (2011). G-CSF prevents the progression of structural disintegration of white matter tracts in amyotrophic lateral sclerosis: a pilot trial. *PLoS ONE* 6:e17770. doi: 10.1371/journal.pone.0017770

Dusing, S. C., Brown, S. E., Van Drew, C. M., Thacker, L. R., and Hendricks-Munoz, K. D. (2015). Supporting play exploration and early development intervention from NICU to home: a feasibility study. *Pediatr. Phys. Ther.* 27, 267–274. doi: 10.1097/PEP.0000000000000161

Dusing, S. C., Tripathi, T., Marcinowski, E. C., Thacker, L. R., Brown, L. F., and Hendricks-Munoz, K. D. (2018). Supporting play exploration and early developmental intervention versus usual care to enhance development outcomes during the transition from the neonatal intensive care unit to home: a pilot randomized controlled trial. *BMC Pediatr.* 18:46. doi: 10.1186/s12887-018-1011-4

Edwards, A. D., Redshaw, M. E., Kennea, N., Rivero-Arias, O., Gonzales-Cinca, N., Nongena, P., et al. (2018). Effect of MRI on preterm infants and their families: a randomised trial with nested diagnostic and economic evaluation. *Arch. Dis. Childh. Fetal* 103, F15–F21. doi: 10.1136/archdischild-2017-313102

Eliasson, A. C., Nordstrand, L., Ek, L., Lennartsson, F., Sjostrand, L., Tedroff, K., et al. (2018). The effectiveness of Baby-CIMT in infants younger than 12 months with clinical signs of unilateral-cerebral palsy; an explorative study with randomized design. *Res. Dev. Disabil.* 72, 191–201. doi: 10.1016/j.ridd.2017.11.006

England, T. J., Sprigg, N., Alasheev, A. M., Belkin, A. A., Kumar, A., Prasad, K., et al. (2016). Granulocyte-colony stimulating factor (G-CSF) for stroke: an individual patient data meta-analysis. *Sci. Rep.* 6:36567. doi: 10.1038/srep36567

Fan, L. W., Pang, Y., Lin, S., Tien, L. T., Ma, T., Rhodes, P. G., et al. (2005). Minocycline reduces lipopolysaccharide-induced neurological dysfunction and brain injury in the neonatal rat. *J. Neurosci. Res.* 82, 71–82. doi: 10.1002/jnr.20623

Fan, Z. Z., Cai, H. B., Ge, Z. M., Wang, L. Q., Zhang, X. D., Li, L., et al. (2015). The efficacy and safety of granulocyte colony-stimulating factor for patients with stroke. *J. Stroke Cerebrovasc. Dis.* 24, 1701–1708. doi: 10.1016/j.jstrokecerebrovasdis.2014.11.033

Ferriero, D. M. (2016). The vulnerable newborn brain: imaging patterns of acquired perinatal injury. *Neonatology* 109, 345–351. doi: 10.1159/000444896

Fischer, H. S., Reibel, N. J., Buhrer, C., and Dame, C. (2017). Prophylactic early erythropoietin for neuroprotection in preterm infants: a meta-analysis. *Pediatrics* 139, 556–566. doi: 10.1542/peds.2016-4317

Fleiss, B., and Gressens, P. (2012). Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? *Lancet Neurol.* 11, 556–566. doi: 10.1016/s1474-4422(12)70058-3

Fleiss, B., Guillot, P. V., Titomanlio, L., Baud, O., Hagberg, H., and Gressens, P. (2014). Stem cell therapy for neonatal brain injury. *Clin. Perinatol.* 41, 133–148. doi: 10.1016/j.clp.2013.09.002

Fogal, B., Mcclaskey, C., Yan, S., Yan, H., and Rivkees, S. A. (2010). Diazoxide promotes oligodendrocyte precursor cell proliferation and myelination. *PLoS ONE* 5:e10906. doi: 10.1371/journal.pone.0010906

Fox, N. A., Almas, A. N., Degnan, K. A., Nelson, C. A., and Zeanah, C. H. (2011). The effects of severe psychosocial deprivation and foster care intervention on cognitive development at 8 years of age: findings from the Bucharest early intervention project. *J. Child Psychol. Psychiatry* 52, 919–928. doi: 10.1111/j.1469-7610.2010.02355.x

Friel, K., Chakrabarty, S., Kuo, H. C., and Martin, J. (2012). Using motor behavior during an early critical period to restore skilled limb movement after damage to the corticospinal system during development. *J. Neurosci.* 32, 9265–9276. doi: 10.1523/JNEUROSCI.1198-12.2012

Friesema, E. C., Ganguly, S., Abdalla, A., Manning Fox, J. E., Halestrap, A. P., and Visser, T. J. (2003). Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. *J. Biol. Chem.* 278, 40128–40135. doi: 10.1074/jbc.M300909200

Galea, C., Mcintyre, S., Smithers-Sheedy, H., Reid, S. M., Gibson, C., Delacy, M., et al. (2018). Cerebral palsy trends in Australia (1995–2009): a population-based observational study. *Dev. Med. Child Neurol.* 61, 186–193. doi: 10.1111/dmcn.14011

Galvez-Contreras, A., Quinones-Hinojosa, A., and Gonzalez-Perez, O. (2013). The role of EGFR and ErbB family related proteins in the oligodendrocyte specification in germinal niches of the adult mammalian brain. *Front. Cell. Neurosci.* 7, 337–352. doi: 10.3389/fncel.2013.00258

Garrido-Mesa, N., Zarzuelo, A., and Galvez, J. (2013). Minocycline: far beyond an antibiotic. *Br. J. Pharmacol.* 169, 337–352. doi: 10.1111/bph.12139

Gordon, P. H., Moore, D. H., Miller, R. G., Florence, J. M., Verheijde, J. L., Doorish, C., et al. (2007). Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. *Lancet Neurol.* 6, 1045–1053. doi: 10.1016/S1474-4422(07)70270-3

Graham, E. M., Everett, A. D., Delpech, J. C., and Northington, F. J. (2018). Blood biomarkers for evaluation of perinatal encephalopathy: state of the art. *Curr. Opin. Pediatr.* 30, 199–203. doi: 10.1097/MOP.0000000000000591

Gressens, P., Marret, S., Hill, J. M., Brenneman, D. E., Gozes, I., Fridkin, M., et al. (1997). Vasoactive intestinal peptide prevents excitotoxic cell death in the murine developing brain. *J. Clin. Invest.* 100, 390–397. doi: 10.1172/JCI119545

Guzzetta, A., Baldini, S., Bancale, A., Baroncelli, L., Ciucci, F., Ghirri, P., et al. (2009). Massage accelerates brain development and the maturation of visual function. *J. Neurosci.* 29, 6042–6051. doi: 10.1523/JNEUROSCI.5548-08.2009

Hall, J., Prabhakar, S., Balaj, L., Lai, C. P., Cerione, R. A., and Breakefield, X. O. (2016). Delivery of therapeutic proteins via extracellular vesicles: review and potential treatments for Parkinson's disease, glioma, and schwannoma. *Cell Mol. Neurobiol.* 36, 417–427. doi: 10.1007/s10571-015-0309-0

Himpens, E., Van Den Broeck, C., Oostra, A., Calders, P., and Vanhaesebrouck, P. (2008). Prevalence, type, distribution, and severity of cerebral palsy in relation to gestational age: a meta-analytic review. *Dev. Med. Child Neurol.* 50, 334–340. doi: 10.1111/j.1469-8749.2008.02047.x

Hintz, S. R., Slovis, T., Bulas, D., Van Meurs, K. P., Perritt, R., Stevenson, D. K., et al. (2007). Interobserver reliability and accuracy of cranial ultrasound scanning interpretation in premature infants. *J. Pediatr.* 150, 592–596.e5. doi: 10.1016/j.jpeds.2007.02.012

Howell, B. R., Styner, M. A., Gao, W., Yap, P. T., Wang, L., Baluyot, K., et al. (2019). The UNC/UMN Baby Connectome Project (BCP): an overview of the study design and protocol development. *Neuroimage* 185, 891–905. doi: 10.1016/j.neuroimage.2018.03.049

Huang, X., Liu, Y., Bai, S., Peng, L., Zhang, B., and Lu, H. (2017). Granulocyte colony stimulating factor therapy for stroke: a pairwise meta-analysis of randomized controlled trial. *PLoS ONE* 12:e0175774. doi: 10.1371/journal.pone.0175774

Huntington Study Group Domino Investigators (2010). A futility study of minocycline in Huntington's disease. *Mov. Disord.* 25, 2219–2224. doi: 10.1002/mds.23236

Husson, I., Mesples, B., Bac, P., Vamecq, J., Evrard, P., and Gressens, P. (2002). Melatoninergic neuroprotection of the murine periventricular white matter against neonatal excitotoxic challenge. *Ann. Neurol.* 51, 82–92. doi: 10.1002/ana.10072

Husson, I., Rangon, C. M., Lelievre, V., Bemelmans, A. P., Sachs, P., Mallet, J., et al. (2005). BDNF-induced white matter neuroprotection and stage-dependent neuronal survival following a neonatal excitotoxic challenge. *Cereb. Cortex.* 15, 250–261. doi: 10.1093/cercor/bhh127

Inder, T. E., Warfield, S. K., Wang, H., Huppi, P. S., and Volpe, J. J. (2005). Abnormal cerebral structure is present at term in premature infants. *Pediatrics* 115, 286–294. doi: 10.1542/peds.2004-0326

Inder, T. E., Wells, S. J., Mogridge, N. B., Spencer, C., and Volpe, J. J. (2003). Defining the nature of the cerebral abnormalities in the premature infant: a qualitative magnetic resonance imaging study. *J. Pediatr.* 143, 171–179. doi: 10.1067/S0022-3476(03)00357-3

Ireland, Z., Castillo-Melendez, M., Dickinson, H., Snow, R., and Walker, D. W. (2011). A maternal diet supplemented with creatine from mid-pregnancy protects the newborn spiny mouse brain from birth hypoxia. *Neuroscience* 194, 372–379. doi: 10.1016/j.neuroscience.2011.05.012

Ireland, Z., Dickinson, H., Snow, R., and Walker, D. W. (2008). Maternal creatine: does it reach the fetus and improve survival after an acute hypoxic episode in the spiny mouse (*Acomys cahirinus*)? *Am. J. Obstet. Gynecol.* 198, 431.e1–6. doi: 10.1016/j.ajog.2007.10.790

Jantzie, L. L., Oppong, A. Y., Conteh, F. S., Yellowhair, T. R., Kim, J., Fink, G., et al. (2018). Repetitive neonatal erythropoietin and melatonin combinatorial

treatment provides sustained repair of functional deficits in a rat model of cerebral palsy. *Front. Neurol.* 9:233. doi: 10.3389/fneur.2018.00233

Jellema, R. K., Lima Passos, V., Ophelders, D. R., Wolfs, T. G., Zwanenburg, A., De Munter, S., et al. (2013). Systemic G-CSF attenuates cerebral inflammation and hypomyelination but does not reduce seizure burden in preterm sheep exposed to global hypoxia-ischemia. *Exp. Neurol.* 250, 293–303. doi: 10.1016/j.expneurol.2013.09.026

Jin, C., Londono, I., Mallard, C., and Lodygensky, G. A. (2015). New means to assess neonatal inflammatory brain injury. *J. Neuroinflammation* 12:180. doi: 10.1186/s12974-015-0397-2

Johnston, M. V. (2009). Plasticity in the developing brain: implications for rehabilitation. *Dev. Disabil. Res. Rev.* 15, 94–101. doi: 10.1002/ddrr.64

Juul, S. E., Mayock, D. E., Comstock, B. A., and Heagerty, P. J. (2015). Neuroprotective potential of erythropoietin in neonates; design of a randomized trial. *Matern. Health Neonatol. Perinatol.* 1:27. doi: 10.1186/s40748-015-0028-z

Kannan, S., Dai, H., Navath, R. S., Balakrishnan, B., Jyoti, A., Janisse, J., et al. (2012). Dendrimer-based postnatal therapy for neuroinflammation and cerebral palsy in a rabbit model. *Sci. Transl. Med.* 4:130ra46. doi: 10.1126/scitranslmed.3003162

Keller, M., Simbruner, G., Gorna, A., Urbanek, M., Tinhofer, I., Griesmaier, E., et al. (2006). Systemic application of granulocyte-colony stimulating factor and stem cell factor exacerbates excitotoxic brain injury in newborn mice. *Pediatr. Res.* 59, 549–553. doi: 10.1203/01.pdr.0000205152.38692.81

Kenner, C., Lott, J. W., and Flandermeyer, A. A. (eds.). (1998). *Comprehensive Neonatal Care: A physiologic Perspective, 2nd Edn.* Philadelphia, PA: W.B. Saunders.

Khwaja, O., and Volpe, J. J. (2008). Pathogenesis of cerebral white matter injury of prematurity. Archives of disease in childhood. *Fetal Neonatal Edition.* 93, F153–F161. doi: 10.1136/adc.2006.108837

Kidokoro, H., Anderson, P. J., Doyle, L. W., Woodward, L. J., Neil, J. J., and Inder, T. E. (2014). Brain injury and altered brain growth in preterm infants: predictors and prognosis. *Pediatrics* 134, e444–e453. doi: 10.1542/peds.2013-2336

Kim, B. R., Shim, J. W., Sung, D. K., Kim, S. S., Jeon, G. W., Kim, M. J., et al. (2008). Granulocyte stimulating factor attenuates hypoxic-ischemic brain injury by inhibiting apoptosis in neonatal rats. *Yonsei Med. J.* 49, 836–842. doi: 10.3349/ymj.2008.49.5.836

Kim, D. K., Nishida, H., An, S. Y., Shetty, A. K., Bartosh, T. J., and Prockop, D. J. (2016). Chromatographically isolated CD63+CD81+ extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI. *Proc. Natl. Acad. Sci. U.S.A.* 113, 170–175. doi: 10.1073/pnas.1522297113

Kleim, J. A., and Jones, T. A. (2008). Principles of experience-dependent neural plasticity: implications for rehabilitation after brain damage. *J. Speech Lang. Hear. Res.* 51, S225–S239. doi: 10.1044/1092-4388(2008/018)

Kolb, B., Harker, A., and Gibb, R. (2017). Principles of plasticity in the developing brain. *Dev. Med. Child. Neurol.* 59, 1218–1223. doi: 10.1111/dmcn.13546

Kolb, B., Mychasiuk, R., Williams, P., and Gibb, R. (2011). Brain plasticity and recovery from early cortical injury. *Dev. Med. Child. Neurol.* 53(Suppl 4), 4–8. doi: 10.1111/j.1469-8749.2011.04054.x

Kordelas, L., Rebmann, V., Ludwig, A. K., Radtke, S., Ruesing, J., Doeppner, T. R., et al. (2014). MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 28:970. doi: 10.1038/leu.2014.41

Lee, H. K., Finniss, S., Cazacu, S., Xiang, C., and Brodie, C. (2014). Mesenchymal stem cells deliver exogenous miRNAs to neural cells and induce their differentiation and glutamate transporter expression. *Stem Cells Dev.* 23, 2851–2861. doi: 10.1089/scd.2014.0146

Lei, J., Rosenzweig, J. M., Mishra, M. K., Alshehri, W., Brancusi, F., Mclane, M., et al. (2017). Maternal dendrimer-based therapy for inflammation-induced preterm birth and perinatal brain injury. *Sci. Rep.* 7:6106. doi: 10.1038/s41598-017-06113-2

Leijser, L. M., Liauw, L., Veen, S., De Boer, I. P., Walther, F. J., and Van Wezel-Meijler, G. (2008). Comparing brain white matter on sequential cranial ultrasound and MRI in very preterm infants. *Neuroradiology* 50:799. doi: 10.1007/s00234-008-0408-4

Lekic, T., Manaenko, A., Rolland, W., Virbel, K., Hartman, R., Tang, J., et al. (2011). Neuroprotection by melatonin after germinal matrix hemorrhage in neonatal rats. *Acta Neurochir. Suppl.* 111, 201–206. doi: 10.1007/978-3-7091-0693-8_34

Li, L., Mcbride, D. W., Doycheva, D., Dixon, B. J., Krafft, P. R., Zhang, J. H., et al. (2015). G-CSF attenuates neuroinflammation and stabilizes the blood-brain barrier via the PI3K/Akt/GSK-3beta signaling pathway following neonatal hypoxia-ischemia in rats. *Exp. Neurol.* 272, 135–144. doi: 10.1016/j.expneurol.2014.12.020

Li, Y., Yang, Y. Y., Ren, J. L., Xu, F., Chen, F. M., and Li, A. (2017). Exosomes secreted by stem cells from human exfoliated deciduous teeth contribute to functional recovery after traumatic brain injury by shifting microglia M1/M2 polarization in rats. *Stem Cell Res. Ther.* 8:198. doi: 10.1186/s13287-017-0648-5

Lim, R., Malhotra, A., Mockler, J., and Wallace, E. (2018). Allogeneic amniotic epithelial cells for established bronchopulmonary dysplasia in premature, low birthweight infants: a first-in-human safety trial. *Cytotherapy* 20:S21. doi: 10.1016/j.jcyt.2018.02.046

Linsell, L., Malouf, R., Morris, J., Kurinczuk, J. J., and Marlow, N. (2016). Prognostic factors for cerebral palsy and motor impairment in children born very preterm or very low birthweight: a systematic review. *Dev. Med. Child Neurol.* 58, 554–569. doi: 10.1111/dmcn.12972

Liu, X., Fu, X., Dai, G., Wang, X., Zhang, Z., Cheng, H., et al. (2017). Comparative analysis of curative effect of bone marrow mesenchymal stem cell and bone marrow mononuclear cell transplantation for spastic cerebral palsy. *J. Transl. Med.* 15:48. doi: 10.1186/s12967-017-1149-0

Lubetzky, R., Mimouni, F. B., Dollberg, S., Reifen, R., Ashbel, G., and Mandel, D. (2010). Effect of music by Mozart on energy expenditure in growing preterm infants. *Pediatrics.* 125, e24–e28. doi: 10.1542/peds.2009-0990

Malcolm, W. F. (ed.). (2014). *Beyond the NICU: Comprehensive Care of the High-Risk Infant.* New York, NY: McGraw-Hill Professional Publication.

Malhotra, K., Chang, J. J., Khunger, A., Blacker, D., Switzer, J. A., Goyal, N., et al. (2018). Minocycline for acute stroke treatment: a systematic review and meta-analysis of randomized clinical trials. *J. Neurol.* 265, 1871–1879. doi: 10.1007/s00415-018-8935-3

Martin, J., Hamilton, B., Osterman, M., Driscoll, A., and Drake, P. (2018). *Births: Final Data for 2016, National Vital Statistics Reports.* Hyattsville, MD: National Center for Health Statistics.

Martinez-Biarge, M., Groenendaal, F., Kersbergen, K. J., Benders, M. J., Foti, F., Cowan, F. M., et al. (2016). MRI based preterm white matter injury classification: the importance of sequential imaging in determining severity of injury. *PLoS ONE* 11:e0156245. doi: 10.1371/journal.pone.0156245

Merchant, N., Azzopardi, D., Counsell, S., Gressens, P., Dierl, A., Gozar, I., et al. (2014). Melatonin as a novel neuroprotectant in preterm infants – a double blinded randomised controlled trial (mint study). *Arch. Dis. Childhood* 99:A43. doi: 10.1136/archdischild-2014-307384.125

Merchant, N. M., Azzopardi, D. V., Hawwa, A. F., Mcelnay, J. C., Middleton, B., Arendt, J., et al. (2013). Pharmacokinetics of melatonin in preterm infants. *Br. J. Clin. Pharmacol.* 76, 725–733. doi: 10.1111/bcp.12092

Miller, S. L., Yawno, T., Alers, N. O., Castillo-Melendez, M., Supramaniam, V. G., Vanzyl, N., et al. (2014). Antenatal antioxidant treatment with melatonin to decrease newborn neurodevelopmental deficits and brain injury caused by fetal growth restriction. *J. Pineal Res.* 56, 283–294. doi: 10.1111/jpi.12121

Min, K., Song, J., Lee, J. H., Kang, M. S., Jang, S. J., Kim, S. H., et al. (2013). Allogenic umbilical cord blood therapy combined with erythropoietin for patients with severe traumatic brain injury: three case reports. *Restor. Neurol. Neurosci.* 31, 397–410. doi: 10.3233/RNN-120289

Moody, C., Callahan, T. J., Aldrich, H., Gance-Cleveland, B., and Sables-Baus, S. (2017). Early initiation of newborn individualized developmental care and assessment program (NIDCAP) reduces length of stay: a quality improvement project. *J. Pediatr. Nurs.* 32, 59–63. doi: 10.1016/j.pedn.2016.11.001

Morgan, C., Darrah, J., Gordon, A. M., Harbourne, R., Spittle, A., Johnson, R., et al. (2016a). Effectiveness of motor interventions in infants with cerebral palsy: a systematic review. *Dev. Med. Child. Neurol.* 58, 900–909. doi: 10.1111/dmcn.13105

Morgan, C., Novak, I., and Badawi, N. (2013). Enriched environments and motor outcomes in cerebral palsy: systematic review and meta-analysis. *Pediatrics* 132, e735–e746. doi: 10.1542/peds.2012-3985

Morgan, C., Novak, I., Dale, R. C., and Badawi, N. (2015). Optimising motor learning in infants at high risk of cerebral palsy: a pilot study. *BMC Pediatr.* 15:30. doi: 10.1186/s12887-015-0347-2

Morgan, C., Novak, I., Dale, R. C., Guzzetta, A., and Badawi, N. (2014). GAME (Goals - Activity - Motor Enrichment): protocol of a single blind randomised

controlled trial of motor training, parent education and environmental enrichment for infants at high risk of cerebral palsy. *BMC Neurol.* 14:203. doi: 10.1186/s12883-014-0203-2

Morgan, C., Novak, I., Dale, R. C., Guzzetta, A., and Badawi, N. (2016b). Single blind randomised controlled trial of GAME (Goals - Activity - Motor Enrichment) in infants at high risk of cerebral palsy. *Res. Dev. Disabil.* 55, 256–267. doi: 10.1016/j.ridd.2016.04.005

Mukerji, A., Shah, V., and Shah, P. S. (2015). Periventricular/intraventricular hemorrhage and neurodevelopmental outcomes: a meta-analysis. *Pediatrics* 136, 1132–1143. doi: 10.1542/peds.2015-0944

Murphy, B. P., Inder, T. E., Rooks, V., Taylor, G. A., Anderson, N. J., Mogridge, N., et al. (2002). Posthaemorrhagic ventricular dilatation in the premature infant: natural history and predictors of outcome. *Arch. Dis. Child Fetal Neonatal Ed.* 87, F37–F41. doi: 10.1136/fn.87.1.F37

Nance, E., Porambo, M., Zhang, F., Mishra, M. K., Buelow, M., Getzenberg, R., et al. (2015). Systemic dendrimer-drug treatment of ischemia-induced neonatal white matter injury. *J. Control Release* 214, 112–120. doi: 10.1016/j.jconrel.2015.07.009

Nassar, W., El-Ansary, M., Sabry, D., Mostafa, M. A., Fayad, T., Kotb, E., et al. (2016). Umbilical cord mesenchymal stem cells derived extracellular vesicles can safely ameliorate the progression of chronic kidney diseases. *Biomater. Res.* 20:21. doi: 10.1186/s40824-016-0068-0

Nefussy, B., Artamonov, I., Deutsch, V., Naparstek, E., Nagler, A., and Drory, V. E. (2010). Recombinant human granulocyte-colony stimulating factor administration for treating amyotrophic lateral sclerosis: a pilot study. *Amyotroph. Lateral. Scler.* 11, 187–193. doi: 10.3109/17482960902933809

Nelson, M. N., White-Traut, R. C., Vasan, U., Silvestri, J., Comiskey, E., Meleedy-Rey, P., et al. (2001). One-year outcome of auditory-tactile-visual-vestibular intervention in the neonatal intensive care unit: effects of severe prematurity and central nervous system injury. *J. Child Neurol.* 16, 493–498. doi: 10.1177/088307380101600706

Nemeth, C. L., Drummond, G. T., Mishra, M. K., Zhang, F., Carr, P., Garcia, M. S., et al. (2017). Uptake of dendrimer-drug by different cell types in the hippocampus after hypoxic-ischemic insult in neonatal mice: Effects of injury, microglial activation and hypothermia. *Nanomedicine* 13, 2359–2369. doi: 10.1016/j.nano.2017.06.014

Neubauer, V., Wegleiter, K., Posod, A., Urbanek, M., Wechselberger, K., Kiechl-Kohlendorfer, U., et al. (2016). Delayed application of the haematopoietic growth factors G-CSF/SCF and FL reduces neonatal excitotoxic brain injury. *Brain Res.* 1634, 94–103. doi: 10.1016/j.brainres.2015.12.058

Ng, S. M., Turner, M. A., Gamble, C., Didi, M., Victor, S., Atkinson, J., et al. (2014). Effect of thyroxine on brain microstructure in extremely premature babies: magnetic resonance imaging findings in the TIPIT study. *Pediatr. Radiol.* 44, 987–996. doi: 10.1007/s00247-014-2911-6

Ninds Net-Pd Investigators. (2006). A randomized, double-blind, futility clinical trial of creatine and minocycline in early Parkinson disease. *Neurology* 66, 664–671. doi: 10.1212/01.wnl.0000201252.57661.e1

Nithianantharajah, J., and Hannan, A. J. (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nat. Rev. Neurosci.* 7, 697–709. doi: 10.1038/nrn1970

Nordstrand, L., Holmefur, M., Kits, A., and Eliasson, A. C. (2015). Improvements in bimanual hand function after baby-CIMT in two-year old children with unilateral cerebral palsy: a retrospective study. *Res. Dev. Disabil.* 41–42, 86–93. doi: 10.1016/j.ridd.2015.05.003

Novak, I., Hines, M., Goldsmith, S., and Barclay, R. (2012). Clinical prognostic messages from a systematic review on cerebral palsy. *Pediatrics* 130, e1285–e1312. doi: 10.1542/peds.2012-0924

Novak, I., Mcintyre, S., Morgan, C., Campbell, L., Dark, L., Morton, N., et al. (2013). A systematic review of interventions for children with cerebral palsy: state of the evidence. *Dev. Med. Child Neurol.* 55, 885–910. doi: 10.1111/dmcn.12246

Novak, I., Morgan, C., Adde, L., Blackman, J., Boyd, R. N., Brunstrom-Hernandez, J., et al. (2017). Early, accurate diagnosis and early intervention in cerebral palsy: advances in diagnosis and treatment. *JAMA Pediatr.* 171, 897–907. doi: 10.1001/jamapediatrics.2017.1689

Novak, I., Walker, K., Hunt, R. W., Wallace, E. M., Fahey, M., and Badawi, N. (2016). Concise review: stem cell interventions for people with cerebral palsy: systematic review with meta-analysis. *Stem Cells Transl. Med.* 5, 1014–1025. doi: 10.5966/sctm.2015-0372

Ohgi, S., Fukuda, M., Akiyama, T., and Gima, H. (2004). Effect of an early intervention programme on low birthweight infants with cerebral injuries. *J. Paediatr. Child Health.* 40, 689–695. doi: 10.1111/j.1440-1754.2004.00512.x

Ohlsson, A., and Jacobs, S. E. (2013). NIDCAP: a systematic review and meta-analyses of randomized controlled trials. *Pediatrics* 131, e881–e893. doi: 10.1542/peds.2012-2121

Ophelders, D. R., Wolfs, T. G., Jellema, R. K., Zwanenburg, A., Andriessen, P., Delhaas, T., et al. (2016). Mesenchymal stromal cell-derived extracellular vesicles protect the fetal brain after hypoxia-ischemia. *Stem Cells Transl. Med.* 5, 754–763. doi: 10.5966/sctm.2015-0197

Osborn, D. A., and Hunt, R. W. (2007). Prophylactic postnatal thyroid hormones for prevention of morbidity and mortality in preterm infants. *Cochr. Database Syst. Rev.* 1:Cd005948. doi: 10.1002/14651858.CD005948.pub2

Otero-Ortega, L., Gomez De Frutos, M. C., Laso-Garcia, F., Rodriguez-Frutos, B., Medina-Gutierrez, E., Lopez, J. A., et al. (2018). Exosomes promote restoration after an experimental animal model of intracerebral hemorrhage. *J. Cereb. Blood Flow Metab.* 38, 767–779. doi: 10.1177/0271678X17708917

Palmer, F. B., Shapiro, B. K., Wachtel, R. C., Allen, M. C., Hiller, J. E., Harryman, S. E., et al. (1988). The effects of physical therapy on cerebral palsy. *New Engl. J. Med.* 318, 803–808. doi: 10.1056/NEJM198803313181302

Panfoli, I., Ravera, S., Podesta, M., Cossu, C., Santucci, L., Bartolucci, M., et al. (2016). Exosomes from human mesenchymal stem cells conduct aerobic metabolism in term and preterm newborn infants. *FASEB J.* 30, 1416–1424. doi: 10.1096/fj.15-279679

Papile, L. A., Burstein, J., Burstein, R., and Koffler, H. (1978). Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. *J. Pediatr.* 92, 529–534. doi: 10.1016/S0022-3476(78)80282-0

Pastula, D. M., Moore, D. H., and Bedlack, R. S. (2012). Creatine for amyotrophic lateral sclerosis/motor neuron disease. *Cochr. Database Syst. Rev.* 12:Cd005225. doi: 10.1002/14651858.CD005225.pub3

Polin, R. A., and Carlo, W. A. (2014). Surfactant replacement therapy for preterm and term neonates with respiratory distress. *Pediatrics* 133:156. doi: 10.1542/peds.2013-3443

Rah, W. J., Lee, Y. H., Moon, J. H., Jun, H. J., Kang, H. R., Koh, H., et al. (2017). Neuroregenerative potential of intravenous G-CSF and autologous peripheral blood stem cells in children with cerebral palsy: a randomized, double-blind, cross-over study. *J. Transl. Med.* 15:16. doi: 10.1186/s12967-017-1120-0

Reid, L. B., Pagnozzi, A. M., Fiori, S., Boyd, R. N., Dowson, N., and Rose, S. E. (2017). Measuring neuroplasticity associated with cerebral palsy rehabilitation: an MRI based power analysis. *Int. J. Dev. Neurosci.* 58, 17–25. doi: 10.1016/j.ijdevneu.2017.01.010

Reid, L. B., Rose, S. E., and Boyd, R. N. (2015). Rehabilitation and neuroplasticity in children with unilateral cerebral palsy. *Nat. Rev. Neurol.* 11, 390–400. doi: 10.1038/nrneurol.2015.97

Reid, S. M., Dagia, C. D., Ditchfield, M. R., Carlin, J. B., and Reddihough, D. S. (2014). Population-based studies of brain imaging patterns in cerebral palsy. *Dev. Med. Child. Neurol.* 56, 222–232. doi: 10.1111/dmcn.12228

Rodriguez, R. G., and Pattini, A. E. (2016). Neonatal intensive care unit lighting: update and recommendations. *Arch. Argent. Pediatr.* 114, 361–367. doi: 10.5546/aap.2016.361

Rooman, R. P., Du Caju, M. V., De Beeck, L. O., Docx, M., Van Reempts, P., and Van Acker, K. J. (1996). Low thyroxinaemia occurs in the majority of very preterm newborns. *Eur. J. Pediatr.* 155, 211–215. doi: 10.1007/BF01953940

Rudnicki, J., Kawa, M. P., Kotowski, M., Michalczyk, B., Ustianowski, P., Czajka, R., et al. (2015). Clinical evaluation of the safety and feasibility of whole autologous cord blood transplant as a source of stem and progenitor cells for extremely premature neonates: preliminary report. *Exp. Clin. Transplant.* 13, 563–572. doi: 10.6002/ect.2015.0081

Ruegger, C. M., Hagmann, C. F., Buhrer, C., Held, L., Bucher, H. U., and Wellmann, S. (2015). Erythropoietin for the repair of cerebral injury in very preterm infants (EpoRepair). *Neonatology* 108, 198–204. doi: 10.1159/000437248

Sakellaris, G., Kotsiou, M., Tamiolaki, M., Kalostos, G., Tsapaki, E., Spanaki, M., et al. (2006). Prevention of complications related to traumatic brain injury in children and adolescents with creatine administration:

an open label randomized pilot study. *J Trauma.* 61, 322–329. doi: 10.1097/01.ta.0000230269.46108.d5

Sakzewski, L., Gordon, A., and Eliasson, A. C. (2014). The state of the evidence for intensive upper limb therapy approaches for children with unilateral cerebral palsy. *J. Child Neurol.* 29, 1077–1090. doi: 10.1177/0883073814533150

Sale, A., Berardi, N., and Maffei, L. (2009). Enrich the environment to empower the brain. *Trends Neurosci.* 32, 233–239. doi: 10.1016/j.tins.2008.12.004

Sangobowale, M., Nikulina, E., and Bergold, P. J. (2018). Minocycline plus N-acetylcysteine protect oligodendrocytes when first dosed 12 hours after closed head injury in mice. *Neurosci. Lett.* 682, 16–20. doi: 10.1016/j.neulet.2018.06.010

Scafidi, J., Hammond, T. R., Scafidi, S., Ritter, J., Jablonska, B., Roncal, M., et al. (2014). Intranasal epidermal growth factor treatment rescues neonatal brain injury. *Nature* 506, 230–234. doi: 10.1038/nature12880

Schlager, G. W., Griesmaier, E., Wegleiter, K., Neubauer, V., Urbanek, M., Kiechl-Kohlendorfer, U., et al. (2011). Systemic G-CSF treatment does not improve long-term outcomes after neonatal hypoxic-ischaemic brain injury. *Exp. Neurol.* 230, 67–74. doi: 10.1016/j.expneurol.2010.11.021

Scratch, S. E., Hunt, R. W., Thompson, D. K., Ahmadzai, Z. M., Doyle, L. W., Inder, T. E., et al. (2014). Free thyroxine levels after very preterm birth and neurodevelopmental outcomes at age 7 years. *Pediatrics* 133, e955–e963. doi: 10.1542/peds.2013-2425

Shin, Y. K., and Cho, S. R. (2016). Exploring erythropoietin and G-CSF combination therapy in chronic stroke patients. *Int. J. Mol. Sci.* 17:463. doi: 10.3390/ijms17040463

Shoemark, H., Harcourt, E., Arnup, S. J., and Hunt, R. W. (2016). Characterising the ambient sound environment for infants in intensive care wards. *J. Paediatr. Child Health* 52, 436–440. doi: 10.1111/jpc.13084

Simonsen, K. A., Anderson-Berry, A. L., Delair, S. F., and Davies, H. D. (2014). Early-onset neonatal sepsis. *Clin. Microbiol. Rev.* 27, 21–47. doi: 10.1128/CMR.00031-13

Siren, A. L., Fasshauer, T., Bartels, C., and Ehrenreich, H. (2009). Therapeutic potential of erythropoietin and its structural or functional variants in the nervous system. *Neurotherapeutics* 6, 108–127. doi: 10.1016/j.nurt.2008.10.041

Smilga, A. S., Garfinkle, J., Ng, P., Andersen, J., Buckley, D., Fehlings, D., et al. (2018). Neonatal infection in children with cerebral palsy: a registry-based cohort study. *Pediatr. Neurol.* 80, 77–83. doi: 10.1016/j.pediatrneurol.2017.11.006

Sokolov, M. E., Bashirov, F. V., Markosyan, V. A., Povysheva, T. V., Fadeev, F. O., Izmailov, A. A., et al. (2018). Triple-gene therapy for stroke: a proof-of-concept *in vivo* study in rats. *Front. Pharmacol.* 9:111. doi: 10.3389/fphar.2018.00111

Solaroglu, I., Jadhav, V., and Zhang, J. H. (2007). Neuroprotective effect of granulocyte-colony stimulating factor. *Front. Biosci.* 12, 712–724. doi: 10.2741/2095

Spittle, A., Orton, J., Anderson, P. J., Boyd, R., and Doyle, L. W. (2015). Early developmental intervention programmes provided post hospital discharge to prevent motor and cognitive impairment in preterm infants. *Cochr. Datab. Syst. Rev.* 11:Cd005495. doi: 10.1002/14651858.CD005495.pub4

Spittle, A. J., Anderson, P. J., Lee, K. J., Ferretti, C., Eeles, A., Orton, J., et al. (2010). Preventive care at home for very preterm infants improves infant and caregiver outcomes at 2 years. *Pediatrics* 126, e171–e178. doi: 10.1542/peds.2009-3137

Spittle, A. J., Cheong, J., Doyle, L. W., Roberts, G., Lee, K. J., Lim, J., et al. (2011). Neonatal white matter abnormality predicts childhood motor impairment in very preterm children. *Dev. Med. Child. Neurol.* 53, 1000–1006. doi: 10.1111/j.1469-8749.2011.04095.x

Spittle, A. J., Morgan, C., Olsen, J. E., Novak, I., and Cheong, J. L. Y. (2018). Early diagnosis and treatment of cerebral palsy in children with a history of preterm birth. *Clin. Perinatol.* 45, 409–420. doi: 10.1016/j.clp.2018.05.011

Sterling, C., Taub, E., Davis, D., Rickards, T., Gauthier, L. V., Griffin, A., et al. (2013). Structural neuroplastic change after constraint-induced movement therapy in children with cerebral palsy. *Pediatrics* 131, e1664–e1669. doi: 10.1542/peds.2012-2051

Strahan, J. A., Walker, W. H. 2nd, Montgomery, T. R., and Forger, N. G. (2017). Minocycline causes widespread cell death and increases microglial labeling in the neonatal mouse brain. *Dev. Neurobiol.* 77, 753–766. doi: 10.1002/dneu.22457

Stritzke, A., Thomas, S., Amin, H., Fusch, C., and Lodha, A. (2017). Renal consequences of preterm birth. *Mol. Cell. Pediatr.* 4:2. doi: 10.1186/s40348-016-0068-0

Sun, J. M., Song, A. W., Case, L. E., Mikati, M. A., Gustafson, K. E., Simmons, R., et al. (2017). Effect of autologous cord blood infusion on motor function and brain connectivity in young children with cerebral palsy: a randomized, placebo-controlled trial. *Stem Cells Transl Med.* 6, 2071–2078. doi: 10.1002/sctm.17-0102

Sun, Y., Luo, Z.-M., Guo, X.-M., Su, D.-F., and Liu, X. (2015). An updated role of microRNA-124 in central nervous system disorders: a review. *Front. Cell. Neurosci.* 9:193. doi: 10.3389/fncel.2015.00193

Suzumura, H., Nitta, A., Tsuboi, Y., Watabe, Y., Kuribayashi, R., and Arisaka, O. (2011). Thyroxine for transient hypothyroxinemia and cerebral palsy in extremely preterm infants. *Pediatr. Int.* 53, 463–467. doi: 10.1111/j.1442-200X.2010.03287.x

Towsley, K., Shevell, M. I., and Dagenais, L. (2011). Population-based study of neuroimaging findings in children with cerebral palsy. *Eur. J. Paediatr. Neurol.* 15, 29–35. doi: 10.1016/j.ejpn.2010.07.005

Trounson, A., and Dewitt, N. D. (2016). Pluripotent stem cells progressing to the clinic. *Nat. Rev. Mol. Cell. Biol.* 17, 194–200. doi: 10.1038/nrm.2016.10

Tsuji, M., Wilson, M. A., Lange, M. S., and Johnston, M. V. (2004). Minocycline worsens hypoxic-ischemic brain injury in a neonatal mouse model. *Exp. Neurol.* 189, 58–65. doi: 10.1016/j.expneurol.2004.01.011

Ustad, T., Evensen, K. A., Campbell, S. K., Girolami, G. L., Helbostad, J., Jorgensen, L., et al. (2016). Early parent-administered physical therapy for preterm infants: a randomized controlled trial. *Pediatrics* 138, F190–F192. doi: 10.1542/peds.2016-0271

Vaccarino, F. M., and Ment, L. R. (2004). Injury and repair in developing brain. *Arch. Dis. Child Fetal Neonatal Ed.* 89, F190–F192. doi: 10.1136/adc.2003.043661

Van Haastert, I. C., Groenendaal, F., Uiterwaal, C. S., Termote, J. U., Van Der Heide-Jalving, M., Eijsermans, M. J., et al. (2011). Decreasing incidence and severity of cerebral palsy in prematurely born children. *J. Pediatr.* 159, 86–91.e1. doi: 10.1016/j.jpeds.2010.12.053

Van Wassenaer-Leemhuis, A., Ares, S., Golombek, S., Kok, J., Paneth, N., Kase, J., et al. (2014). Thyroid hormone supplementation in preterm infants born before 28 weeks gestational age and neurodevelopmental outcome at age 36 months. *Thyroid* 24, 1162–1169. doi: 10.1089/thy.2013.0618

Vinukonda, G., Hu, F., Mehdizadeh, R., Dohare, P., Kidwai, A., Juneja, A., et al. (2016). Epidermal growth factor preserves myelin and promotes astrogliosis after intraventricular hemorrhage. *Glia* 64, 1987–2004. doi: 10.1002/glia.23037

Volpe, J. J. (2009). The encephalopathy of prematurity–brain injury and impaired brain development inextricably intertwined. *Semin. Pediatr. Neurol.* 16, 167–178. doi: 10.1016/j.spen.2009.09.005

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., and Eppenberger, H. M. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem. J.* 281(Pt 1), 21–40. doi: 10.1042/bj2810021

Wang, H., Zhang, L., and Jin, Y. (2015). A meta-analysis of the protective effect of recombinant human erythropoietin (rhEPO) for neurodevelopment in preterm infants. *Cell Biochem. Biophys.* 71, 795–802. doi: 10.1007/s12013-014-0265-1

Wang, Q., Duan, F., Wang, M. X., Wang, X. D., Liu, P., and Ma, L. Z. (2016). Effect of stem cell-based therapy for ischemic stroke treatment: a meta-analysis. *Clin. Neurol. Neurosurg.* 146, 1–11. doi: 10.1016/j.clineuro.2016.04.011

Watanabe, K., Hamada, F., Wakatsuki, A., Nagai, R., Shinohara, K., Hayashi, Y., et al. (2012). Prophylactic administration of melatonin to the mother throughout pregnancy can protect against oxidative cerebral damage in neonatal rats. *J. Matern. Fetal Neonatal. Med.* 25, 1254–1259. doi: 10.3109/14767058.2011.636094

Watson, N., Diamandis, T., Gonzales-Portillo, C., Reyes, S., and Borlongan, C. V. (2016). Melatonin as an antioxidant for stroke neuroprotection. *Cell Transplant.* 25, 883–891. doi: 10.3727/096368915X689749

Weindling, A. M., Hallam, P., Gregg, J., Klenka, H., Rosenbloom, L., and Hutton, J. L. (1996). A randomized controlled trial of early physiotherapy for high-risk infants. *Acta Paediatr.* 85, 1107–1111. doi: 10.1111/j.1651-2227.1996.tb14226.x

Welin, A. K., Svedin, P., Lapatto, R., Sultan, B., Hagberg, H., Gressens, P., et al. (2007). Melatonin reduces inflammation and cell death in white matter in the

mid-gestation fetal sheep following umbilical cord occlusion. *Pediatr. Res.* 61, 153–158. doi: 10.1203/01.pdr.0000252546.20451.1a

Whittingham, K., Boyd, R. N., Sanders, M. R., and Colditz, P. (2014). Parenting and prematurity: understanding parent experience and preferences for support. *J. Child Family Stud.* 23, 1050–1061. doi: 10.1007/s10826-013-9762-x

Wilkinson, D., Shepherd, E., and Wallace, E. M. (2016). Melatonin for women in pregnancy for neuroprotection of the fetus. *Cochr Datab Syst Rev.* 3:Cd010527. doi: 10.1002/14651858.CD010527.pub2

Williams, A. M., Dennahy, I. S., Bhatti, U. F., Halaweish, I., Xiong, Y., Chang, P., et al. (2019). Mesenchymal stem cell-derived exosomes provide neuroprotection and improve long-term neurologic outcomes in a swine model of traumatic brain injury and hemorrhagic shock. *J. Neurotrauma.* 36, 54–60. doi: 10.1089/neu.2018.5711

Williams, G. R. (2008). Neurodevelopmental and neurophysiological actions of thyroid hormone. *J. Neuroendocrinol.* 20, 784–794. doi: 10.1111/j.1365-2826.2008.01733.x

Willis, G. R., Kourembanas, S., and Mitsialis, S. A. (2017). "Therapeutic applications of extracellular vesicles: perspectives from newborn medicine," in *Extracellular Vesicles: Methods and Protocols*, editors W. P. Kuo, and S. Jia (New York, NY: Springer New York), 409–432. doi: 10.1007/978-1-4939-7253-1_34

Wittmann, G., Szabon, J., Mohácsik, P., Nouriel, S. S., Gereben, B., Fekete, C., et al. (2015). Parallel regulation of thyroid hormone transporters OATP1c1 and MCT8 during and after endotoxemia at the blood-brain barrier of male rodents. *Endocrinology* 156, 1552–1564. doi: 10.1210/en.2014-1830

Wongprayoon, P., and Govitrapong, P. (2017). Melatonin as a mitochondrial protector in neurodegenerative diseases. *Cell Mol. Life Sci.* 74, 3999–4014. doi: 10.1007/s00018-017-2614-x

Woodward, L. J., Anderson, P. J., Austin, N. C., Howard, K., and Inder, T. E. (2006). Neonatal MRI to predict neurodevelopmental outcomes in preterm infants. *New Engl. J. Med.* 355, 685–694. doi: 10.1056/NEJMoa053792

Wu, Y. W., Bauer, L. A., Ballard, R. A., Ferriero, D. M., Glidden, D. V., Mayock, D. E., et al. (2012). Erythropoietin for neuroprotection in neonatal encephalopathy: safety and pharmacokinetics. *Pediatrics* 130, 683–691. doi: 10.1542/peds.2012-0498

Xiao, Y., Luo, M., Luo, H., and Wang, J. (2014). Creatine for Parkinson's disease. *Cochr. Datab. Syst. Rev.* 6:Cd009646. doi: 10.1002/14651858.CD009646.pub2

Yata, K., Matchett, G. A., Tsubokawa, T., Tang, J., Kanamaru, K., and Zhang, J. H. (2007). Granulocyte-colony stimulating factor inhibits apoptotic neuron loss after neonatal hypoxia-ischemia in rats. *Brain Res.* 1145, 227–238. doi: 10.1016/j.brainres.2007.01.144

Yokobori, S., Hosein, K., Burks, S., Sharma, I., Gajavelli, S., and Bullock, R. (2013). Biomarkers for the clinical differential diagnosis in traumatic brain injury–a systematic review. *CNS Neurosci. Ther.* 19, 556–565. doi: 10.1111/cns.12127

Yong, V. W., Wells, J., Giuliani, F., Casha, S., Power, C., and Metz, L. M. (2004). The promise of minocycline in neurology. *Lancet Neurol.* 3, 744–751. doi: 10.1016/S1474-4422(04)00937-8

Zhang, C., Ling, C. L., Pang, L., Wang, Q., Liu, J. X., Wang, B. S., et al. (2017a). Direct macromolecular drug delivery to cerebral ischemia area using neutrophil-mediated nanoparticles. *Theranostics* 7, 3260–3275. doi: 10.7150/thno.19979

Zhang, J., Wang, Q., Xiang, H., Xin, Y., Chang, M., and Lu, H. (2014). Neuroprotection with erythropoietin in preterm and/or low birth weight infants. *J. Clin. Neurosci.* 21, 1283–1287. doi: 10.1016/j.jocn.2013.10.040

Zhang, Y., Chopp, M., Meng, Y., Katakowski, M., Xin, H., Mahmood, A., et al. (2015). Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. *J. Neurosurg.* 122, 856–867. doi: 10.3171/2014.11.JNS14770

Zhang, Y., Chopp, M., Zhang, Z. G., Katakowski, M., Xin, H., Qu, C., et al. (2017b). Systemic administration of cell-free exosomes generated by human bone marrow derived mesenchymal stem cells cultured under 2D and 3D conditions improves functional recovery in rats after traumatic brain injury. *Neurochem. Int.* 111, 69–81. doi: 10.1016/j.neuint.2016.08.003

Zhu, Y., Wendler, C. C., Shi, O., and Rivkees, S. A. (2014). Diazoxide promotes oligodendrocyte differentiation in neonatal brain in normoxia and chronic sublethal hypoxia. *Brain Res.* 1586, 64–72. doi: 10.1016/j.brainres.2014.08.046

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Finch-Edmondson, Morgan, Hunt and Novak. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 29 January 2019
doi: 10.3389/fphys.2019.00023

Bestrophin-3 Expression in a Subpopulation of Astrocytes in the Neonatal Brain After Hypoxic-Ischemic Injury

Veronika Golubinskaya[1], Regina Vontell[2], Veena Supramaniam[2], Josephine Wyatt-Ashmead[3], Helena Gustafsson[1], Carina Mallard[1] and Holger Nilsson[1]*

[1] Department of Physiology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [2] Division of Imaging Sciences & Biomedical Engineering, Centre for the Developing Brain, King's College London, King's Health Partners, St Thomas' Hospital, London, United Kingdom, [3] Wigglesworth Perinatal-Padiatric Pathology Service, Imperial College Healthcare NHS Trust, London, United Kingdom

OPEN ACCESS

Edited by:
Charles Evans Wood, University of Florida, United States

Reviewed by:
Jayanth Ramadoss, Texas A&M University, United States
Dean A. Myers, The University of Oklahoma Health Sciences Center, United States
Eileen I. Chang, University of Colorado Denver, United States

***Correspondence:**
Veronika Golubinskaya
veronika.golubinskaya@gu.se

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *20 August 2018*
Accepted: *10 January 2019*
Published: *29 January 2019*

Citation:
Golubinskaya V, Vontell R, Supramaniam V, Wyatt-Ashmead J, Gustafsson H, Mallard C and Nilsson H (2019) Bestrophin-3 Expression in a Subpopulation of Astrocytes in the Neonatal Brain After Hypoxic-Ischemic Injury. Front. Physiol. 10:23. doi: 10.3389/fphys.2019.00023

Bestrophin-3, a potential candidate for a calcium-activated chloride channel, recently was suggested to have cell-protective functions. We studied the expression and alternative splicing of bestrophin-3 in neonatal mouse brain and after hypoxic-ischemic (HI) injury and in human neonatal brain samples. HI brain injury was induced in 9-day old mice by unilateral permanent common carotid artery occlusion in combination with exposure to 10% oxygen for 50 min. Endoplasmic reticulum stress was induced by thapsigargin treatment in primary culture of mouse brain astrocytes. We also investigated expression of bestrophin-3 protein in a sample of human neonatal brain tissue. Bestrophin-3 protein expression was detected with immunohistochemical methods and western blot; mRNA expression and splicing were analyzed by RT-PCR. HI induced a brain tissue infarct and a pronounced increase in the endoplasmic reticulum-associated marker CHOP. Three days after HI a population of astrocytes co-expressed bestrophin-3 and nestin in a penumbra-like area of the injured hemisphere. However, total levels of Bestrophin-3 protein in mouse cortex were reduced after injury. Mouse astrocytes in primary culture also expressed bestrophin-3 protein, the amount of which was reduced by endoplasmic reticulum stress. Bestrophin-3 protein was detected in astrocytes in the hippocampal region of the human neonatal brain which had patchy white matter gliosis and neuronal loss in the Sommer's sector of the Ammon's horn (CA1). Analysis of bestrophin-3 mRNA in mouse brain with and without injury showed the presence of two truncated spliced variants, but no full-length mRNA. Total amount of bestrophin-3 mRNA increased after HI, but showed only minor injury-related change. However, the splice variants of bestrophin-3 mRNA were differentially regulated after HI depending on the presence of tissue injury. Our results show that bestrophin-3 is expressed in neonatal mouse brain after injury and in the human neonatal brain with pathology. In mouse brain bestrophin-3 protein is upregulated in a specific astrocyte population after injury and is co-expressed with nestin. Splice variants of bestrophin-3 mRNA respond differently to HI, which might indicate their different roles in tissue injury.

Keywords: bestrophin-3 (Best3), alternative splicing, astroglia, endoplasmic reticulum (ER) stress, hypoxia-ischemia

INTRODUCTION

Bestrophins are anion channels that generally serve as chloride channels involved in regulation of cell excitability or cell volume regulation (Duran et al., 2010). Mainly three isoforms have been identified, and on the mRNA level all have been shown to be expressed in the brain (Krämer et al., 2004). Best1 is the most studied, and can function not only as a chloride channel, but also as a release mechanism for glutamate (Oh et al., 2012; Han et al., 2013) and GABA (Park et al., 2009; Lee et al., 2010) in brain astrocytes. It seems to be of special importance in reactive astrocytes, where redistribution of Best1 parallels enhanced GABA production (Oh and Lee, 2017). Data on Best2 and Best3 in brain are scarce.

Best3 mRNA is subject to alternative splicing in mouse and human, resulting in a number of splice variants of unclear function. Some splice variants of Best3 have the channel pore region spliced out, likely causing them to lose ability to act as ion channels (Srivastava et al., 2008). A specific splice variant has been suggested to control intracellular calcium release in myoblasts, possibly preventing calcium overload (Wu et al., 2016). Best3 has recently also been implicated in various cell-protective mechanisms outside the brain. This mainly stems from three studies: (a) Best3 has anti-apoptotic function in basilar artery smooth muscle cells stressed by hydrogen peroxide (Jiang et al., 2013), (b) Best3 ameliorates TNFα-induced inflammation in endothelial cells (Song et al., 2014), and (c) Best3 decides cell fate in an endoplasmic reticulum (ER) stress model in renal proximal tubule cells (Lee et al., 2012).

One of the consequences of cerebral ischemia is ER stress and the unfolded protein response (UPR) in brain cells. These processes have a complex role in cell injury, promoting either survival or death of the cells in the brain, depending on injury conditions (DeGracia and Montie, 2004). ER stress has been demonstrated also in astrocytes, resulting in increased production of cytokines and less neurotropic factors (Singh et al., 2018), potentially enhancing tissue injury. At the same time ER stress can have a protective preconditioning effect in cell injury (Lehotský et al., 2009; Leonard et al., 2014). Both ER stress (Chavez-Valdez et al., 2016) and apoptosis (Zhu et al., 2005) are important mechanisms following injury in the developing brain.

The role of Best3 in brain injury is unknown. Since Best3 has been implicated in cell protection after ER stress, we hypothesized that it could serve as a marker for cells involved in the response to brain injury. We used a well-established model of neonatal hypoxia-ischemia in mice. Unilateral brain injury was induced by a combination of hypoxia and ischemia (HI) (Rice et al., 1981; Sheldon et al., 1998; Hedtjärn et al., 2002). The resultant brain injury is characterized by increased gliosis and apoptosis-dependent brain injury (Blomgren et al., 2001).

Our work shows for the first time the expression of Best3 in neonatal brain in mice and humans, and describes changes in Best3 expression and alternative splicing following brain injury.

MATERIALS AND METHODS

Animals

C57Bl6J mice were obtained from the Jackson Laboratory, United States, and bred in-house at the laboratory of Experimental Biomedicine (EBM) at Sahlgrenska Academy, University of Gothenburg, Sweden.

In total 76 mouse pups of either sex were used in the experiments. Animals were randomly allocated into groups, and different animals were used for mRNA and protein expression analysis. For the mRNA expression analysis, a total of 41 pups (21 male, 20 females) were used, among those 26 pups in the HI group (12 males, 14 females) and 15 pups in the sham-operated group (9 males, 6 females). These were divided into separate groups for the various time points. The 6 h time point: 5 pups in the HI group (3 males, 2 females) and 3 pups in the sham group (2 males, 1 female); 12 h time point: HI 6 pups (3 males, 3 females), sham 4 pups (2 males, 2 females); 24 h time point: HI 6 pups (3 males, 3 females), sham 3 pups (2 males, 1 female); 72 h time point: HI 5 pups (2 males, 3 females), sham 3 pups (1 male, 2 females); 7 days time point: HI 4 pups (1 male, 3 females), sham 2 pups (2 males). For protein expression analysis by western blot a total of 18 pups were used, HI 14 pups (8 males, 6 females), sham 4 pups (2 males and 2 females). In experiments with immunostaining 9 pups of either sex were used. In experiments with astrocyte primary culture 8 pups of either sex were used.

Animals were euthanised either by an overdose of barbiturates or by direct decapitation (in experiments with primary cell culture of brain cells). All animal experiments and methods for animal euthanasia were approved by the Animal Ethics Committee in Gothenburg.

Model of Neonatal Hypoxia-Ischemia (HI) Injury

Nine-day old mice of either sex were exposed to HI treatment according to the Rice-Vanucci model (Rice et al., 1981; Sheldon et al., 1998). Under isoflurane anesthesia (1.5% in a 1:1 mixture of N_2 and O_2) the left common carotid artery was permanently ligated with proline 6.0 suture, then the incision was closed and treated with local anesthetic. The pups were returned to their mothers for 1 h recovery. After that pups were placed for 50 min in a 36° C -heated incubator containing a humidified gas mixture of O_2 (10%) in N_2. Subsequently pups stayed with their mothers until removed for tissue sampling. No mortality was observed after HI under the conditions used. Control sham-operated animals underwent the same surgical procedure except for the ligation of the carotid artery, and later they were kept under warm conditions in normal air at the same time as HI treatment was performed. Animals were euthanised at the time points of 6, 12, 24 and 72 h after HI.

In this model, the left hemisphere, ipsilateral to the artery ligation, receives a reduced blood flow, which in combination with hypoxia results in unilateral injury to this hemisphere. The contralateral (right) hemisphere does not experience a fall in cerebral blood flow (Ek et al., 2015), and no gross morphological injury is observed in this hemisphere

(Vannucci and Hagberg, 2004). Left and right hemispheres from sham-operated mice were used as non-treated controls.

Primary Culture of Mouse Brain Astrocytes

Experiments were based on previously described procedures for primary culture of murine brain cells (Lund et al., 2006; de Vellis et al., 2010). 1–2 days old mouse pups were decapitated, the brain was quickly removed and washed in cold Hanks' balanced salt solution (HBSS, Sigma-Aldrich) containing 1% antibiotics (penicillin-streptomycin, Sigma-Aldrich). Then the cerebellum was removed, and the rest of the brain tissue was mechanically homogenized by pipetting in warm Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, D5796) containing 1% antibiotics and 20% fetal bovine serum (FBS, Gibco, Life Technologies). Further culture medium always contained 1% of antibiotics. A homogenate of 1–2 brains was filtered through a 70 μm-diameter filter and diluted up to the final volume of 24 ml in DMEM with 20% FBS, and finally transferred to 75 cm^2 flask (Sarstedt AB, Helsingborg, Sweden). Cells were left growing in 5% CO_2/95% air at 37°C for 7 days, then medium was changed to DMEM with 10% FBS, and cells were allowed to grow for an additional week. After that flasks were shaken for 24 h at 250 rpm in 5% CO_2/95% air at 37°C. The medium was removed, cells were washed 2–3 times with warm HBSS and incubated in 0.05% Trypsin+EDTA (in HBSS) for 20-30 min in 5% CO_2/95% air at 37°C with gentle shaking, until the cells fully detached from the flask bottom. Then trypsin activity was blocked by adding an equivalent volume of DMEM with 10% FBS. After that cell suspension was transferred to 50 ml tubes and centrifuged at 250 g for 5 min. The supernatant was discarded, the cell pellet was resuspended in DMEM with 10% FBS, and the number of cells per 1 ml was measured (Scepter TM Automated Cell Counter, Merck Millipore). Cells were plated either 50,000 cells per well in 12-well plate (Corning Inc., United States) for further studying the mRNA or protein expression, or 10,000 cells per well in 8-well glass slide chamber (BD Falcon TM culture slides, BD Biosciences, United States) for further immunostaining. Cells were left to attach and grow for 3 days, and then the treatments were performed. The resulting cell culture contained about 95% of GFAP-positive astrocytes, among the remaining cells microglia could be detected (de Vellis et al., 2010).

The ER stress was induced in the cells by treatment with thapsigargin (TG, 200 nM, Sigma-Aldrich) in serum-free DMEM; control cells received the same medium. In this concentration TG induces ER stress in cultured astrocytes and increases ER-stress markers (Johnson et al., 2014). Forty-eight hours after the treatment cells were harvested for mRNA and western blot analysis.

Preparation of the Samples for Immunostaining in Mouse Brain Tissue

For brain tissue samples the animals were euthanised by an overdose of barbiturates i.p. and immediately transcardially perfused first with saline, then with PFA (Sigma-Aldrich) 4% in PBS. Brains were then removed and kept in 4% PFA for 1 day at 4°C. Then tissues were dehydrated in graded ethanol and xylene substitute Tissue-Clear® (Tissue-Tek® Sacura) and embedded in paraffin. Coronal sections (5 μm) of the brain were prepared and mounted on glass slides.

For immunochemistry in primary astrocyte culture the cell medium was replaced with 4% PFA, and the cells were fixed for 15 min at room temperature and stored in PBS at 4°C for 1–2 weeks until the staining was performed.

Immunostaining

Immunostaining in mouse brain sections was performed as described in Golubinskaya et al. (2015). In short, PFA-fixed tissue and cell preparations were deparaffinised and received standard antigen retrieval treatment: the preparations on the glass slides were placed in 50 mM citrate buffer (pH 6.0) and heated to 98–99°C for 30 min. Then the preparations were permeabilized and incubated with primary antibody for 24–36 h at 4°C. One of the specimens in each experiment was processed the same way, but without primary antibodies in the incubation buffer, to control for possible non-specific binding of the secondary antibody.

The primary anti-mouse antibodies used were obtained from commercial sources: Best3 (Rabbit polyclonal, IgG, Bst-301AP, lot# A532.Fb.AP, FabGennix International Inc., United States), Nestin (Goat polyclonal, IgG, G-20: sc-21248, Santa Cruz Biotechnology Inc., United States), GFAP (Mouse monoclonal IgG, G3893, Sigma-Aldrich), Iba-1 (Goat polyclonal IgG, orb19198, Biorbyt), NeuN (Mouse monoclonal IgG, MAB377, Millipore). The anti-Best3 antibody does not distinguish between Best3 splice isoforms studied in this paper, as the antigen is localized at the very end of the C-terminal region. An antibody directed to the same region in human Best3 (BEST-312AP, FabGennix), which differs in amino acid sequence, was used as a non-specific IgG control. We have previously shown the specificity of staining with BEST-301AP as it was inhibited by blocking peptide (Golubinskaya et al., 2015).

Secondary antibodies (488 or 594 DyLight-labeled secondary antibody made in donkey, Jackson Immunoresearch, United Kingdom) were incubated with specimens for 60 min at room temperature. Nuclei were detected by DAPI (Invitrogen) in dilution 1:75000. ProLongTM Gold antifade reagent (Invitrogen, Molecular Probes) was used for mounting the slides.

Conventional images were obtained by Olympus BX60 conventional fluorescence microscope (camera Olympus DP50) and Zeiss Imager.Z2 conventional fluorescence microscope (camera AxioCam MRM, Zeiss). Confocal images were obtained with a Zeiss LSM 800 microscope.

mRNA Expression Analysis

The animals were transcardially perfused with saline. Brains were quickly removed and dissected: cerebellum and the frontal part with olfactory bulbs were removed, then on the coronal projection of the brain the sector containing the cortex and hippocampus injury was dissected out. Tissue was immediately frozen in dry ice and stored for further investigation. Primary astrocyte cells were briefly washed three times with cold PBS and then lysed directly in the wells by RLT buffer (Qiagen GmbH,

Germany) containing 1% of beta-mercaptoethanol (Sigma-Aldrich) and then frozen for storage.

For total RNA extraction brain samples were briefly homogenized mechanically on ice in RNAase-free PBS, then a third of the sample was transferred to QIAzol Lysis Reagent (Qiagen GmbH, Germany) and homogenized additionally. Cultured astrocytes were homogenized in beta-mercaptoethanol-containing RLT buffer by intense vortexing. The samples were further processed according to RNeasy Lipid tissue Mini Kit (brain samples) or RNeasy Mini Kit protocol (cultured cells), both with DNAse digestion (QIAGEN GmbH, Germany). Concentration of the extracted total RNA was detected spectrophotometrically as the absorbance at 260 and 280 nm (NanoDrop 1000 spectrophotometer, Wilmington, Del., United States). For cDNA synthesis QuantiTect Rev.Transcription Kit (QIAGEN GmbH, Germany) was used, total amount of RNA used in RT reaction for each sample was 1 μg.

Primers and analysis of PCR products (**Figure 2A**) were made as described previously (Golubinskaya et al., 2015) in accordance with the predicted mRNA sequence for mouse bestrophin-3 (NM_001007583.1). Also PCR and qPCR analysis of alternative splicing of Best3 mRNA was performed as described by Golubinskaya et al. (2015). For qPCR analysis of Best3, expression primer pairs C (for detecting total mRNA of Best3), G (for variant with exon 6 is present, "+6" variant) and I (for variant missing exon 6, "−6" variant) were used. For quantification of nestin (Mm_Nes_2_SG), CHOP (Mm_Ddit3_2_SG) and a housekeeping gene GAPDH (Mm_Gapdh_3_SG) the commercial primers (QuantiTect Primer Assays from QIAGEN GmbH, Germany) were used.

For each sample the results were calculated as the difference in crossing-point (*C*p) numbers between the housekeeping gene and the gene of interest (delta *C*p). Then from each individual delta *C*p the delta *C*p value of the corresponding control samples was subtracted as shown below (delta delta *C*p). The resulting values are equal to $\log_2$ of the concentration ratio of the gene of interest between treated and control groups and reflect the change in treated samples in relation to the corresponding control samples.

(1) Effect of HI:
$\log_2$(fold change) = delta *C*p (left hemisphere of HI-treated or sham-operated mouse) − average delta *C*p in the left hemisphere of time point-matched sham-operated mice;
(2) Effect of HI in the uninjured hemisphere:
$\log_2$(fold change) = delta *C*p (right hemisphere of HI-treated mouse or right hemisphere of sham-operated mouse) − average delta *C*p in the right hemisphere of time point-matched sham-operated mice;
(3) Effect of TG in the cell culture:
$\log_2$(fold change) = delta *C*p (sample of treated or control cells) − average delta *C*p in all control samples.

Protein Expression Analysis in Mouse Brain After HI by Western Blot

Brain tissue was obtained and dissected in the same way as in experiments with mRNA expression analysis. Then the tissue samples were either frozen for further analysis of total protein or were directly homogenized on ice for further subcellular fractionation of proteins. Samples from right (control) and left (injured) hemispheres were processed separately for each animal.

For subcellular fractionation of the proteins the simplified variant of protocol described by Cox and Emili (2006) was used. Briefly, tissue was homogenized in 250-STMDPS buffer (250 mM sucrose, 50 mM TrisBase, 5 mM $MgCl_2$, DTT 1 mM, spermine 50 μg/μl, spermidine 50 μg/μl, PMSF 0,1 mM, pH 7.4) in glass Dounce homogenizer tube. The aliquot of homogenate was saved frozen for later total protein analysis. The rest of homogenate was centrifuged at 800 × *g*, 15 min at 4°C, twice after which the supernatant representing the cytoplasmic fraction was saved frozen.

For extraction of total protein from brains after HI, frozen brain tissue and the total protein aliquot from subcellular fractionation were homogenized in the lysis buffer containing Tris-HCl 50 mM, NaCl 150 mM, Triton X-100 1%, Igepal 0,25%, phosphatase inhibitor and protease inhibitor cocktails (Roche, Germany), pH 7.5.

For extraction of total protein from primary astrocytes the cells were washed 2–3 times in PBS, then collected in PBS containing Triton X-100 0.5%, Igepal 0,12%, PMSF 0.1% and protease inhibitor cocktail 0.5% and then stored frozen.

Then all the samples (total protein from HI brains and from primary astrocytes, cytoplasmic and total protein fractions) were additionally mechanically homogenized by vortexing and by passing through 27–29G syringe needle. After that protein concentration in the samples was measured by BCA method. Then samples were prepared in Laemmli sample buffer 4× (Bio-Rad Laboratories, United States) with 10% DTT, heated for 5 min at 95°C and run through the 4–20% Criterion TGS Stain-free gel in amounts of 10 μg/well for total protein and cytoplasmic fractions and 1 μg/well for total protein samples from primary astrocytes. Protein was transferred to 0.2 μm PVDF membrane (Bio-Rad) by Trans-Blot Turbo Transfer System (Bio-Rad). Then membrane was washed in TBS-T buffer, blocked by 5% milk solution and stained with primary antibody against Best3 (5000 dilution in PBS with 3% BSA and 0.1% sodium azide). Bst-301AP (FabGennix International Inc., United States) was used for detection of Best3 in primary astrocyte samples, and ARP50108_P050 (Aviva Systems Biology, United States) was used for analysis of Best3 in HI brain tissue samples. We have previously shown specificity of Western blot detection with Bst-301AP by inhibition with blocking peptide (Matchkov et al., 2008); **Supplementary Image S2** here shows the same for ARP50108_P050. For visualization of Best3 peroxidase-labeled secondary antibody (Vector Laboratories, United States) and SuperSignalR West Dura Extended Duration Substrate (Thermo Scientific, United States) were used. The images were captured by ChemiDoc™ Imaging System, Bio-Rad. Precision Plus Unstained and Precision Plus All blue Protein Ladders from Bio-Rad were used to estimate the molecular weight of visualized proteins. Bands were identified and their area-intensity product normalized to total protein using ImageLab software (Bio-Rad).

Statistical Analysis

In the experiments *n* equals number of animals (brain tissue samples) or wells (cell culture studies). Data of qPCR experiments (delta *C*p values) was analyzed by three-way mixed ANOVA (analyzing brain hemispheres (within subjects), treatment and time (between subjects) using GLM for repeated measurements using IBM SPS Statistics 25; simple effects were analyzed from estimated marginal means with Bonferroni adjustment. Data from western blot were analyzed by two-way mixed ANOVA. Analyses of two groups were by means of Student's *t*-test. Data is presented on the graphs as individual values with group means ± SEM. A *P*-value of less than 0.05 was regarded as significant. In **Figure 3** values for treated animals are plotted as differences from the corresponding means of sham animals; the detailed statistical results for **Figure 3** are given in the **Supplementary Table S1**.

Immunostaining in Human Neonatal Post-mortem Brain Tissue With Pathology

Informed parental consent was acquired in accordance with the National Health Services United Kingdom guidelines and research study ethics was obtained from the National Research Ethics Services (West London), United Kingdom (ethics number 07/H0707/139; Postmortem Magnetic Imaging Study of the Developing Brain). The postmortem case assessed in this study was a term stillbirth/intrauterine death (at 41.7 weeks

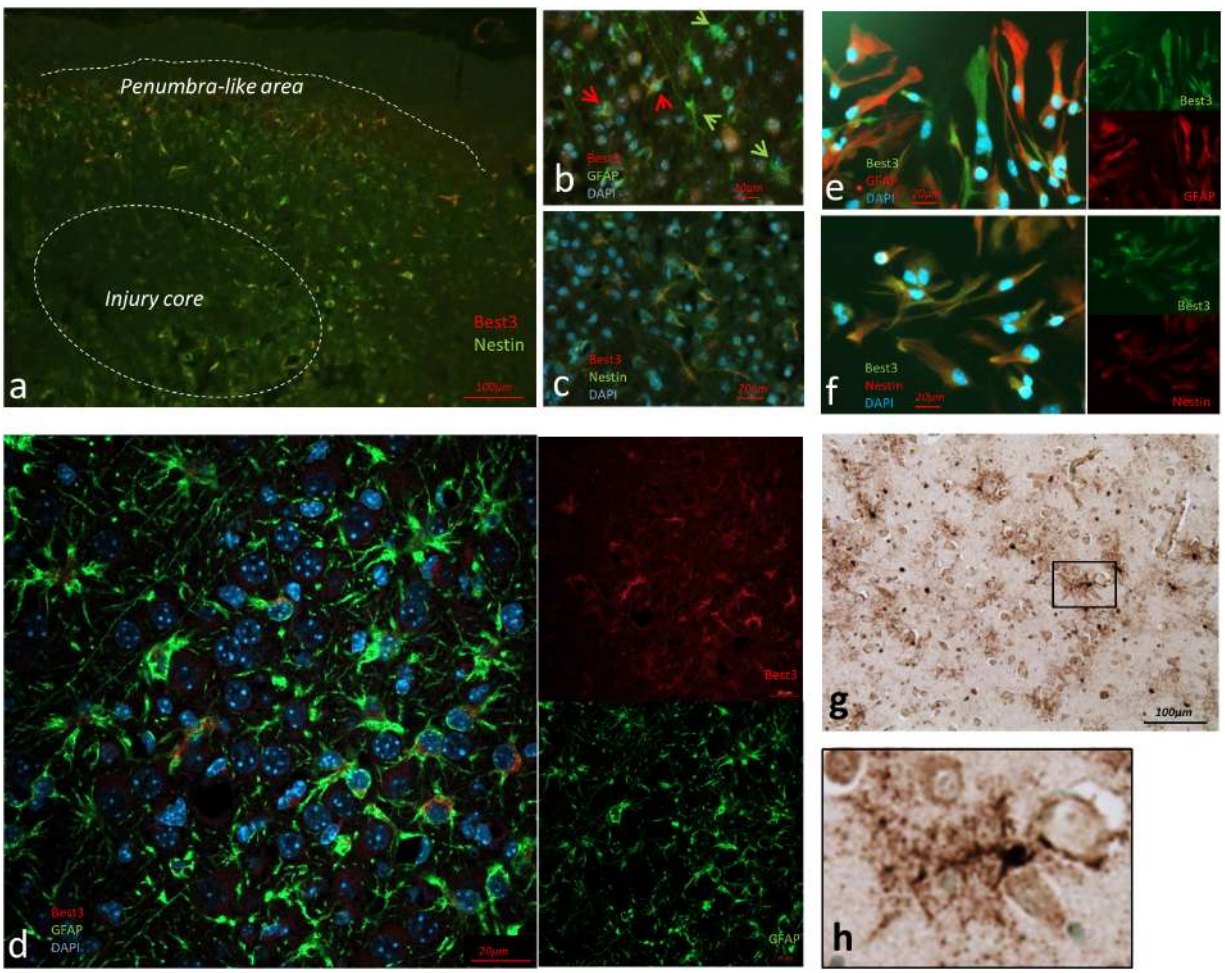


FIGURE 1 | Immunohistochemical evidence for Best3 expression in newborn mouse and human brain after injury. Best3 is expressed in mouse brain cortex after HI **(a–d)**, in mouse primary astrocyte culture **(e,f)** and in brain of newborn child with birth pathology **(g,h)**. Best3 was expressed in the injured mouse brain 3 days after HI **(a)** in a subpopulation of GFAP-positive cells **(b,d)**. Some cells expressed both Best3 and GFAP (**b**, red arrows), while other GFAP-positive cells did not express Best3 (**b**, green arrows). Best3-positive cells also expressed the intermediate filament nestin and were localized mostly in cortex and hippocampus in a penumbra-like area of the injury **(a,c)**. Confocal image of the penumbra-like area **(d)** shows Best3 intracellularly in the perinuclear area. Similar to the brain tissue astrocytes, some, but not all GFAP-positive astrocytes in a primary culture were expressing Best3 **(e)**. These cells also expressed the intermediate filament nestin **(f)**. Best3 is stained red, nestin and GFAP green **(a–d)**, Best3 is stained green, nestin and GFAP red **(e,f)**, nuclear staining with DAPI blue, magnification 10× **(a)**, 40× **(b,c)**, 60× **(d)**. Best3 was also expressed in astrocyte-like cells in cortical areas of the brain of a term infant with white matter gliosis (**g**, enlarged Best3-positive structure in **h**). Peroxidase-based immunostaining captured at magnification 20×.

gestational age; male) associated with amniotic fluid infection. The pregnancy was otherwise uncomplicated and there were no major congenital anomalies, chromosomal defects or overt culture-positive sepsis evident in the infant. The clinical pathologist noted that there was slight congestion and oedema seen throughout the brain and there was evidence of patchy white matter gliosis. However, except for the hippocampal region which had overt neuronal loss in the Sommer's sector of the Ammon's Horn, the rest of the brain was unremarkable (i.e., no hypoxic-ischemic neurons or apoptotic neurons were seen). Samples containing the Ammon's Horn, thalamus and lentiform nucleus were used in this study. Postmortem tissue preparation has previously been described (Vontell et al., 2015) and the standard immunohistochemistry procedure for neonatal brain tissue has been described elsewhere (Supramaniam et al., 2013; Vontell et al., 2013). The tissue slides were incubated with the human-specific antiBest3 primary antibody (1:200 rabbit polyclonal, ab101828 from Abcam, Cambridge, United Kingdom) overnight, followed by incubation with secondary biotinylated antibody (1:1000 goat-anti-rabbit, BA-1000, Vector Laboratories, Burlingame, CA, United States) for 1 h, and then processed as described in Vontell et al. (2015).

RESULTS

Expression of Best3 Is Seen in the Ventricles of Non-injured Mouse Brain

In non-injured mouse brains (in sham-operated animals and in the right, contralateral hemisphere of the brain of HI mice) immunohistochemical analysis revealed presence of Best3 protein in ependymal cells and occasionally in radial-glia on the surface of the brain ventricles (**Supplementary Image S1**), but no obvious staining in other brain cells was detected. A similar

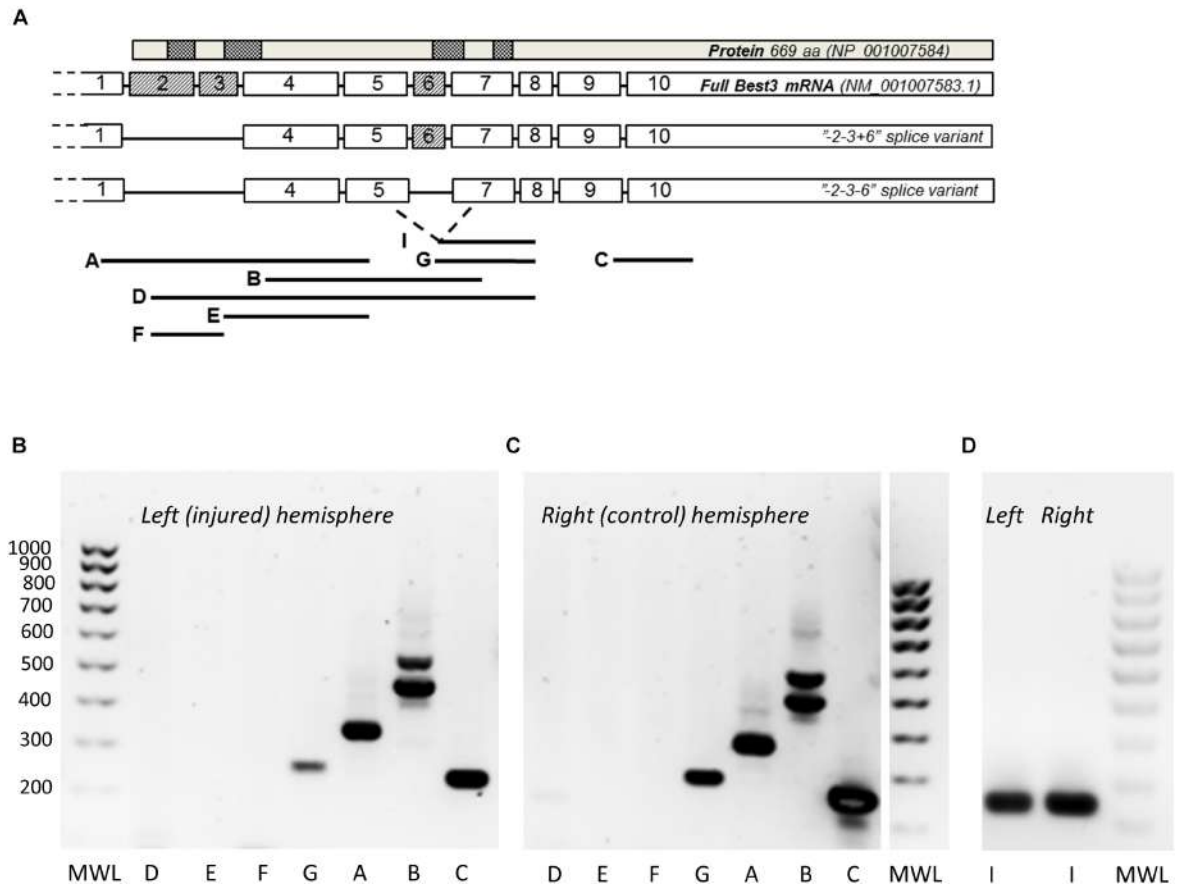


FIGURE 2 | PCR analysis of Best3 mRNA alternative splicing in mouse brain with and without HI injury. Alternative splicing of Best3 mRNA was studied by a set of primers **(A)** spanning the areas of expected splicing of exons 2, 3, and 6 in different combinations as described in detail previously (Golubinskaya et al., 2015). The results of these PCR experiments are presented in **(B–D)**. Total Best3 mRNA can be detected by primer pair C localized in exons 9 and 10 in both healthy **(C)** and injured brain **(B)**. Splice variants of Best3 with exons 2 and 3 present were not detected, neither in healthy brain nor in injury (pairs of primers: A, localized in exons 1 and 5; D in exons 2 and 7; E in exons 3 and 5; F in exons 2 and 3). Exon 6 can be either present or spliced out in Best3 mRNA. Primer pair B spanning exons 4–7 gives two bands, the heavier band corresponds to "+6" and the lighter one to "–6" splice variants. The pair of primers G localized in exons 6 and 8 shows a band corresponding to "+6" splice variant. Primer pair I detects the "–6" variant **(D)**. Primer pairs G and I can potentially detect more than one transcript corresponding to "+6" and "–6" variants depending on possible splicing of exon 10. Positive controls for these primers have been published previously by our group (Golubinskaya et al., 2015). MWL, molecular weight ladder.

pattern was seen in HI-injured mouse brain (left, ipsilateral hemisphere). We also have a preliminary observation of similar staining for Best3 in brain ependymal cells in the brains of adult naive mice.

Immunofluorescent Analysis Shows Best3 Protein Expression in Mouse Brain Astrocytes After Injury

After HI Best3-positive cells were detected in the injured hemisphere, primarily in the cortex and hippocampus in the penumbra-like area of injury (**Figure 1a**) and this expression pattern was similar in male and female pups. Best3-positive cells were first seen at 24 h after injury, but the staining was most pronounced 72 h after the HI event. Seven days after injury almost no injury-associated Best3-positive cells were evident. Best3 immunoreactivity was not detected in the contralateral non-injured hemisphere in cortical and hippocampal regions.

Best3-positive cells also expressed the intermediate filament nestin (**Figures 1a,c**). Best3 expression in astrocytes was supported by the observation that Best3 staining did not overlap with neuronal markers NeuN or calretinin, nor with Iba-1, a marker for microglia. With regard to the astrocyte marker GFAP, it was clear that Best3 was expressed in some but not in all GFAP-positive cells (**Figures 1b,d**). This suggests that after injury Best3 is expressed in a subpopulation of astrocytes that express nestin. The possibility that some of these cells could be of other type (neural progenitors etc.) cannot be neglected, but the combination of astrocyte markers and cell size and morphology suggests that the majority of Best3-positive cells were astrocytes.

Immunofluorescent Analysis Shows Best3 Protein Expression in Mouse Astrocytes in Primary Culture

Best3 expression in brain astrocytes was further confirmed by Best3-positive staining in a primary culture of mouse astrocytes (**Figures 1e,f**). As in the brain tissue, Best3 in cultured astrocytes was co-expressed with nestin (**Figure 1h**), while overlap with GFAP was not complete (**Figure 1e**).

Expression of Best3 Is Seen in the Human Brain

Immunohistochemical analysis showed expression of Best3 protein in astrocytes (**Figures 1g,h**) in the thalamic and hippocampal region of the post-mortem neonatal human brain. Best3 was highly expressed in hypertrophic astrocytes, where it was observed in the cell bodies and extended into the filamentous processes.

Expression and Alternative Splicing of Best3 mRNA in the Mouse Brain Tissue Is Changed After HI in the Injured and Non-injured Hemispheres

Alternative Splicing of Best3 mRNA

The PCR analysis of Best3 mRNA was made by using specifically made primer pairs published previously (Golubinskaya et al., 2015) spanning the expected areas of splicing of exons 2, 3, and 6 (**Figure 2A**) based on earlier published data on alternative splicing in Best3 mRNA in mouse tissues (Krämer et al., 2004; Srivastava et al., 2008). We did not investigate the possible splicing of exon 10 that recently has been described by Wu et al. (2016), so in our PCR experiments the "−2−3+6" and "−2−3−6" splice variants of mRNA could represent corresponding groups of splice variants where also exon 10 is alternatively spliced. Analysis of the ipsilateral, injured hemisphere and the contralateral non-injured hemisphere (**Figures 2B–D**) showed the presence of two splice variants of Best3 in both hemispheres: one longer variant where exon 6 was present ("+6" variant) and another shorter one, "−6" variant, where exon 6 was spliced out. This conclusion was made from the observation that primer pair B, covering exons 4–7, gave two bands of corresponding weights. The presence of the "+6" splice isoform was confirmed by the band corresponding to the primer pair G with forward primer placed in exon 6, and the presence of the "−6" isoform by the band corresponding to primer pair I targeting the junction of exons 5 and 7. The "+6" variant was dominating in brain tissue.

At the same time no full-length mRNA for Best3 could be detected in either hemisphere, as exons 2 and 3 were absent in both "−6" and "+6" splice variants. Primer pair A, spanning exons 1 and 5, gave only one band with a weight corresponding to the splice variant with both exons 2 and 3 absent. Neither primer pairs D and E, where one of the primers in each pair was complementary to the sequence in exon 2 or 3, nor primer pair F spanning exons 2 and 3, revealed any band by PCR analysis.

Time Course of Changes in Best3 mRNA Expression in the Non-injured Brain Hemisphere After HI

By comparing the right contralateral hemispheres of HI-treated mice to the right hemispheres of sham-operated mice, one can estimate the effect of HI on the uninjured hemisphere (**Figure 3**, blue marking compared to the zero level). In our experiments HI induced early changes in Best3 mRNA expression in the contralateral (right) hemisphere. Total Best3 (**Figure 3A**) showed a biphasic response with an early increase at 6 and 12 h and down-regulation at 72 h. The two splice variants followed similar initial time courses, increasing at 6 and 12 h after HI, but after 24 h the values were back to control level and no further changes occurred during the later time points (**Figures 3B,C**).

Nestin expression was transiently downregulated at 6, 12, and 24 h, returning to its control values at 72 h after HI (**Figure 3D**). There were no signs of pronounced ER stress in the non-injured brain hemisphere after HI, as expression of the ER-stress marker CHOP did not increase, but rather slightly decreased at the early time points after HI (**Figure 3E**).

Time Course of Changes in Best3 mRNA Expression in Brain Injured After HI

The total effect of HI can be studied by comparing the left ipsilateral (injured) hemisphere of HI-exposed mice with the left hemisphere of sham-operated animals (**Figure 3**, red marking compared to the zero level). Total mRNA for Best3 increased transiently at 6 h after HI, and then remained at control level

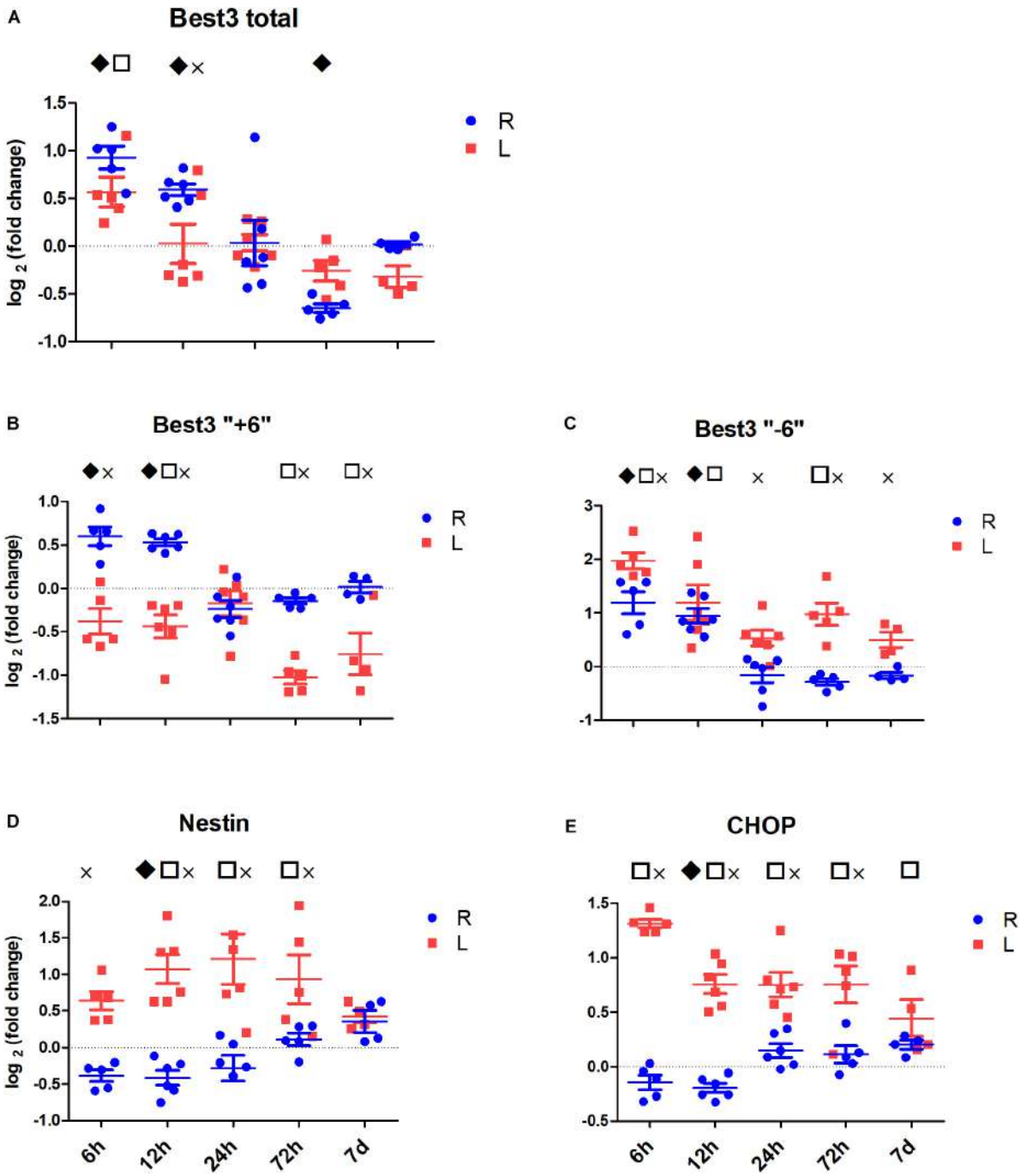


FIGURE 3 | The expression of Best3 mRNA in mouse brain tissue at different time points after HI. Figures show relative expression of the studied mRNAs in uninjured (R, blue) and injured (L, red) hemispheres in relation to the average expression in corresponding hemispheres of sham animals at the respective time points (sham group mean set to zero in the figure). Statistical analysis was performed by mixed-model three-way ANOVA on *Cp* values; main effects were based on subsequently estimated marginal means. Symbols above graphs indicate significant difference between uninjured and sham (R vs. sham group at zero, ♦), between injured and sham (L vs. sham group at zero, □), and between uninjured and injured (R vs. L, ×), all symbols indicate $P < 0.05$. The total mRNA for Best3 increased in both hemispheres at early time points after HI, but returned to control values already 24 h after HI, and even showed a transient decrease at 72 h **(A)**. The long "+6" splice variant increased only in the uninjured hemisphere, while in the injured hemisphere it decreased and stayed decreased through all time point except 24 h **(B)**. The short "–6" splice variant transiently increased at 6 and 12 h, and in the injured hemisphere also again at 72 h **(C)**. Expression of mRNA for nestin **(D)** decreased at early time points in non-injured hemisphere, but returned to control values at 72 h after HI, while it increased in the injured hemisphere during this period to return to control values at 7 days after injury. The ER-stress marker CHOP slightly decreased during the early period after HI in the non-injured hemisphere, but was elevated throughout the studied period in the injured hemisphere **(E)**. A total of 41 pups (26 HI, 15 sham) were used; for details see section "Materials and Methods." Detailed statistical results are given in the (**Supplementary Table S1**).

(**Figure 3A**). The long and the short splice variants of Best3 showed opposite changes: the "+6" variant was downregulated (**Figure 3B**), and "−6" was markedly upregulated (**Figure 3C**). At 24 h after injury the expression of both splice variants was close to the control values (**Figures 3B,C**).

Both nestin (**Figure 3D**) and ER-stress marker CHOP (**Figure 3E**) were upregulated already at 6 h and stayed upregulated through most of the investigated period. Nestin developed its maximal expression between 12 and 72 h, while CHOP showed maximum expression at 6 h after HI, after which its expression decreased over time toward baseline.

Time Course of Injury-Related Changes in Best3 mRNA Expression in the Brain After HI

By comparing the left ipsilateral (injured; **Figure 3**, red marking) with the right contralateral (non-injured; **Figure 3**, blue marking) hemisphere in the same animal the changes produced by the development of tissue injury can be estimated. **Figure 3** shows that injury reduced the expression of total Best3 mRNA at 12 h only (**Figure 3A**). Best3 splice variants changed in opposite directions: the long "+6" splice variant was downregulated in a biphasic manner with a transient return to control values at 24 h after HI (**Figure 3B**), while the expression of the short "−6" splice variant, on the other hand, slightly increased at 6 h and from 24 h onwards compared to the non-injured hemisphere (**Figure 3C**).

Nestin gradually increased in response to injury, reaching maximal expression between 12 and 24 h, and returning to its control values by 7 days after HI (**Figure 3D**). Injury was associated with ER stress as indicated by CHOP expression, which was maximally increased at 6 h, and then gradually decreased, returning to its control values at 7 days after HI (**Figure 3E**). Details of the statistical analysis are given in the **Supplementary Table S1**.

ER Stress Alters Expression of Best3 mRNA in Mouse Cultured Astrocytes

The results of the experiments are presented in **Figure 4**. To confirm that Best3 is altered in astrocytes after injury we investigated mRNA expression for Best3 in cultured astrocytes after inducing ER stress by TG. Before inducing ER stress the "−6" variant was dominating. ER stress caused an increase in expression of total Best3 and both its splice variants (**Figures 4A–C**). The increase in the long "+6" splice variant was the most pronounced ($P < 0.01$; **Figure 4B**). Nestin also increased after TG ($P < 0.01$; **Figure 4D**), and CHOP, as an ER-stress marker, was dramatically upregulated ($P < 0.001$; **Figure 4E**).

Best3 Protein Expression Analysis by Western Blot

Protein analysis by western blot was performed in brain tissue using ARP50108_P050 antibody and in astrocyte culture by Bst-301AP. It revealed Best3-related protein bands in the range of 55–80 kDa (**Figure 5**). In homogenates of parts of cortex from each hemisphere, typically Best3 staining revealed a pair of bands with molecular weight differing by approximately 2–3 kDa, which might correspond to the predicted difference between "+6" and "−6" splice variants of Best3. As there is no antibody specifically recognizing splice variants of Best3, we calculated the density of both bands together in our analysis.

Lighter pairs of bands at about 65 kDa could often be seen. The appearance of these additional bands likely might be related to the homogenization protocol, by which possibly posttranslational modifications or tightly bound proteins, for example a protein phosphatase (Marmorstein et al., 2002) could be partially removed. It is interesting to note that in cultured astrocytes the Best3-related band had a weight of approximately 52–55 kDa, closer to the theoretically predicted weight for "+6" and "−6" splice variants of Best3. Whether the lower molecular weight in cultured cells relates to a lack of modifications/associations remains to be determined.

Quantification of western blot results showed downregulation of Best3 protein expression in brain cortex and hippocampus after HI ($P < 0.05$) and in cultured mouse astrocytes in ER stress ($P < 0.001$; **Figure 5**), although this was not apparent in the cytoplasmic fraction of the brain tissue after HI injury ($P > 0.05$). The same result was observed whether Best3-related protein bands were normalized to total protein (**Figure 5**) or to GAPDH (data not shown).

DISCUSSION

Best3 protein has previously not been described in the brain, and there are only a few reports where Best3 mRNA was detected in the whole adult mouse brain, although without identification of the cells expressing it (Krämer et al., 2004; Srivastava et al., 2008), and only weakly detected in the normal adult human brain (Stöhr et al., 2002). We show for the first time that Best3 protein and mRNA are expressed in normal and injured brain in newborn mouse pups and in a term infant with white matter gliosis.

Our main focus in this study was to investigate Best3 in cell injury as recent studies suggest a novel role for Best3 in apoptosis and ER-stress. In our mouse experiments we describe for the first time a subpopulation of nestin-positive astrocytes appearing after the HI injury, which expresses Best3 and can be visualized primarily in the penumbra-like area (**Figure 1a**). These cells have an astrocyte morphology, are positive for GFAP, a classic marker of astrocytes (**Figures 1b,d**) and for nestin (**Figures 1a,c**), and do not co-express neuronal or microglial markers. Under normal conditions nestin expression in the brain is more characteristic for progenitor cells than for astrocytes. We cannot exclude the possibility that some of the Best3-positive cells were neural progenitor cells, yet we did not see Best3 expression in the uninjured brain. However, the possibility that progenitor cell proliferation, triggered by injury, contributes to the Best3 expression cannot be ruled out. After injury a subpopulation of activated astrocytes start expressing nestin (Gilyarov, 2008), and these cells have been suggested to be in an early stage of activation preceding hypertrophic changes (Cho et al., 2013). Functionally, these cells have been shown to be proliferating astrocytes that have a positive influence on tissue recovery (Suzuki et al., 2012). The appearance of nestin+/GFAP+ cells has been described in the neonatal rat brain after HI, and these cells are suggested to be

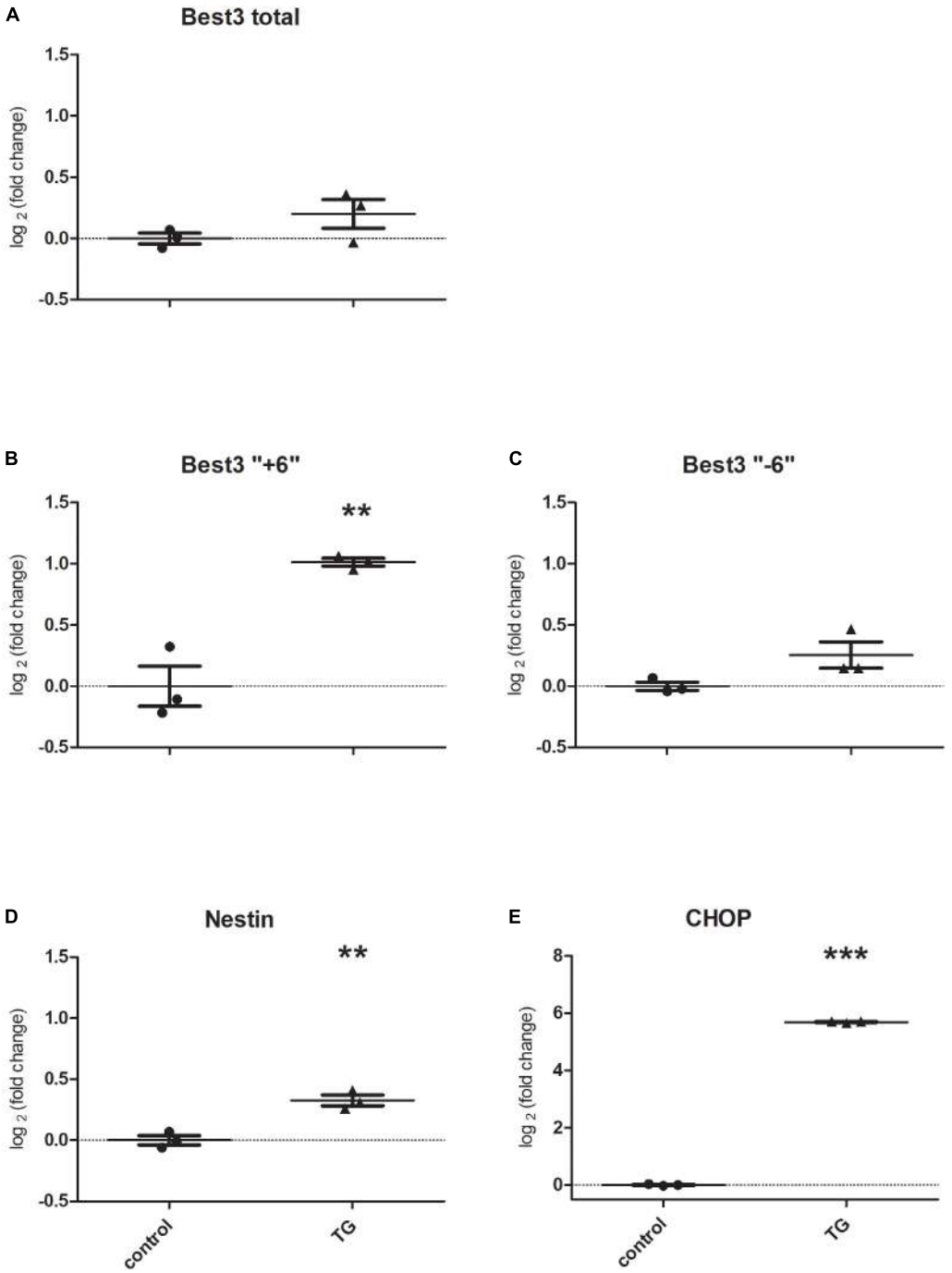


FIGURE 4 | Best3 mRNA is expressed in the primary culture of mouse astrocytes. TG caused very small changes in total Best3 **(A)** and in its short splice variant expression **(C)**, but induced a noticeable increase in expression of the long "+6" splice variant of Best3 **(B)**. TG-treated cells showed a pronounced ER-stress **(E)** and increase in nestin expression **(D)**. $^{**}P < 0.01$, $^{***}P < 0.001$ *t*-test control vs. treatment.

in a transition state from nestin-positive radial glia into GFAP-expressing mature astrocytes (Sizonenko et al., 2008). Mouse astrocytes in primary culture in the present study showed a similar pattern of Best3 protein expression as after HI *in vivo*: partial overlap with GFAP and high degree of co-expression with nestin (**Figures 1e,f**). These results confirm the presence of Best3 in astrocytes, and the pronounced expression of both nestin and Best3 in the majority of cells suggests that astrocytes in culture are to a large extent in an activated state.

We also show that Best3 is expressed in astrocytes in the human term neonatal brain with neuropathology as a result of an acute event leading to intrauterine death (**Figures 1g,h**).

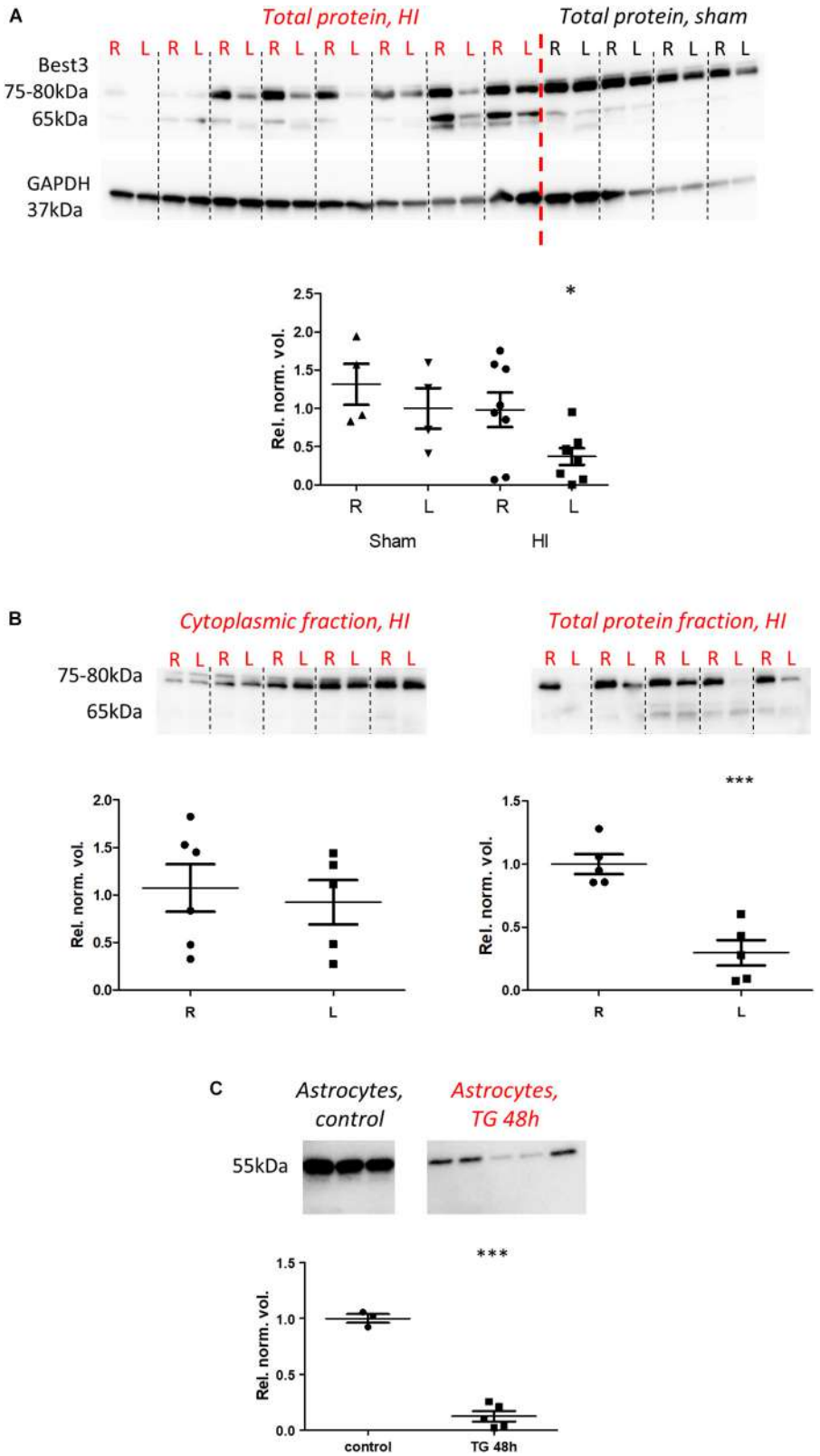


FIGURE 5 | Best3 protein is downregulated in mouse brain tissue and cultured mouse astrocytes after injury. Western blot analysis of brain tissue **(A,B)** and cultured astrocytes **(C)**. Brain specimens from right uninjured (R) and left injured (L) hemispheres were alternately placed on the gels in **(A,B)**; the same was done for

(Continued)

FIGURE 5 | Continued
sham-operated animals **(A)**. Two neighboring R and L lanes belong to the same animal. In total protein samples from brain **(A)** at approximately 75–80 kDa two bands with close molecular weights could be detected; their density was summarized in the analysis. These bands were reduced in injury. Additional lighter pairs of bands could be observed at approximately 65 kDa. The result of subcellular fractionation of brain tissue is shown in **(B)**, where two bands of approximately 75–80 kDa were detected in the cytoplasmic fraction as well as in the total protein fraction. The content of Best3 in the cytoplasm did not change significantly in injury, while Best3 in the total protein fraction was downregulated. In cultured astrocytes **(C)** treatment with thapsigargin for 48 h (TG 48 h) also caused downregulation of Best3. *$P < 0.05$ by two-way ANOVA and estimated marginal means; ***$P < 0.001$ *t*-test R vs. L or treated vs. untreated cells. A total of 18 pups (14 HI, 4 sham) were used; for details see section "Materials and Methods."

This indicates the relevance of our observations in mice to situations with brain injury in humans. This term neonate very likely had injury to brain cells as a result of different traumatic events leading to death. As a result, the brain tissue shows signs of pronounced gliosis, in particular strong activation of astrocytes, and it was interesting to see expression of Best3 in this clinical case. Best3-positive cells were reminiscent of activated astrocytes by their morphology and developed cell processes. Based on the morphology we can suggest that Best3 can be expressed in a subpopulation of activated astrocytes in situations of neuropathology in neonates. The specific relevance of this discovery to hypoxic-ischemic brain injury in neonates, however, is not determined, and a more detailed study of the co-localization of Best3 with various cell markers is required to characterize the Best3-positive human cells.

In the HI model exposure to hypoxia alone does not induce brain injury, while its combination with ischemia causes appearance of an infarct area and cell apoptosis (Hedtjärn et al., 2002). Total Best3 mRNA was upregulated early after HI in both hemispheres. This change thus is related either to hypoxemia or to some influence of the injury on both hemispheres, whether neural or humoral. Interestingly, the rise in Best3 mRNA lasted longer in the uninjured hemisphere. Whether this relates to that hemisphere coping with the hypoxemia, and the other one succumbing, remains to be investigated.

The alternative splicing of Best3 mRNA after the HI injury in mouse brain was also analyzed. In mouse Best3 mRNA exons 2, 3, and 6 can be spliced out in different combinations in different organs, and the whole brain homogenate from the adult mouse was reported to have two splice variants "−2−3+6" and "−2−3−6", but not the full-length mRNA (Krämer et al., 2004). Similar results were obtained in our PCR analysis of Best3 splicing in the brains of neonatal mice (**Figure 2**), and the composition of splice variants was similar in contralateral non-injured and ipsilateral injured brain hemispheres. However, there is also recent data on possible splicing of the C-terminal region of Best3 mRNA and protein in mouse myoblasts (Wu et al., 2016). If this is the case also in brain, the "+6" and "−6" variants seen in our experiments could represent more than one transcript each. A more detailed analysis of alternative splicing of Best3 in situations with brain injury may therefore require RNA sequencing.

In contrast to total Best3, expression of the long "+6" splice variant changed in an injury-related manner, it was downregulated in the injured hemisphere in parallel with developing ER stress, but it was upregulated on the non-injured side (**Figure 3**). The short "−6" was less injury-dependent and was mostly upregulated in both hemispheres. These changes in splicing started already at 6 h after HI, and it is interesting to note that the changes in expression of both splice variants showed biphasic dynamics: the values always tended to transiently return to control levels 24 h after the injury.

Increased expression of the transcription factor CHOP and nestin mRNA was present in the injured hemisphere only (**Figure 3**) suggesting pro-apoptotic ER stress in this tissue (Xu et al., 2005), although the degree of apoptosis was not examined in the present study. In non-injured brain tissue CHOP and nestin were actually downregulated after HI, which might be related to compensatory changes in blood flow and is an interesting observation for future investigation.

In mouse astrocyte cell culture (**Figure 4**) direct induction of strong ER stress by blocking the SERCA pump showed, together with a dramatic increase in CHOP expression, also a tendency for total Best3 mRNA to increase as well as mRNA for both its splice variants, with the most pronounced change in "+6" splice variant expression in contrast to the injury in the brain tissue, where "+6" variant was downregulated. This may indicate that different origins of ER stress can influence Best3 expression differently, but it could also show that cultured astrocytes, being already in an activated state, respond to ER stress differently from astrocytes *in vivo*.

Our results of Best3 mRNA expression show that it is important to study not only the total expression of the gene, but also to analyze individual splice variants, as such proteins can have different functions. Alternative splicing of mRNA can be changed by cell injury and inflammation, and this phenomenon is well described for the protein family Bcl2 (Bcl2, Bcl2L1, Bax, and other), which can act as pro- or antiapoptotic proteins depending on their splicing (Glasgow et al., 2001; Akgul et al., 2004; Miura et al., 2012). A change in the ratio between the antiapoptotic long Bcl-xL and the proapoptotic short Bcl-xS splice variants of one of the members of this family, Bcl2L1 (also called BclX), was also described in neonatal HI injury (Xiao et al., 2012). The results of the present work and of our previous studies (Golubinskaya et al., 2015) reveal a new protein, Best3, alternative splicing of which changes in the situation of tissue injury.

Analysis of Best3 protein expression by western blot showed that the general expression of Best3 protein in brain cortex and hippocampus was reduced after HI injury, and yet in our IHC experiments Best3 appeared in a specific cell population around the injured area. This data suggests that the western blot shows the overall expression in all cell types of the brain and cannot detect an increase in a small sub-population of

Best3-positive astrocytes. At the same time expression of Best3 protein was strongly reduced in cultured astrocytes after 48 h with thapsigargin treatment. Both HI injury and thapsigargin treatment caused ER stress, judging from the increase in CHOP. The Best3 protein was downregulated in cultured cells during ER stress, which calls into question whether Best3 is actually involved in resolving the ER-stress response in astrocytes. On the other hand, the severity of ER stress may differ between the brain astrocytes in HI and thapsigargin-treated cultured astrocytes, where thapsigargin presumably is a much stronger inducer of ER stress or induces ER stress by different mechanisms. Another reason may be that ER stress as such may cause downregulation of Best3 protein, whereas signals related to tissue injury and coming from other types of the cells cause upregulation of Best3 in astrocytes close to the injured area. It is also interesting to note that Best3 protein levels did not change significantly in the cytoplasmic fraction of brain cortex, suggesting the reduction in protein must occur in other compartments of the cells.

In summary, our study demonstrates expression of Best3 in a nestin-positive subpopulation of astrocytes in neonatal mouse brain after hypoxic-ischemic injury, with a general suppression of its expression in the cortex as a whole. Best3 also shows localized expression in astrocyte-like cells in human neonatal brain with pathologies. Analysis of alternative splicing for Best3 mRNA in mouse brain after HI injury showed that even if total expression of Best3 mRNA may not show much change, its splice variants respond differently and thus might have different roles in development of injury in brain tissue. The shorter splice variants are unlikely to function as ion channels (Srivastava et al., 2008) although they may have both intracellular and membrane localization. Their function is so far unknown. If Best3 actually functions as a regulator of apoptosis, it is possible that this property is associated with one variant as it is with Bcl-X, however, this is highly speculative and needs further investigation. Best3 expression in astrocytes near injury in the neonatal brain is a novel and important finding. As a marker for a certain cell population it can help understanding mechanisms of brain injury, where glial cells play an important role. A definitive determination of the role for Best3 in pathophysiology hinges upon the development of a knock-out model.

AUTHOR CONTRIBUTIONS

VG, HG, CM, and HN designed the study. VG, RV, and JW-A collected the data. VG, RV, JW-A, CM, and HN analyzed the data. VG, CM, and HN drafted the manuscript. All authors revised and approved the manuscript.

FUNDING

This study was supported by grants from Vetenskapsrådet (2012-2992), CM, http://www.vr.se, Swedish state under the agreement between the Swedish Government and the county councils, the ALF-agreement (ALFGBG 432291), CM, http://www.fou.nu/is/alfgbg, Leducq Foundation (DSRR_P34404), CM, https://www.fondationleducq.org, Swedish Brain Foundation (FO2014-008), CM, http://www.hjarnfonden.se, Torsten Söderberg Foundation (M98/15), CM, http://www.torstensoderbergsstiftelse.se, Åhlén Foundation (CM and HN) http://www.ahlen-stiftelsen.se/index.html, Stiftelsen Wilhelm och Martina Lundgrens Vetenskapsfond (VG, CM, and HN) http://www.wmlundgren.se, Stiftelsen Fru Mary von Sydows, född Wijk, donationsfond (VG) http://www.maryvonsydowstiftelsen.se, Svenska Frimurare Barnhusdirektionen i Göteborg (VG) https://www.frimurarorden.se/loger/tredje-fordelningen/gota-provinsialloge/caritas. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank the families who consented to the "Postmortem MRI study" and our colleagues in the Perinatal Pathology Services at St Thomas' Hospital.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00023/full#supplementary-material

REFERENCES

Akgul, C., Moulding, D. A., and Edwards, S. W. (2004). Alternative splicing of Bcl-2-related genes: functional consequences and potential therapeutic applications. *Cell. Mol. Life Sci.* 61, 2189–2199. doi: 10.1007/s00018-004-4001-7

Blomgren, K., Zhu, C., Wang, X., Karlsson, J.-O., Leverin, A.-L., Bahr, B. A., et al. (2001). Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia a mechanism of "pathological apoptosis"? *J. Biol. Chem.* 276, 10191–10198. doi: 10.1074/jbc.M007807200

Chavez-Valdez, R., Flock, D. L., Martin, L. J., and Northington, F. J. (2016). Endoplasmic reticulum pathology and stress response in neurons precede programmed necrosis after neonatal hypoxia-ischemia. *Int. J. Dev. Neurosci.* 48, 58–70. doi: 10.1016/j.ijdevneu.2015.11.007

Cho, J. M., Shin, Y. J., Park, J. M., Kim, J., and Lee, M. Y. (2013). Characterization of nestin expression in astrocytes in the rat hippocampal CA1 region following transient forebrain ischemia. *Anat. Cell Biol.* 46, 131–140. doi: 10.5115/acb.2013.46.2.131

Cox, B., and Emili, A. (2006). Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. *Nat. Protoc.* 1, 1872–1878. doi: 10.1038/nprot.2006.273

de Vellis, J., Ghiani, C. A., Wanner, I. B., and Cole, R. (2010). "Preparation of normal and reactive astrocyte cultures," in *Protocols for Neural Cell Culture Springer Protocols Handbooks*, ed. L. C. Doering (New York, NY: Humana Press), 193–215.

DeGracia, D. J., and Montie, H. L. (2004). Cerebral ischemia and the unfolded protein response. *J. Neurochem.* 91, 1–8. doi: 10.1111/j.1471-4159.2004.02703.x

Duran, C., Thompson, C. H., Xiao, Q., and Hartzell, H. C. (2010). Chloride channels: often enigmatic, rarely predictable. *Annu. Rev. Physiol.* 72, 95–121. doi: 10.1146/annurev-physiol-021909-135811

Ek, C. J., D'Angelo, B., Baburamani, A. A., Lehner, C., Leverin, A.-L., Smith, P. L., et al. (2015). Brain barrier properties and cerebral blood flow in neonatal mice exposed to cerebral hypoxia-ischemia. *J. Cereb. Blood Flow Metab.* 35, 818–827. doi: 10.1038/jcbfm.2014.255

Gilyarov, A. V. (2008). Nestin in central nervous system cells. *Neurosci. Behav. Physiol.* 38, 165–169. doi: 10.1007/s11055-008-0025-z

Glasgow, J. N., Qiu, J., Rassin, D., Grafe, M., Wood, T., and Perez-Pol, J. R. (2001). Transcriptional regulation of the BCL-X gene by NF-kappaB is an element of hypoxic responses in the rat brain. *Neurochem. Res.* 26, 647–659. doi: 10.1023/A:1010987220034

Golubinskaya, V., Elvin, J., Ebefors, K., Gustafsson, H., Mallard, C., Nyström, J., et al. (2015). Bestrophin-3 is differently expressed in normal and injured mouse glomerular podocytes. *Acta Physiol.* 214, 481–496. doi: 10.1111/apha.12516

Han, K. S., Woo, J., Park, H., Yoon, B. J., Choi, S., and Lee, C. J. (2013). Channel-mediated astrocytic glutamate release via Bestrophin-1 targets synaptic NMDARs. *Mol. Brain* 6:4. doi: 10.1186/1756-6606-6-4

Hedtjärn, M., Leverin, A.-L., Eriksson, K., Blomgren, K., Mallard, C., and Hagberg, H. (2002). Interleukin-18 involvement in hypoxic–ischemic brain injury. *J. Neurosci.* 22, 5910–5919. doi: 10.1523/JNEUROSCI.22-14-05910.2002

Jiang, L., Liu, Y., Ma, M. M., Tang, Y. B., Zhou, J. G., and Guan, Y. Y. (2013). Mitochondria dependent pathway is involved in the protective effect of bestrophin-3 on hydrogen peroxide-induced apoptosis in basilar artery smooth muscle cells. *Apoptosis* 18, 556–565. doi: 10.1007/s10495-013-0828-4

Johnson, G. G., White, M. C., Wu, J.-H., Vallejo, M., and Grimaldi, M. (2014). The deadly connection between endoplasmic reticulum, Ca^{2+}, protein synthesis, and the endoplasmic reticulum stress response in malignant glioma cells. *Neuro Oncol.* 16, 1086–1099. doi: 10.1093/neuonc/nou012

Krämer, F., Stöhr, H., and Weber, B. H. (2004). Cloning and characterization of the murine Vmd2 RFP-TM gene family. *Cytogenet. Res.* 105, 107–114. doi: 10.1159/000078016

Lee, S., Yoon, B. E., Berglund, K., Oh, S. J., Park, H., Shin, H. S., et al. (2010). Channel-mediated tonic GABA release from glia. *Science* 330, 790–796. doi: 10.1126/science.1184334

Lee, W. K., Chakraborty, P. K., Roussa, E., Wolff, N. A., and Thevenod, F. (2012). ERK1/2-dependent bestrophin-3 expression prevents ER-stress-induced cell death in renal epithelial cells by reducing CHOP. *Biochim. Biophys. Acta* 1823, 1864–1876. doi: 10.1016/j.bbamcr.2012.06.003

Lehotský, J., Urban, P., Pavlíková, M., Tatarková, Z., Kaminska, B., and Kaplán, P. (2009). Molecular mechanisms leading to neuroprotection/ischemic tolerance: effect of preconditioning on the stress reaction of endoplasmic reticulum. *Cell. Mol. Neurobiol.* 29, 917–925. doi: 10.1007/s10571-009-9376-4

Leonard, A., Paton, A. W., El-Quadi, M., Paton, J. C., and Fazal, F. (2014). Preconditioning with endoplasmic reticulum stress ameliorates endothelial cell inflammation. *PLoS One* 9:e110949. doi: 10.1371/journal.pone.0110949

Lund, S., Christensen, K. V., Hedtjärn, M., Mortensen, A. L., Hagberg, H., Falsig, J., et al. (2006). The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and in vivo conditions. *J. Neuroimmunol.* 180, 71–87. doi: 10.1016/j.jneuroim.2006.07.007

Marmorstein, L. Y., McLaughlin, P. J., Stanton, J. B., Yan, L., Crabb, J. W., and Marmorstein, A. D. (2002). Bestrophin interacts physically and functionally with protein phosphatase 2A. *J. Biol. Chem.* 277, 30591–30597. doi: 10.1074/jbc.M204269200

Matchkov, V. V., Larsen, P., Bouzinova, E. V., Rojek, A., Boedtkjer, D. M., Golubinskaya, V., et al. (2008). Bestrophin-3 (vitelliform macular dystrophy 2-like 3 protein) is essential for the cGMP-dependent calcium-activated chloride conductance in vascular smooth muscle cells. *Circ. Res.* 103, 864–872. doi: 10.1161/CIRCRESAHA.108.178517

Miura, K., Fujibuchi, W., and Unno, M. (2012). Splice variants in apoptotic pathway. *Exp. Oncol.* 34, 212–217.

Oh, S. J., Han, K. S., Park, H., Woo, D. H., Kim, H. Y., Traynelis, S. F., et al. (2012). Protease activated receptor 1-induced glutamate release in cultured astrocytes is mediated by Bestrophin-1 channel but not by vesicular exocytosis. *Mol. Brain* 5:38. doi: 10.1186/1756-6606-5-38

Oh, S.-J., and Lee, C. J. (2017). Distribution and function of the bestrophin-1 (Best1) channel in the brain. *Exp. Neurobiol.* 26, 113–121. doi: 10.5607/en.2017.26.3.113

Park, H., Oh, S. J., Han, K. S., Woo, D. H., Park, H., Mannaioni, G., et al. (2009). Bestrophin-1 encodes for the Ca^{2+}-activated anion channel in hippocampal astrocytes. *J. Neurosci.* 29, 13063–13073. doi: 10.1523/JNEUROSCI.3193-09.2009

Rice, J. E. III, Vannucci, R. C., and Brierley, J. B. (1981). The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann. Neurol.* 9, 131–141. doi: 10.1002/ana.410090206

Sheldon, R. A., Sedik, C., and Ferriero, D. M. (1998). Strain-related brain injury in neonatal mice subjected to hypoxia-ischemia. *Brain Res.* 810, 114–122. doi: 10.1016/S0006-8993(98)00892-0

Singh, K., Han, K., Tilve, S., Wu, K., Geller, H. M., and Sack, M. N. (2018). Parkin targets NOD2 to regulate astrocyte endoplasmic reticulum stress and inflammation. *Glia* 66, 2427–2437. doi: 10.1002/glia.23482

Sizonenko, S. V., Camm, E. J., Dayer, A., and Kiss, J. Z. (2008). Glial responses to neonatal hypoxic-ischemic injury in the rat cerebral cortex. *Int. J. Dev. Neurosci.* 26, 37–45. doi: 10.1016/j.ijdevneu.2007.08.014

Song, W., Yang, Z., and He, B. (2014). Bestrophin 3 ameliorates TNFα-induced inflammation by inhibiting NF-κB activation in endothelial cells. *PLoS One* 9:e111093. doi: 10.1371/journal.pone.0111093

Srivastava, A., Romanenko, V. G., Gonzalez-Begne, M., Catalan, M. A., and Melvin, J. E. (2008). A variant of the Ca^{2+}-activated Cl channel Best3 is expressed in mouse exocrine glands. *J. Membr. Biol.* 222, 43–54. doi: 10.1007/s00232-008-9098-4

Stöhr, H., Marquardt, A., Nanda, I., Schmid, M., and Weber, B. H. (2002). Three novel human VMD2-like genes are members of the evolutionary highly conserved RFP-TM family. *Eur. J. Hum. Genet.* 10, 281–284. doi: 10.1038/sj.ejhg.5200796

Supramaniam, V., Vontell, R., Srinivasan, L., Wyatt-Ashmead, J., Hagberg, H., and Rutherford, M. (2013). Microglia activation in the extremely preterm human brain. *Pediatr. Res.* 73, 301–309. doi: 10.1038/pr.2012.186

Suzuki, T., Sakata, H., Kato, C., Connor, J. A., and Morita, M. (2012). Astrocyte activation and wound healing in intact-skull mouse after focal brain injury. *Eur. J. Neurosci.* 36, 3653–3664. doi: 10.1111/j.1460-9568.2012.08280.x

Vannucci, S. J., and Hagberg, H. (2004). Hypoxia-ischemia in the immature brain. *J. Exp. Biol.* 207, 3149–3154. doi: 10.1242/jeb.01064

Vontell, R., Supramaniam, V., Thornton, C., Wyatt-Ashmead, J., Mallard, C., Gressens, P., et al. (2013). Toll-like receptor 3 expression in glia and neurons alters in response to white matter injury in preterm infants. *Dev. Neurosci.* 35, 130–139. doi: 10.1159/000346158

Vontell, R., Supramaniam, V., Wyatt-Ashmead, J., Gressens, P., Rutherford, M., Hagberg, H., et al. (2015). Cellular mechanisms of toll-like receptor-3 activation in the thalamus are associated with white matter injury in the developing brain. *J. Neuropathol. Exp. Neurol.* 74, 273–285. doi: 10.1097/NEN.0000000000000172

Wu, L., Sun, Y., Ma, L., Zhu, J., Zhang, B., Pan, Q., et al. (2016). A C-terminally truncated mouse Best3 splice variant targets and alters the ion balance in lysosome-endosome hybrids and the endoplasmic reticulum. *Sci. Rep.* 6:27332. doi: 10.1038/srep27332

Xiao, Q., Ford, A. L., Xu, J., Yan, P., Lee, K. Y., Gonzales, E., et al. (2012). Bcl-x pre-mRNA splicing regulates brain injury after neonatal hypoxia-ischemia. *J. Neurosci.* 32, 13587–13596. doi: 10.1523/JNEUROSCI.2617-12.2012

Xu, C., Bailly-Maitre, B., and Reed, J. C. (2005). Endoplasmic reticulum stress: cell life and death decisions. *J. Clin. Invest.* 115, 2656–2664. doi: 10.1172/JCI26373

Zhu, C., Wang, X., Xu, F., Bahr, B. A., Shibata, M., Uchiyama, Y., et al. (2005). The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia–ischemia. *Cell Death Differ.* 12, 162–176. doi: 10.1038/sj.cdd.4401545

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Golubinskaya, Vontell, Supramaniam, Wyatt-Ashmead, Gustafsson, Mallard and Nilsson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 11 February 2019
doi: 10.3389/fphys.2019.00064

The Relationship Between Clinical Imaging and Neurobehavioral Assessment in Posthemorrhagic Ventricular Dilation of Prematurity

Rebecca A. Dorner[1,2], Bruno P. Soares[2,3], Shenandoah Robinson[2,4], Marilee C. Allen[1,2], Jamie Perin[5] and Vera Joanna Burton[6,7]*

[1] Neonatology, Johns Hopkins Hospital, Baltimore, MD, United States, [2] Neurosciences Intensive Care Nursery, Johns Hopkins Hospital, Baltimore, MD, United States, [3] Pediatric Radiology and Pediatric Neuroradiology, Johns Hopkins Hospital, Baltimore, MD, United States, [4] Pediatric Neurosurgery, Johns Hopkins Hospital, Baltimore, MD, United States, [5] Biostatistics, Epidemiology, and Data Management Core, Johns Hopkins Hospital, Baltimore, MD, United States, [6] Neurology and Developmental Medicine, Kennedy Krieger Institute, Baltimore, MD, United States, [7] Department of Neurology, The Johns Hopkins School of Medicine, Baltimore, MD, United States

Introduction: Neonatal intraventricular hemorrhage (IVH) and subsequent posthemorrhagic ventricular dilation and hydrocephalus of prematurity are associated with brain injury and neurodevelopmental impairment in the preterm population. Neuroimaging assesses cerebral injury and guides neurosurgical intervention; however, the relationship of head ultrasound (HUS) and magnetic resonance imaging (MRI) parameters to neonatal exams in this group has not been well described. The NICU Network Neurobehavioral Scale (NNNS) is a reproducible, highly reliable battery with motor and cognitive domain scores.

Objective: To evaluate the relationship between neonatal neurobehavioral findings on the NNNS and measures of ventricular dilation and associated brain injury on HUS and MRI.

Materials and Methods: Neonates with IVH and ventricular dilatation with and without posthemorrhagic hydrocephalus were enrolled. NNNS exams were performed at approximately term age equivalent. HUS indices were measured on the last HUS before initial neurosurgical procedure or that with worst ventriculomegaly if no intervention. The posterior fossa was assessed with MRI at term. Descriptive statistics including medians, interquartile ranges, means, and percentages were performed. Correlations were estimated using Pearson's method.

Results: 28 patients had NNNS and HUS, and 18 patients also had an MRI. Ventricle size measures for the cohort were significantly above normal. Motor and cognitive subscores on the NNNS exam varied from established baseline scores for postmenstrual age. Children who required neurosurgical intervention had higher ventricle/brain ratios and worse NNNS habituation scores. Ventricle sizes were modestly correlated with motor abnormalities (0.24–0.59); larger anterior horn width correlated with nonoptimal reflexes, hypertonicity and hypotonicity. Ventricle sizes were modestly correlated with cognitive scores (−0.44 to 0.27); larger ventricular index correlated with worse attention. Periventricular hemorrhagic infarction correlated with worse habituation.

OPEN ACCESS

Edited by:
Mary Tolcos,
RMIT University, Australia

Reviewed by:
Angela Leigh Cumberland,
RMIT University, Australia
Sandrine de Ribaupierre,
University of Western Ontario, Canada

****Correspondence:***
Rebecca A. Dorner
rdorner1@jhmi.edu

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *15 October 2018*
Accepted: *21 January 2019*
Published: *11 February 2019*

Citation:
Dorner RA, Soares BP, Robinson S, Allen MC, Perin J and Burton VJ (2019) The Relationship Between Clinical Imaging and Neurobehavioral Assessment in Posthemorrhagic Ventricular Dilation of Prematurity. Front. Physiol. 10:64. doi: 10.3389/fphys.2019.00064

Conclusion: For this cohort of preterm infants with IVH, surgical intervention for posthemorrhagic hydrocephalus correlated with both larger degrees of ventriculomegaly and worse NNNS exams. Findings on both HUS and MRI correlated with motor and cognitive abnormalities on neonatal neurobehavioral exam, suggesting that larger neonatal ventricle sizes and white matter injury have detectable correlates on exam. The NNNS exam provides important additional information when assessing posthemorrhagic ventricular dilation and hydrocephalus of prematurity.

Keywords: intraventricular hemorrhage, prematurity, ventriculomegaly, hydrocephalus, development, neuroimaging

BACKGROUND

Despite advances in neonatal care, intraventricular hemorrhage (IVH) remains a serious complication of prematurity occurring in up to 30% of premature infants. It is estimated that one-third to half of infants with severe IVH (grades 3 or 4) develop posthemorrhagic ventricular dilatation (Alan et al., 2012; Robinson, 2012). Approximately 10% of all neonates with IVH and 20% of infants with severe IVH will need surgical intervention due to posthemorrhagic hydrocephalus (Alan et al., 2012; Robinson, 2012). Preterm children with posthemorrhagic ventricular dilatation, and especially posthemorrhagic hydrocephalus requiring surgical intervention, are at high risk for future neurodevelopmental challenges. Abnormal motor outcomes include a spectrum from cerebral palsy to minor neuromotor dysfunction/developmental coordination disorder (Spittle and Orton, 2014). Other areas of neurodevelopment impacted include: intellectual disability, fine motor coordination problems, memory and executive function deficits, chronic pain, behavior problems, depression and anxiety, attention deficit/hyperactivity disorder and cortical visual impairment (Ment et al., 1999; Brouwer et al., 2008; Roze et al., 2009; Goldstein et al., 2013; Guzzetta et al., 2013; Tsai et al., 2014; Holwerda et al., 2016). Despite these risks, limited data is available regarding observable neurologic abnormalities in the neonatal period in children with ventriculomegaly and hydrocephalus.

The NNNS, or NICU Network Neurobehavioral Scale, is a standardized assessment tool used to measure neurologic integrity and organization (i.e., active and passive tone, reflexes), behavioral and state regulation, and stress/abstinence (Lester et al., 2004). It has strong psychometric qualities and good validity in the assessment of motor and cognitive deficits in the newborn period (Noble and Boyd, 2012) and normative values are available (Tronick et al., 2004). Administration of the exam requires certification after formal instruction and reliability testing. The examination consists of 45 administration and 70 observation items. Summary scores are created for 13 neurobehavioral domains including: habituation, attention, handling, quality of movement, regulation, nonoptimal reflexes, asymmetrical reflexes, stress/abstinence, arousal, hypertonicity, hypotonicity, excitability, and lethargy (Tronick et al., 2004). See **Table 1** for descriptions of each summary score (Tronick et al., 2004). NNNS performance at various time points including birth, term age equivalent, and time of neonatal admission discharge, have been independently correlated with later developmental outcomes in children with a variety of high-risk conditions, including neonatal abstinence syndrome(for which it was originally developed) (Lester et al., 2004), premature and low birthweight babies (Lester et al., 2011; El-Dib et al., 2012), term babies with fetal risk factors (Appleton et al., 2016) and more recently children with hypoxic ischemic encephalopathy (Massaro et al., 2015). Importantly, the NNNS is especially equipped to assess early cognitive function and state regulation. Regulation and adaptation to negative stimuli in the NNNS can begin to predict behavioral regulation, "a higher function that would be missed by other routine neurological examinations" (Lester et al., 2011).

There are no studies in the literature on NNNS exams in children with posthemorrhagic hydrocephalus of prematurity. The NNNS may help caregivers better understand the infant by providing information about early cognitive and motor function. Additionally, exams can be used serially to recognize subtle changes indicative of worsening neurologic status to guide neuroprotective and rehabilitative interventions.

The aim of our study was to evaluate the association between quantitative measures of neurobehavioral performance in the neonatal period with current gold standard measures of cerebral injury, head ultrasound imaging (HUS) and adjunctive magnetic resonance imaging (MRI). We hypothesized that children with more ventricular dilation, white matter injury, and those requiring intervention for posthemorrhagic hydrocephalus would have worse neonatal neurobehavioral assessments in cognitive and motor domains. Neonatal neurobehavioral exams, if associated with both imaging and later developmental milestones, could function as a bridge between commonly-performed imaging and early milestone attainment.

Abbreviations: AHW, anterior horn width; BPD, Bronchopulmonary dysplasia; HUS, Head ultrasound; IVH, Intraventricular hemorrhage; MRI, Magnetic Resonance Imaging; NEC, Necrotizing Enterocolitis; NICU, Neonatal Intensive Care Unit; NNNS, NICU Network Neurobehavioral Scale; PVHI, Periventricular hemorrhagic infarction; RI, Resistive Index; ROP, Retinopathy of Prematurity; TOD, Thalamooccipital Distance; V/B Ratio, Ventricular/Brain Ratio; VI, Ventricular Index; VPS, Ventriculoperitoneal shunt; VSGS, Ventriculosubgaleal shunt.

TABLE 1 | NNNS summary score descriptions (Tronick et al., 2004).

MOTOR SUBSCORES	
Excitability	Measure of high levels of motor, state and physiologic reactivity; sum of items for excitable behaviors
Lethargy	Measure of low levels of motor, state and physiologic reactivity; sum of items for lethargic behaviors
Nonoptimal reflexes	Any nonoptimal response to reflex excitation; sum of items for nonoptimal reflexes
Asymmetric reflexes	Any asymmetric response to reflex excitation; sum of items for asymmetric reflexes
Hypertonicity	Hypertonic response in arms, legs, trunk, or in general tone, sum of items for hypertonic indicators
Hypotonicity	Hypotonic response in arms, legs, trunk, or in general tone, sum of items for hypotonic indicators
Quality of movement	Measurement of motor control including smoothness, maturity, lack of startles and tremors; mean of items recoded for good motor control
COGNITIVE SUBSCORES	
Habituation	Response decrement to repeated auditory and visual stimuli; mean of items
Attention	Response to animate and inanimate auditory and visual stimuli; mean of items
Handling	Handling strategies used during orientation to maintain alert state; mean number of strategies needed
Regulation	Capacity to organize motor activity, physiology, and state during the examination and to respond to cuddling, consoling, and negative stimuli; mean of items recoded for good regulation
Arousal	Level of arousal including state and motor activity during the examination; mean of items for high arousal
Stress/abstinence	Mean amount of observed stress signs

MATERIALS AND METHODS

Recruitment for this prospective cohort study was conducted from July 1, 2016–July 31, 2018. Eligible patients were preterm infants born in this time frame with IVH and ventricular dilatation, with or without posthemorrhagic hydrocephalus. Babies were either in-born or referred to the Johns Hopkins Hospital Level IIIB and IIIC neonatal intensive care units. Infants were excluded if they had suspected or confirmed genetic anomalies. Infants were identified via neurodevelopmental consultation from the Neurosciences Intensive Care Nursery or by request of the clinical team as part of our routine practice for infants with IVH and ventricular dilatation. After NNNS examination was performed for clinical purposes, parents were approached to consent to join the research cohort. Clinically obtained standard-of-care neuroimaging were recorded in addition to NNNS exam results (see **Figure 1**). Twenty-Eight eligible infants were enrolled in this time frame, including 88% (16/18) of infants admitted with posthemorrhagic hydrocephalus.

Baseline demographics and comorbidities, including IVH grade, sepsis, necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD) severity (Jobe and Bancalari, 2001) and retinopathy of prematurity (ROP), were recorded (**Table 2**). Covariates included postmenstrual age at time of NNNS, HUS, and MRI.

NNNS exams were performed at the closest time point to term age equivalent, ideally no sooner than 34 weeks or less than 10 days from any surgical procedure. Although NNNS exams can be performed in infants from 30 to 48 weeks, we wanted to compare our infant exams to well-established norms at term age equivalent. We chose no sooner than 10 days after a procedure, neurosurgical or otherwise, as we found that with any less time infants were excessively irritable. Per exam criteria, NNNS scores were only performed in medically stable infants with the agreement of the treating medical team. NNNS summary scores were grouped into motor and cognitive categories. Motor subscores include hypertonicity, hypotonicity, excitability, lethargy, nonoptimal reflexes, asymmetrical reflexes, and quality of movement. Cognitive subscores include habituation, attention, handling, regulation, stress/abstinence, and arousal.

The last HUS prior to surgical intervention was selected for measurements as it typically represents the most severe ventricular dilation. If not requiring surgical intervention, the HUS with worst ventriculomegaly was selected. A pediatric neuroradiologist (B.P.S) blinded to clinical course measured the following HUS indices of ventricular size: left and right ventricular index (VI), anterior horn width (AHW), thalamooccipital distance (TOD), and ventricle/brain (V/B) ratio. The left (L) is recorded for each sided measurement for brevity as there was no significant difference amongst right- and left- sided values. Doppler resistive indices (RI) in the anterior cerebral artery, both with and without gentle manual pressure on the transducer, as well as presence of periventricular hemorrhagic infarction (PVHI) and cystic changes were evaluated. The posterior fossa was assessed with MRI; the degree of fourth ventricle dilatation was qualitatively scored (0- none; 1-mild, fourth ventricle compresses vermis only; 2-moderate, compresses dorsal brainstem; 3-severe, compresses ventral brainstem against clivus; 4- massive, fourth ventricle extends superiorly to supratentorial compartment), and the anterior-posterior diameter of the cerebellum from the fastigial point to the posterior vermis (pyramid) was measured. Due to the anatomical distortion of brain parenchyma in the majority of the patients, we were not able to complete typical preterm MRI scoring systems such as the Kidokoro system (Kidokoro et al., 2013). For example, due to the severity of fourth ventricle dilation, cerebellar height and transcerebellar diameter were unable to be completed for the majority of patients. Additionally, HUS measurements of ventricular size agreed with MRI measures, and the sample size of MRIs was more limited than HUS. For this reason, the majority of our statistics analyze HUS measures, with the exception of the fourth ventricle size and anterior-posterior diameter of the cerebellum by MRI. Descriptive statistics including medians, interquartile ranges (IQRs), means, and percentages were used to summarize the cohort. Correlations were estimated using Pearson's method. All analyses were conducted in R version 3.5.0 (R Core Development Team, 2018).

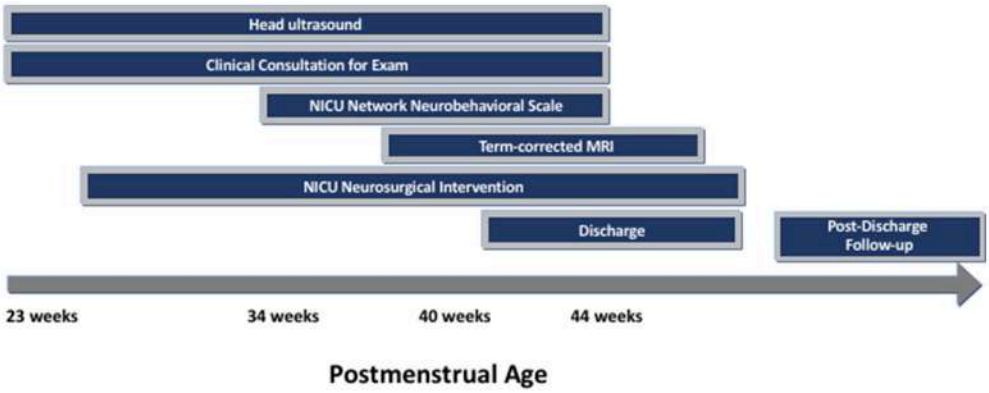


FIGURE 1 | Cohort Timeline of Clinical Milestones, NNNS exam, and Neuroimaging.

RESULTS

In total, 28 patients were consented and had the NNNS and HUS performed. Of these 28, 18 patients had the NNNS, HUS and MRI performed. Average gestational age at birth was 27.1(range 23.4–34.2) weeks and average birthweight 1,115 (range 500–3816) g (**Table 2**). Mean postmenstrual age (PMA) at the time of the HUS was 32.2 weeks, NNNS exam was 37.2 weeks, and MRI was 39.5 weeks (**Table 2**). Ventricle size measures for the cohort were significantly above normal (**Table 3**). Motor and cognitive subscores on the NNNS exam (**Table 4**) varied from established baseline scores for term-corrected age (Lester and Tronick, 2004; Lester et al., 2011; Appleton et al., 2016). The only significant difference between infants with and without MRI was that babies with MRIs were more likely to have a 5 min APGAR score less than 7 (59 vs. 10%, $p = 0.018$).

Significant associations between imaging parameters and NNNS motor and cognitive subscores are summarized in **Figure 2**.

Abnormal radiology measures were associated with other abnormal radiology markers. Ventricle size measures (VI, AHW, TOD, and V/B ratio) were positively associated with one another (**Figure 2**). This is important as many studies have used different measurements to define ventriculomegaly (Sondhi et al., 2008; Maunu et al., 2011; Brouwer et al., 2012; Dorner et al., 2018). Larger AHW (but not other ventricle size measures) and larger fourth ventricle sizes were associated with smaller anterior-posterior diameter of the cerebellum (**Figure 2**). PVHI and cystic changes were associated; the odds ratio between PVHI and cystic changes was infinite (95% CI: 7.22—Infinity, $p = < 0.001$).

Features on both the HUS and NNNS exam were associated with receipt of neurosurgical intervention, where intervention was defined as either ventriculosubgaleal shunt (VSGS) placement or ventriculoperitoneal shunt (VPS) placement (**Table 5**). Fifty-Seven Percent of the cohort (16/28) required shunt placement; 2 patients required a VSGS, 11 had an initial VSGS followed by a VPS, and 3 patients had a VPS alone. Children receiving neurosurgical intervention were more likely to have larger ventricle sizes; means of ventricular size for those receiving neurosurgical intervention versus those not receiving intervention were statistically different as measured by: TOD (45.42 mm vs. 27.67, respectively, $p = < 0.001$), AHW (23.85 vs. 11.53 mm, respectively, $p = 0.001$), VI (24.62 vs. 14.20, respectively, $p = 0.001$), and V/B Ratio (0.615 vs. 0.378, respectively, $p = 0.022$) (**Table 5**). Neurosurgical intervention was most strongly associated with V/B ratio, with a correlation coefficient of 0.78 (**Figure 2**). The NNNS habituation subscore (response decrement to repeated auditory and visual stimuli) was the only additional parameter that differed between those infants receiving intervention and not receiving intervention (**Table 5**). Doppler RI values, with and without compression, did not correlate with receiving surgery.

Ventricle sizes were modestly correlated with motor abnormalities on NNNS exam (estimated correlations −0.72 to 0.39, **Figure 2**). Larger AHW sizes on HUS were associated with higher scores for nonoptimal reflexes (number of nonoptimal responses to reflex elicitation, such as excessive clonus), hypotonicity (number of hypotonic responses in arm, legs, trunk, or general tone) and hypertonicity (number of hypertonic responses in arm, legs, trunk, or general tone). Correlations between AHW and hypertonicity, hypotonicity, and nonoptimal reflexes subscores from the NNNS exam were: 0.59 (95% CI: 0.09–0.70, $p = 0.018$), 0.24 (95% CI: 0.08–0.70, $p = 0.018$), and 0.44 (95% CI: 0.08–0.70, $p = 0.018$), respectively (**Figure 2**).

Ventricle sizes were modestly inversely correlated with cognitive scores on NNNS exam as well (estimated correlations from −0.44 to 0.27, **Figure 2**). NNNS attention was negatively correlated with VI, where a larger VI was related to a lower average NNNS attention score (−0.06, 95% CI: −0.70 to −0.04, $p = 0.034$).

Of the 16 children in the cohort requiring intervention for posthemorrhagic hydrocephalus, 8 had antecedent Grade 3 IVH and 8 had PVHI (formerly called Grade 4 IVH). Presence of PVHI was associated with decreased or inadequate habituation to stimuli; the mean (standard deviation) for habituation for those with PVHI was 5.41 (1.75) and for no PVHI was 7.73 (1.18), $p = 0.012$.

TABLE 2 | Characteristics of 28 Preterm Neonates with IVH with and without Hydrocephalus ($n = 28$).

Mean gestational age at birth	27.2 weeks (range: 23.4–34.2 weeks)
Mean birthweight	1,130 g (500–3,816 g)
Mean PMA at NNNS Number of exams performed in PMA category	37.2 weeks (32.4–45.1 weeks) <34 weeks: 3 34.0–34.6 weeks: 5 35.0–35.6 weeks: 3 36.0–36.6 weeks: 4 37+ weeks: 13
Mean PMA at HUS	37.2 weeks (25.6–41.3 weeks)
Mean PMA at MRI	39.5 weeks (33.5–49.2 weeks)
Sex	16 (57%) male, 12 (43%) female
Ethnicity	9 (32%) African-American, 16(57%) Caucasian, 1(4%) Asian, 2 (7%) Hispanic
Adequate prenatal betamethasone*	18 (64%)
Grade of Intraventricular hemorrhage	Grade 1: 4 (14%) Grade 2: 3 (11%) Grade 3: 11 (39%) Grade 4 (PVHI): 10 (36%)
Moderate/severe BPD^{+}	29 (68%)
Severe ROP (Stage 3 or surgical intervention/biologic medication)	7(25%)
Necrotizing enterocolitis (NEC)^	2 patients Stage 3B, 1 patient Stage 3B after spontaneous intestinal perforation, 1 patient Stage 1B
Treated for culture positive or negative sepsis	20 (71%)
5-min Apgar <7	11(39%)

PMA, Postmenstrual Age.
**Completed 24 h in-utero after second dose.*
$^{+}$Per NICHD criteria (Jobe and Bancalari, 2001); moderate BPD as O_2 for ≥28 days plus treatment with <30% FiO_2 at 36 weeks' PMA and severe BPD as O_2 for ≥ 28 days plus ≥ 30% FiO_2 and/or positive pressure at 36 weeks' PMA.
^Modified bell staging criteria for NEC (Lee and Polin, 2003).

There was clustering of NNNS exam findings in preterm children with IVH. In terms of motor development, those with abnormally high levels of motor, state, and physiologic reactivity (higher excitability scores) were more likely to have abnormal movement (quality of movement score), less regulation, and higher stress/abstinence and arousal scores. Those with low levels of activity, or lethargy, were more hypotonic, and exhibited lower attention and arousal scores. Interestingly, hypotonicity was correlated with hypertonicity (correlation 0.46, 95% CI: 0.11–0.71, $p = 0.014$) and both were correlated with nonoptimal reflexes: (hypertonicity—0.71, 95% CI: 0.46–0.86, $p = 0.00$) and (hypotonicity—0.45, 95% CI: 0.10–0.71, $p = 0.015$) (**Figure 2**).

NNNS scores were also associated with medical morbidities. Infants with severe ROP had higher NNNS Excitability [average (SD) 4.6 (1.9) vs. 2.3 (1.9), $p = 0.006$). Severe BPD was also associated with higher nonoptimal reflexes [5.4 (1.9) for those with BPD, 3.6 (1.6) without, $p = 0.027$] and lower movement [3.7 (0.8) for those with BPD and 4.7 (0.6) without, $p = 0.003$].

Of note, BPD does not confound the relationship of non-optimal reflexes with AHW; the mean AHW for infants without BPD is 14.4 mm and with moderate/severe BPD was 18.2, $p = 0.308$. No NNNS items were independently associated with Sepsis or NEC.

DISCUSSION

This is the first study to analyze the relationship between HUS and MRI imaging with neonatal neurobehavioral exams in posthemorrhagic ventricular dilation and hydrocephalus of prematurity. It is also the first to describe the range of NNNS findings in this group. Our patient population had severe ventriculomegaly, with means for all ventricular size measurements on HUS far above norms for age. As intended, the NNNS exams were performed around term equivalent age (average 37.3 weeks) in the attempt to correlate with published norms for term babies (Tronick et al., 2004). MRIs were also performed at term age equivalent (39.5 weeks), but only fourth ventricle dilation and anterior-posterior diameter of the cerebellum were analyzed due to technical challenges and lack of standardization of MRI reads for posthemorrhagic ventricular dilatation.

For this cohort of 28 preterm infants, the degree of ventriculomegaly—using precise imaging parameters such as V/B ratio and AHW—correlated with surgical intervention, as expected. This suggests that decisions regarding intervention are made either on the basis of ventricular size alone or due to changes in ventricular size in combination with the clinical course. Importantly, different radiologic measurements of the lateral ventricles were all related to each other, suggesting that the choice of ventricular measurement may be less important than choosing a standard measure. Specific ventricular measurements may be superior, however, for different purposes. In this study, intervention had the highest association with V/B ratio, AHW with NNNS motor subscores and the VI with NNNS cognitive subscores. The NNNS cognitive exam, specifically the habituation subscore, also correlated with receipt of surgical intervention. The relationship of intervention and NNNS exam suggests that posthemorrhagic hydrocephalus and/or intervention itself may create a detectable signal of reactivity.

In this participant group, V/B ratio best correlated with receipt of intervention. V/B ratios > 0.35 on ultrasound have been associated with smaller cerebrum and cerebellar volumes on term-corrected MRI (Govaert and de Vries, 1997). Decreased cerebral and cerebellar parenchymal volumes are associated with lower cognitive and language scores, abnormal motor outcome and processing speed (Nosarti et al., 2008; Maunu et al., 2009). Smaller cerebellar size, which has previously been shown to correlate with worse future cognitive outcomes (de Vries et al., 2019), was associated both with larger AHW and more fourth ventricle dilatation in our population.

Higher AHWs were associated with worse motor exams, specifically a higher number of nonoptimal reflexes and more hypotonicity. An increase in AHW has been suggested to be a more sensitive marker for early worsening of hydrocephalus and is seen subjectively as rounding of the frontal horns (Brouwer et al., 2010). Both lower (>6 mm) and higher (>10mm) AHW

TABLE 3 | Head ultrasound and magnetic resonance imaging parameters.

Measure	Number patients	Cohort median (IQR)	**Normal** (Sondhi et al., 2008; Brouwer et al., 2010, 2012; Maunu et al., 2011; Graca et al., 2013; Kidokoro et al., 2013)
Ventricular index (mm)	28	21.00 (10.75–24.25)	10–13
Anterior horn width (mm)	28	19.00 (8.50–24.25)	Less than 3
Thalamooccipital distance (mm)	28	40.00 (23.75–45.25)	5–25
Ventricle/brain ratio	28	0.52 (0.32–0.60)	Less than 0.35
Resistive Index (with pressure)	28	0.87 (0.78–0.93)	0.5–0.8
Resistive Index (without pressure)	28	0.81 (0.75–0.89)	0.5–0.8
AP diameter cerebellum (mm)	18	14.00 (10.25–17.25)	22–24
4th ventricle dilatation (qualitative score)*	18	1.00 (0.00–1.75)	0
	Number patients	**Observed (%)**	
PVHI (present)	28	10 (36%)	None
Cystic changes (present)	28	8 (29%)	None

PVHI, Periventricular Hemorrhagic Infarction.
**4th ventricle dilatation: 0-none, 1- compresses vermis, 2-compresses dorsal brainstem, 3-compresses ventral brainstem against clivus, 4- to supratentorial compartment.*

size cutoffs for surgical intervention have been used (Leijser et al., 2018; de Vries et al., 2019). The fact that early AHW dilatation correlates to exam findings in the neonatal period provides an opportunity to follow this parameter in a more multifaceted way.

Ventricle sizes and white matter injury were associated with worst neonatal cognitive assessment scores. Children with larger ventricles had lower attention scores; larger VI measurements were negatively correlated with the NNNS attention cognitive subscore. Attention is the ability to pay attention to salient stimuli in the environment. Additionally, PVHI, present in 10 of our children, correlated with decreased habituation to stimuli. Habituation is the ability to stop responding to (i.e., to ignore) repetitive stimuli. An inability to inhibit a response is one of the earliest measures of executive function and is well-developed by 6–7 months of age in typically developing infants (Diamond et al., 1994). In general, the children with larger VI and with PVHI were more irritable and responded more continuously to noxious stimuli, at the expense of paying attention to important stimuli such as a face or voice. It is possible that difficulty with attention and habituation in this early time frame are signs of later difficulty with inhibition and attention and a marker for later executive dysfunction (Diamond et al., 1994). Executive function and attention difficulties are well described in the hydrocephalus population (Holwerda et al., 2016). This early internal disorganization and over-responsiveness may be early markers of such difficulties.

Fourth ventricle dilatation on MRI was not associated with changes in NNNS exam (**Table 5**). While this attempt to evaluate fourth ventricle dilatation did not correlate with neonatal exams, this parameter has not yet been adequately assessed in this population. Further data are needed to understand the impact of fourth ventricular dilatation and on exams and outcomes.

We also saw informative patterns in the NNNS exam. Both ends of the arousal spectrum were seen; abnormally high excitability, stress and arousal score subgroups were present as were lethargy, hypotonia, and lower attention and arousal scores.

TABLE 4 | NICU Network Neurobehavioral Scale (NNNS) measurements.

	Cohort mean (SD)	Standardized relative to typical scores	Typical mean* (SD)
MOTOR SUBSCORES			
Excitability	3.43 (2.32)	−0.59 (−1.06 to 0.84)	4.23 (2.10)
Lethargy	4.79 (2.18)	−0.72 (−1.02 to −0.02)	6.32 (3.24)
Nonoptimal reflexes	4.96 (1.97)	0.39 (−0.18 to 0.97)	4.32 (1.73)
Asymmetric reflexes	1.41 (1.55)	−0.70 (−1.45 to 0.05)	1.93 (1.33)
Hypertonicity	0.29 (0.53)	−0.27 (−0.27 to 0.69)	0.07 (0.26)
Hypotonicity	0.64 (0.73)	−0.07 (−0.72 to 0.59)	0.55 (0.76)
Quality of movement	3.95 (0.86)	0.24 (−0.30 to 1.10)	3.81 (0.78)
COGNITIVE SUBSCORES			
Habituation	6.77 (1.82)	−0.80 (−2.26 to 0.08)	7.91 (1.14)
Attention	5.25 (1.16)	0.26 (−0.84 to 0.81)	5.30 (1.04)
Handling	0.47 (0.22)	0.85 (0.16 to 1.32)	0.27 (0.27)
Regulation	4.95 (0.74)	−0.28 (−0.74 to 0.67)	5.0 (0.82)
Arousal	3.63 (0.91)	−0.81 (−1.43 to 0.16)	4.16 (0.81)
Stress/ Abstinence	0.19 (0.05)	0.80 (0.20 to 1.50)	0.15 (0.05)

**, per Maternal Lifestyle Study (Lester et al., 2011).*
SD, Standard deviation.

These groups were not mutually exclusive; indeed, many children scored high on both lethargy and excitability, meaning during the exam they were often either too sleepy or too irritable. Both result in a failure to interact and pay attention to informative stimuli. Along the same lines, hypotonicity within our cohort was correlated with hypertonicity (correlation 0.46, 0.11–0.71, $p = 0.014$) and both were correlated with nonoptimal reflexes: (hypertonicity- 0.71, 0.46–0.86, $p = 0.00$) and (hypotonicity- 0.45 (0.10–0.71, $p = 0.015$). These patterns fit what we often see as a

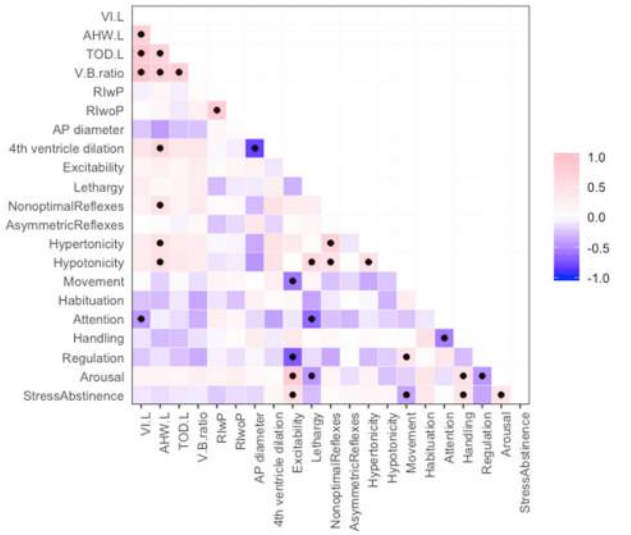


FIGURE 2 | Correlations among ventricular size measurements and NNNS motor and cognitive subscores, where pink indicates positive correlation and purple negative correlation. Significant correlations with $p < 0.05$ are marked with a black dot.

TABLE 5 | Means of HUS and MRI parameters, NNNS subscales by infants with and without neurosurgical intervention for hydrocephalus.

Feature	Mean (SD) or *N* (%) for infants who received intervention (*n* = 16)	Mean (SD) or *N* (%) for infants with no intervention (*n* = 12)	Test for difference (*p**)
VI (mm)	23.88 (5.48)	12.58 (5.62)	< 0.001*
AHW (mm)	22.88 (6.01)	9.75 (11.54)	0.003*
TOD (mm)	43.90 (9.00)	25.25 (14.95)	0.001*
V/B ratio	0.60 (0.08)	0.34 (0.08)	< 0.001*
RI w/P	0.87 (0.11)	0.85 (0.10)	0.532
RI w/o P	0.83 (0.10)	0.79 (0.08)	0.209
AP diameter cerebellum (mm)	11.62 (7.43)	16.40 (3.65)	0.089
4th ventricle dilatation (mm)	1.31 (1.25)	0.60 (0.55)	0.116
PVHI (present)	8 (50%)	2 (17%)	0.114
Cystic changes (present)	7 (44%)	1 (8%)	0.088
Excitability	2.92 (1.98)	3.81 (2.54)	0.303
Lethargy	4.17 (1.70)	5.25 (2.44)	0.177
Nonoptimal reflexes	4.42 (1.51)	5.38 (2.22)	0.185
Asymmetric reflexes	1.17 (1.64)	1.60 (1.50)	0.487
Hypertonicity	0.17 (0.39)	0.38 (0.62)	0.286
Hypotonicity	0.50 (0.67)	0.75 (0.77)	0.371
Movement	4.06 (0.93)	3.87 (0.82)	0.569
Habituation	7.72 (1.68)	5.93 (1.57)	0.039*
Attention	5.50 (0.94)	5.08 (1.29)	0.359
Handling	0.54 (0.20)	0.43 (0.23)	0.217
Regulation	5.18 (0.76)	4.76 (0.71)	0.160
Arousal	3.46 (0.81)	3.75 (0.98)	0.403
Stress/abstinence	0.21 (0.05)	0.18 (0.06)	0.253

**Significance determined by Student's t-test or by Fisher's exact test for categorical factors.*

concerning pattern in ventricular dilatation; axial hypotonia and poor head control with high appendicular tone in the extremities. Together, these patterns of extreme reactions reflect a limited ability of the injured brain to regulate responses.

It is important to consider that ventricular dilatation and hydrocephalus are not isolated in preterm infants; the relationship of HUS and MRI measures to NNNS scores may be influenced by comorbidities. We found that severe ROP and moderate/severe BPD were associated with abnormalities in NNNS items regardless of ventricular size. Severe ROP was associated with higher NNNS excitability scores and moderate/severe BPD was associated with more nonoptimal reflexes and lower movement scores. Although both moderate/severe BPD and AHW were both associated with worse non-optimal reflexes, on further analysis BPD did not confound the relationship of non-optimal reflexes with AHW. It will be important in the future to consider both effects from hydrocephalus and comorbid conditions to better describe the contribution of hydrocephalus to neurodevelopmental delay.

Our data suggests that ventricular size is associated with neonatal exam as larger ventricle sizes were related to poorer scores on both motor and cognitive sections of the NNNS exam. The observed modest correlations are not unexpected; likely other factors in the infant's clinical course might also affect exam performance. Given that aspects of the neonatal neurobehavioral exam are associated with ventricular size measurements, our data suggests that exam and imaging may provide complementary and additive information on existing neurodevelopmental status.

There are limitations to the present study. Although a large portion of infants with posthemorrhagic hydrocephalus were captured, not all consecutive admissions to the NICU with IVH and some ventricular dilatation completed a NNNS evaluation; this may introduce selection bias. The average postmenstrual age at NNNS examination was 37.2 weeks, but some infants had exams earlier, as seen in **Table 2**. The nature of our NICUs are such that convalescing preterm infants, even after neurosurgery, are often transferred to lower acuity locations. As such, some infants were examined before term-corrected age due to imminent discharge. The impact of postnatal age on the NNNS is unclear in this study; previous studies have shown that postmenstrual age has an effect on NNNS subscores (Spittle et al., 2016). Additionally, given our small sample size, inclusion and analysis of all potential covariates was not feasible and we could not evaluate the cumulative impact of measured co-morbidities, such as NEC, on NNNS scores. Adequately powered larger samples are needed to address this important issue.

CONCLUSION

In summary, for this cohort of 28 preterm infants with IVH and ventricular dilatation, surgical intervention was associated with degree of ventriculomegaly, using precise imaging parameters, as well as NNNS exam. Findings on both HUS and MRI imaging correlated with motor and cognitive abnormalities on neonatal neurobehavioral exam. These results suggest that larger neonatal ventricle sizes and white matter injury have detectable correlates on exam in this population. The NNNS exam may be additive with imaging in assessment of a common form of neonatal hydrocephalus. Importantly, the NNNS is identifying deficits at term equivalence, and even earlier, during a preterm infant's NICU stay. This risk identification is much earlier than typical neurodevelopmental exams done in follow-up care. This can allow for better developmental care during the NICU as changes can be made to the environment and rehabilitative interventions begun as soon as neurobehavioral deficits are identified (Mahoney and Cohen, 2005; Symington and Pinelli, 2006; King et al., 2008; Als and McAnulty, 2011; Fucile et al., 2011). It will be important to achieve longer-term follow-up in these infants to determine if these findings correlate with later neurodevelopmental disability.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations and approval of the Institutional Review Board at Johns Hopkins Hospital. Parents of all pediatric subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

RD and VB conceived of and designed the study, performed exams, supervised data entry and analysis, analyzed and interpreted data, and wrote and edited manuscript. BS performed neuroimaging analysis. BS, JP, SR, and MA analyzed data and edited manuscript.

ACKNOWLEDGMENTS

We would like to thank Veena Billioux for her help with statistical work conceptualizing this project. This project was supported by the Thomas Wilson Sanitarium for Children of Baltimore City awarded to RD and MA and the NIH T32 Training Grant (T32HL125239-03) awarded to RD.

REFERENCES

Alan, N., Manjila, S., Minich, N., Bass, N., Cohen, A. R., Walsh, M., et al. (2012). Reduced ventricular shunt rate in very preterm infants with severe intraventricular hemorrhage: an institutional experience. *J. Neurosurg. Pediatr.* 10, 357–364. doi: 10.3171/2012.7.PEDS11504

Als, H., and McAnulty, G. B. (2011). The Newborn Individualized Developmental Care and Assessment Program (NIDCAP) with Kangaroo Mother Care (KMC): comprehensive care for preterm infants. *Curr. Women Health Rev.* 7, 288–301. doi: 10.2174/157340411796355216

Appleton, A. A., Murphy, M. A., Koestler, D. C., Lesseur, C., Paquette, A. G., Padbury, J. F., et al. (2016). Prenatal programming of infant neurobehaviour in a healthy population. *Paediatr. Perinat. Epidemiol.* 30, 367–375. doi: 10.1111/ppe.12294

Brouwer, A., Groenendaal, F., van Haastert, I. L., Rademaker, K., Hanlo, P., and de Vries, L. (2008). Neurodevelopmental outcome of preterm infants with severe intraventricular hemorrhage and therapy for post-hemorrhagic ventricular dilatation. *J. Pediatr.* 152, 648–654. doi: 10.1016/j.jpeds.2007.10.005

Brouwer, M. J., de Vries, L. S., Groenendaal, F., Koopman, C., Pistorius, L. R., Mulder, E. J., et al. (2012). New reference values for the neonatal cerebral ventricles. *Radiology* 262, 224–233. doi: 10.1148/radiol.11110334

Brouwer, M. J., de Vries, L. S., Pistorius, L., Rademaker, K. J., Groenendaal, F., and Benders, M. J. (2010). Ultrasound measurements of the lateral ventricles in neonates: why, how and when? A systematic review. *Acta Paediatr.* 99, 1298–1306. doi: 10.1111/j.1651-2227.2010.01830.x

de Vries, L. S., Groenendaal, F., Liem, K. D., Heep, A., Brouwer, A. J., van 't Verlaat, E., et al. (2019). Treatment thresholds for intervention in posthaemorrhagic ventricular dilation: a randomised controlled trial. *Arch. Dis. Childh. Fetal Neonatal Ed.* 104, F70–F75. doi: 10.1136/archdischild-2017-314206

Diamond, A., Werker, J. F., and Lalonde, C. (1994). "Toward understanding commonalities in the development of object search, detour navigation, categorization, and speech perception," in *Human Behavior and the Developing Brain*, eds G. Dawson and K. W. Fischer (New York, NY: Guilford Press), 380–426.

Dorner, R. A., Burton, V. J., Allen, M. C., Robinson, S., and Soares, B. P. (2018). Preterm neuroimaging and neurodevelopmental outcome: a focus on intraventricular hemorrhage, post-hemorrhagic hydrocephalus, and associated brain injury. *J. Perinatol.* 38, 1431–1443. doi: 10.1038/s41372-018-0209-5

El-Dib, M., Massaro, A. N., Glass, P., and Aly, H. (2012). Neurobehavioral assessment as a predictor of neurodevelopmental outcome in preterm infants. *J. Perinatol.* 32, 299–303. doi: 10.1038/jp.2011.100

Fucile, S., Gisel, E. G., McFarland, D. H., and Lau, C. (2011). Oral and non-oral sensorimotor interventions enhance oral feeding performance in preterm infants. *Dev. Med. Child Neurol.* 53, 829–835. doi: 10.1111/j.1469-8749.2011.04023.x

Goldstein, R. F., Cotten, C. M., Shankaran, S., Gantz, M. G., and Poole, W. K. (2013). Influence of gestational age on death and neurodevelopmental outcome in premature infants with severe intracranial hemorrhage. *J. Perinatol.* 33, 25–32. doi: 10.1038/jp.2012.91

Govaert, P., and de Vries, L. S. (1997). *An Atlas of Neonatal Brain Sonography*. London: Mac Keith Press.

Graca, A. M., Geraldo, A. F., Cardoso, K., and Cowan, F. M. (2013). Preterm cerebellum at term age: ultrasound measurements are not different from infants born at term. *Pediatr. Res.* 74, 698–704. doi: 10.1038/pr.2013.154

Guzzetta, A., Fiori, S., Scelfo, D., Conti, E., and Bancale, A. (2013). Reorganization of visual fields after periventricular haemorrhagic infarction: potentials and limitations. *Dev. Med. Child Neurol.* 55(Suppl. 4), 23–26. doi: 10.1111/dmcn.12302

Holwerda, J. C., Van Braeckel, K., Roze, E., Hoving, E. W., Maathuis, C. G. B., Brouwer, O. F., et al. (2016). Functional outcome at school age of neonatal post-hemorrhagic ventricular dilatation. *Early Hum. Dev.* 96, 15–20. doi: 10.1016/j.earlhumdev.2016.02.005

Jobe, A. H., and Bancalari, E. (2001). Bronchopulmonary dysplasia. *Am. J. Respir. Crit. Care Med.* 163, 1723–1729. doi: 10.1164/ajrccm.163.7.2011060

Kidokoro, H., Neil, J. J., and Inder, T. E. (2013). New MR imaging assessment tool to define brain abnormalities in very preterm infants at term. *Am. J. Neuroradiol.* 34, 2208–2214. doi: 10.3174/ajnr.A3521

King, G., Currie, M., Bartlett, D. J., Strachan, D., Tucker, M. A., and Willoughby, C. (2008). The development of expertise in paediatric rehabilitation therapists: the roles of motivation, openness to experience, and types of caseload experience. *Aust. Occup. Ther. J.* 55, 108–122. doi: 10.1111/j.1440-1630.2007.00681.x

Lee, J. S., and Polin, R. A. (2003). Treatment and prevention of necrotizing enterocolitis. *Semin. Neonatol.* SN8, 449–459. doi: 10.1016/S1084-2756(03)00123-4

Leijser, L. M., Miller, S. P., van Wezel-Meijler, G., Brouwer, A. J., Traubici, J., van Haastert, I. C., et al. (2018). Posthemorrhagic ventricular dilatation in preterm infants: when best to intervene? *Neurology* 90, e698–e706. doi: 10.1212/WNL.0000000000004984

Lester, B. M., Miller, R. J., Hawes, K., Salisbury, A., Bigsby, R., Sullivan, M. C., et al. (2011). Infant neurobehavioral development. *Semin. Perinatol.* 35, 8–19. doi: 10.1053/j.semperi.2010.10.003

Lester, B. M., and Tronick, E. Z. (eds) (2004). *NICU Network Neurobehavioral Scale, (NNNS) Manual*. Baltimore, MD: Paul H. Brookes Pub. Co.

Lester, B. M., Tronick, E. Z., and Brazelton, T. B. (2004). The neonatal intensive care unit network neurobehavioral scale procedures. *Pediatrics* 113 (3 Pt 2), 641–667.

Mahoney, M. C., and Cohen, M. I. (2005). Effectiveness of developmental intervention in the neonatal intensive care unit: implications for neonatal physical therapy. *Pediatr. Phys. Ther.* 17, 194–208.

Massaro, A. N., Evangelou, I., Brown, J., Fatemi, A., Vezina, G., McCarter, R., et al. (2015). Neonatal neurobehavior after therapeutic hypothermia for hypoxic ischemic encephalopathy. *Early Hum. Dev.* 91, 593–599. doi: 10.1016/j.earlhumdev.2015.07.008

Maunu, J., Lehtonen, L., Lapinleimu, H., Matomaki, J., Munck, P., Rikalainen, H., et al. (2011). Ventricular dilatation in relation to outcome at 2 years of age in very preterm infants: a prospective Finnish cohort study. *Dev. Med. Child Neurol.* 53, 48–54. doi: 10.1111/j.1469-8749.2010.03785.x

Maunu, J., Parkkola, R., Rikalainen, H., Lehtonen, L., Haataja, L., and Lapinleimu, H. (2009). Brain and ventricles in very low birth weight infants at term: a comparison among head circumference, ultrasound, and magnetic resonance imaging. *Pediatrics* 123, 617–626. doi: 10.1542/peds.2007-3264

Ment, L. R., Vohr, B., Allan, W., Westerveld, M., Katz, K. H., Schneider, K. C., et al. (1999). The etiology and outcome of cerebral ventriculomegaly at term in very low birth weight preterm infants. *Pediatrics* 104(2 Pt 1), 243–248. doi: 10.1542/peds.104.2.243

Noble, Y., and Boyd, R. (2012). Neonatal assessments for the preterm infant up to 4 months corrected age: a systematic review. *Dev. Med. Child Neurol.* 54, 129–139. doi: 10.1111/j.1469-8749.2010.03903.x

Nosarti, C., Giouroukou, E., Healy, E., Rifkin, L., Walshe, M., Reichenberg, A., et al. (2008). Grey and white matter distribution in very preterm adolescents mediates neurodevelopmental outcome. *Brain* 131 (Pt 1), 205–217. doi: 10.1093/brain/awm282

R Core Development Team (2018). *R: A Language, and Environment for Statistical Computing*. The R Foundation for Statistical Computing.

Robinson, S. (2012). Neonatal posthemorrhagic hydrocephalus from prematurity: pathophysiology and current treatment concepts. *J. Neurosurg. Pediatr.* 9, 242–258. doi: 10.3171/2011.12.PEDS11136

Roze, E., Van Braeckel, K. N., van der Veere, C. N., Maathuis, C. G., Martijn, A., and Bos, A. F. (2009). Functional outcome at school age of preterm infants with periventricular hemorrhagic infarction. *Pediatrics* 123, 1493–1500. doi: 10.1542/peds.2008-1919

Sondhi, V., Gupta, G., Gupta, P. K., Patnaik, S. K., and Tshering, K. (2008). Establishment of nomograms and reference ranges for intra-cranial ventricular dimensions and ventriculo-hemispheric ratio in newborns by ultrasonography. *Acta Paediatr.* 97, 738–744. doi: 10.1111/j.1651-2227.2008.00765.x

Spittle, A. J., and Orton, J. (2014). Cerebral palsy and developmental coordination disorder in children born preterm. *Semin. Fetal Neonatal Med.* 19, 84–89. doi: 10.1016/j.siny.2013.11.005

Spittle, A. J., Walsh, J., Olsen, J. E., McInnes, E., Eeles, A. L., Brown, N. C., et al. (2016). Neurobehaviour and neurological development in the first month after birth for infants born between 32-42 weeks' gestation. *Early Hum. Dev.* 96, 7–14. doi: 10.1016/j.earlhumdev.2016.02.006

Symington, A., and Pinelli, J. (2006). Developmental care for promoting development and preventing morbidity in preterm infants. *Cochr. Datab. Syst. Rev.* CD001814. doi: 10.1002/14651858.CD001814.pub2

Tronick, E. Z., Olson, K., Rosenberg, R., Bohne, L., Lu, J., and Lester, B. M. (2004). Normative neurobehavioral performance of healthy infants on the neonatal intensive care unit network neurobehavioral scale. *Pediatrics* 113(3 Pt 2), 676–678.

Tsai, A. J., Lasky, R. E., John, S. D., Evans, P. W., and Kennedy, K. A. (2014). Predictors of neurodevelopmental outcomes in preterm infants with intraparenchymal hemorrhage. *J. Perinatol.* 34, 399–404. doi: 10.1038/jp.2014.21

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Dorner, Soares, Robinson, Allen, Perin and Burton. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 18 February 2019
doi: 10.3389/fphys.2019.00065

Measurement of Neurovascular Coupling in Neonates

*Dries Hendrikx[1,2], Anne Smits[3,4], Mario Lavanga[1,2], Ofelie De Wel[1,2], Liesbeth Thewissen[3,4], Katrien Jansen[3,4,5], Alexander Caicedo[6], Sabine Van Huffel[1,2] and Gunnar Naulaers[3,4]**

[1] Department of Electrical Engineering, KU Leuven, Leuven, Belgium, [2] imec, Leuven, Belgium, [3] Department of Development and Regeneration, KU Leuven, Leuven, Belgium, [4] Neonatal Intensive Care Unit, University Hospitals Leuven, Leuven, Belgium, [5] Child Neurology, University Hospitals Leuven, Leuven, Belgium, [6] Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia

Neurovascular coupling refers to the mechanism that links the transient neural activity to the subsequent change in cerebral blood flow, which is regulated by both chemical signals and mechanical effects. Recent studies suggest that neurovascular coupling in neonates and preterm born infants is different compared to adults. The hemodynamic response after a stimulus is later and less pronounced and the stimulus might even result in a negative (hypoxic) signal. In addition, studies both in animals and neonates confirm the presence of a short hypoxic period after a stimulus in preterm infants. In clinical practice, different methodologies exist to study neurovascular coupling. The combination of functional magnetic resonance imaging or functional near-infrared spectroscopy (brain hemodynamics) with EEG (brain function) is most commonly used in neonates. Especially near-infrared spectroscopy is of interest, since it is a non-invasive method that can be integrated easily in clinical care and is able to provide results concerning longer periods of time. Therefore, near-infrared spectroscopy can be used to develop a continuous non-invasive measurement system, that could be used to study neonates in different clinical settings, or neonates with different pathologies. The main challenge for the development of a continuous marker for neurovascular coupling is how the coupling between the signals can be described. In practice, a wide range of signal interaction measures exist. Moreover, biomedical signals often operate on different time scales. In a more general setting, other variables also have to be taken into account, such as oxygen saturation, carbon dioxide and blood pressure in order to describe neurovascular coupling in a concise manner. Recently, new mathematical techniques were developed to give an answer to these questions. This review discusses these recent developments.

Keywords: neurovascular coupling, neonates, cerebral blood flow, EEG, NIRS, graph theory

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Rathinaswamy Bhavanandhan Govindan,
Children's National Health System,
United States
Rebecca Maree Dyson,
University of Otago, New Zealand

***Correspondence:**
Gunnar Naulaers
gunnar.naulaers@uzleuven.be

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *14 October 2018*
Accepted: *21 January 2019*
Published: *18 February 2019*

Citation:
Hendrikx D, Smits A, Lavanga M, De Wel O, Thewissen L, Jansen K, Caicedo A, Van Huffel S and Naulaers G (2019) Measurement of Neurovascular Coupling in Neonates. Front. Physiol. 10:65. doi: 10.3389/fphys.2019.00065

INTRODUCTION

Neurovascular coupling refers to the regulation mechanism that links the transient neural activity to the subsequent change in cerebral blood flow. The first reports on this regulation mechanism date from more than 100 years ago (Donders, 1851). Neurovascular coupling can be studied from different points of view, including a macroscopic and microscopic perspective. At the macroscopic

level, the extremely high vascularization and tight regulation of the cerebral blood flow provides the brain with adequate blood flow for a given metabolic demand. There is a close temporal and regional link between neuronal activity and cerebral blood flow, brain regions with high activity receive an increased amount of blood flow. At the microscopic level, the neurovascular unit is comprised of the vascular smooth muscle, the neuron and the astrocyte glial cell. Glutamate is released upon neuronal activation, which causes both neurons and astrocytes to transmit signals in order to regulate cerebral blood flow. Although it was assumed for a long time that these signals were only associated with a vasodilatory effect, recent studies show a more complex balance of vasodilation and vasoconstriction, in which both chemical and mechanical effects play an important role. Known chemical signals include prostaglandins, nitric oxide and adenosine, secreted by both neurons and astrocytes, which cause a vasodilation of the smooth muscle cell (**Figure 1**). In addition, astrocytes also secrete other vasodilators like potassium and epoxyeicosatrienoic acids. Besides these vasodilators, astrocytes are also known to secrete arachidonic acid, which has a vasoconstrictive effect. All these mediators have a direct impact on the smooth muscle of the arterioles and therefore control cerebral blood flow in a direct way. In addition to these mechanisms present in the neurovascular unit, a second mechanism was recently described by which also pericytes around the capillaries cause vasodilation, probably also playing a role in the local flow distribution. For further description of these mechanisms, we refer to recent reviews (Huneau et al., 2015; Phillips et al., 2016). As a concluding remark, it is also important to mention that most of the studies on neurovascular coupling are done in the adult population; currently, we do not know whether these mechanisms are present in preterm neonates, and if they work in the same way as described in adults.

In general, there are two main categories of studies to describe and investigate neurovascular coupling. First of all, there are *spatiotemporal* studies, which link changes in local blood flow to an artificially applied stimulus. Second, *general* studies are available that investigate resting-state neurovascular coupling using spontaneous electrical activity of the brain.

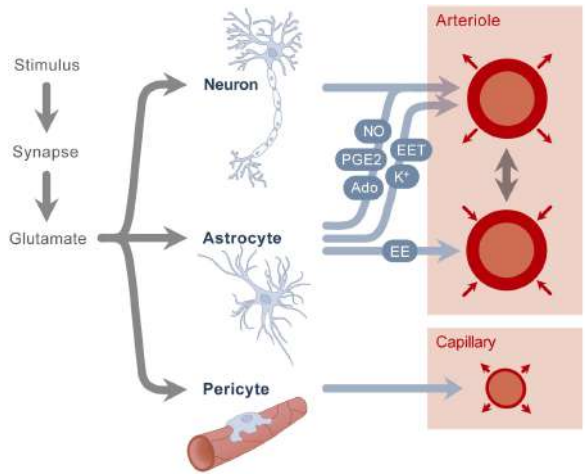


FIGURE 1 | A stimulus causes the secretion of glutamate. Glutamate stimulates neurons and astrocytes, which results in the secretion of nitric oxide (NO), potassium (K^+), adenosine (Ado), epoxyeicosatrienoic acids (EET) and prostaglandins (PGE2), which in turn results in arteriolar vasodilation. Astrocytes also secrete arachidonic acid (AA), which causes vasoconstriction. A second mechanism is the stimulation of pericytes, resulting in capillary vasodilation.

SPATIOTEMPORAL STUDIES

In order to test neurovascular coupling hypotheses in adults, spatiotemporal studies are standard of practice. In such studies, a standardized motor, visual, auditive, or cognitive task is given and simultaneously, the flow and/or activity in the brain is measured. There are different recommendations regarding optimal stimulus and assessment methods for adult research (Huneau et al., 2015). The most widely used stimulus in adults is finger tapping, which is known to cause an increase in blood flow at the contralateral motor cortex (Dettmers et al., 1996). In (preterm) neonates, spatiotemporal studies are also of interest, but they are inherently more difficult to perform. The majority the spatiotemporal studies in neonates makes use of visual stimuli, which includes the projection of checkerboard patterns on LCD displays (Meek et al., 1998; Liao et al., 2010), or flashing LEDs (Karen et al., 2008). The latter has the advantage that the neonates can be kept asleep throughout the measurements. Other less frequently used stimuli in preterm neonates include auditory (Sakatani et al., 1999; Zaramella et al., 2001) and somatosensory stimuli (Erberich et al., 2006; Arichi et al., 2010, 2012). To the best of our knowledge, guidelines regarding optimal stimulus selection for newborns are not available.

The classical response to the stimulus described in adults is a sudden increase in cerebral blood flow and cerebral oxygenation with a secondary, less pronounced decrease (Heekeren et al., 1997). This type of response is typically called 'functional hyperemia,' and is generally referred to as a *positive* response. A *negative* response, on the other hand, occurs when the increase in blood flow to the brain is insufficient to meet the metabolic demand. In this setting, however, the relation of functional hyperemia to neuronal metabolisms is unclear (Hillman, 2014). Since functional hyperemia is observed in hyperoxic, hypoglycemic and hyperglycemic states, it is clear that the blood flow increases are not simply triggered by local sensing of depleted nutrients (Powers et al., 1996; Wolf et al., 1997; Lindauer et al., 2010). The relative delay in the peak of increased blood flow further confirms that neurons do not rely upon functional hyperemia to meet their initial needs for increased oxygen and glucose, since neuronal firing may have ended prior to measurable changes in blood flow (Hillman, 2014).

In practice, different techniques are used to test neurovascular coupling in humans. In *spatiotemporal* studies, functional magnetic resonance imaging (fMRI) and functional near-infrared spectroscopy (fNIRS) are the most frequently used methods. The fMRI studies investigate the response of the blood oxygen level dependent (BOLD) signal. According to the definition,

fMRI and fNIRS studies only measure blood flow changes and thus not neural activity. However, since the change in blood flow occurs mainly at the part of the brain responsible for the imposed activity (verbal, motor, visual), the measured response can be a good surrogate measurement for neurovascular coupling. In addition to the sign of the underlying mechanism (*positive* versus *negative* response), fMRI studies are also used to determine the location of brain activity after a specific stimulus. In healthy humans, cognitive, verbal, and motor tasks will on average lead to a 10–20% increase in cerebral blood flow in the posterior cerebral artery and a 5–8% increase in the middle cerebral artery (Huneau et al., 2015). In fNIRS studies, a positive response is comprised of an increase in oxyhemoglobin and total hemoglobin, together with a slight decrease in deoxygenated hemoglobin, which overall leads to an increase in tissue oxygenation. In adults, impaired neurovascular coupling has been described in different pathological conditions like stroke, hypertension, hypotension, autonomic dysfunction, Alzheimer's disease, diabetes, and traumatic brain injury (Duschek and Schandry, 2004; Girouard and Iadecola, 2006; Azevedo et al., 2011; Vetri et al., 2012; Phillips et al., 2014; Jang et al., 2017). In addition, also smoking and healthy aging are associated with a negative effect on neurovascular coupling (Boms et al., 2010; Tarantini et al., 2017; Nowak-Fluck et al., 2018).

Besides the use of fMRI and fNIRS, other techniques can also be used to carry out spatiotemporal studies. These techniques are however less frequently used – and, to the best of our knowledge – not yet employed in studies on neurovascular coupling in preterm neonates. fMRI BOLD imaging depends on specific coupling relations between cerebral metabolic rate of oxygen, cerebral blood flow and cerebral blood volume, which are not unambiguously described in preterm neonates to date (Muramoto et al., 2002; Kusaka et al., 2004). Furthermore, these relations are altered in case of brain injury (Lin et al., 2016). Perfusion-MRI techniques, such as arterial spin labeling (ASL) MRI, can be used to overcome these limits (Tortora et al., 2017). In addition, oxygen based techniques such as BOLD fMRI and fNIRS only perform indirect measures of functional hyperemia (Hyder, 2009). More precise, direct measurements of cerebral blood flow can be obtained using laser Doppler flowmetry (Dyson et al., 2014) or laser speckle imaging (Farraro et al., 2016). A disadvantage of these techniques is the sensitivity of the probe, which results in signals that are prone to artifacts, and which impedes continuous measurements – typically, laser imaging measurements are taken at discrete points in time. A new promising technique is functional ultrasound imaging, where fast ultrasound sonography is coupled with EEG (Demene et al., 2017).

Recent studies suggest that neurovascular coupling in neonates and preterm born infants can differ compared to adults. This is typically attributed to the fact that many of the components involved in actuating and propagating the hemodynamic response are still in further development, including perivascular cells such as astrocytes and pericytes (Kozberg and Hillman, 2016a). Neural and vascular networks develop, expand and are then selectively pruned over the first year of human life. In addition, the metabolic demands of the newborn brain are still evolving and are vastly different compared to the adult brain. On the first day after birth, very low values of brain oxygenation are observed, in combination with high values of cerebral oxygen extraction (Brew et al., 2014). Brain oxygenation increases during the first days of life, while cerebral oxygen extraction decreases. In extremely preterm infants, there is no correlation between cerebral blood flow and spontaneous changes in the cerebral metabolic rate of oxygen during the first 2 days after birth (Kissack et al., 2005; Wong et al., 2009). Instead, changes in cerebral oxygen extraction rather than cerebral blood flow meet changes in oxygen requirements arising from variations of the cerebral metabolic rate of oxygen. The vast differences in both neural and vascular network structure, as well as substantially different metabolic needs of the preterm brain are highly likely to affect early postnatal neurovascular coupling.

The differences in neurovascular coupling hypotheses in neonates versus adults are confirmed in fMRI studies, which are discussed in this paragraph, and in fNIRS studies, which are summarized in the next paragraph. Heep et al. (2009) described a negative response in preterm infants after stimulation, which was also confirmed by others (Yamada et al., 1997; Born et al., 1998; Erberich et al., 2006). Arichi et al. (2012) found a positive BOLD response in adults, term infants and preterm infants. Furthermore, they identified a systematic maturational trend in terms of decreasing time-to-peak and increasing positive peak amplitude. These findings suggest that in young infants the increase in cerebral oxygen consumption may be relatively greater than the corresponding increase in cerebral blood flow during functional activation. The age-dependency of the neurovascular coupling was also confirmed in a rodent model by Kozberg et al. (2013). The development of the neurovascular coupling alongside neuronal development in the postnatal brain suggests that in the developmental period the brain may be experiencing differences in energy supply and demand dynamics compared to the adult brain (Kozberg and Hillman, 2016a).

Another approach to study the neurovascular coupling is by means of fNIRS. Again, the results and conclusions of the different studies available in literature are not unambiguous. Liao et al. (2010) described a normal hyperemic signal using NIRS in the visual cortex after visual stimulation in a cohort of term neonates in the first weeks of life. Furthermore, no simultaneous reaction was observed at the motor cortex, indicating that the observed response was a local reaction. On the other hand, however, an increase in brain deoxygenation was observed in awake infants in another study (Meek et al., 1998). Verriotis et al. (2016) described the response on noxious (heel lance prick) and innoxious stimuli (tactile cutaneous stimulation) in 30 term infants. In general, noxious stimulation was found to elicit a more pronounced hemodynamic response than innoxious stimulation. However, the hemodynamic pattern after stimulation was observed to be characterized by pronounced inter-patient differences, which might suggest that increased oxygen consumption does not always lead to regional overperfusion, likely due to the immature vascular regulation or to greater metabolic demands of neurotransmission in unmyelinated white matter.

In addition to the fNIRS studies on neonates, other authors have used this technique on animals. Nakamura et al. (2017) studied the neurovascular coupling in newborn lambs. They found subjects with a positive response, i.e., an increase in oxygenated hemoglobin, but also subjects with a negative response after a motor stimulation. Furthermore, blood pressure at the start of the investigation did not seem to have any influence, but the negative response became positive in hypercapnia (Nakamura et al., 2017). Kozberg et al. (2016) studied the neurovascular coupling in rats of different ages. They described a less positive response in rats of postnatal age P10–P13, and even a negative response in rats of postnatal age P7–P8. In order to compare these results to the human population, it is common to assume that the newborn full-term and 1 year old human brain are developmentally equivalent to the postnatal days 7 and 14 in rats, respectively, based on the codevelopment of factors such as eye opening and myelination (Quinn, 2005).

In addition to the uncertainty regarding the sign of the hemodynamic response (*positive* versus *negative*) in neonates, more questions arise when we focus on specific pathologic conditions like bronchopulmonary dysplasia, congenital heart disease and pulmonary hypertension, where a lower oxygen saturation is present (Rosengarten et al., 2006). Furthermore, the effect of anesthetic and sedative drugs, which are frequently used in fMRI studies, needs to be investigated (Franceschini et al., 2010). From a broader perspective, also the effect of other drugs, such as anti-epileptic drugs on neurovascular coupling in neonates is not properly defined to date (Schwartz, 2007). Since an elaborate discussion on these topics is out of the scope of this text, we will not go into further detail.

It is important to mention that studies both in animals and neonates confirm the presence of a short hypoxic period in the brain after a stimulus in preterm infants. These short hypoxic moments are hypothesized to cause an increase in vascular endothelial growth factor and oligodendrocyte-encoded hypoxia-inducible factor function, resulting in new angiogenesis and neurogenesis in the brain (Kozberg and Hillman, 2016b). This hypothesis could explain the mechanisms of the positive effect of sensitive stimulation and the negative effect of overstimulation in newborns (Kozberg and Hillman, 2016a).

Despite the fact that the fMRI and fNIRS studies listed above aim to investigate neurovascular coupling, they actually only describe the hemodynamic changes observed in the brain after applying a stimulus. These studies can be used to describe these hemodynamic changes after a particular stimulus and how these hemodynamic patterns change with age, from preterm neonates to adults. Precise studies on neurovascular coupling are based on concomitant measurements of brain hemodynamics and brain function (electrical activity of the brain). This can be done by combining EEG measurements with fMRI measurements (Mullinger and Bowtell, 2011), with fNIRS measurements (Shin et al., 2018) or with positron emission tomography measurements (Juhász et al., 2000), although such studies are challenging from a technical point of view due to for example electrode heating (Vanhatalo et al., 2014) and the presence of numerous artifacts (Galderisi et al., 2016; Abrue et al., 2018). Therefore, such studies are very scarce, especially in (preterm) neonates. Singh et al. (2014) explored the use of concomitant multichannel EEG-fNIRS measurements during epileptic seizures in neonates and found a hemodynamic response to seizure activity which consists of an initial increase in cortical blood volume prior to a large and extended decrease typically lasting several minutes.

GENERAL STUDIES

Another approach to investigate the neurovascular coupling is to study the relation between the general metabolism of the brain and cerebral blood flow. Obviously, EEG is the most commonly used non-invasive method to assess the electrical activity of the brain, although it remains limited to the cortical activity. Cerebral blood flow can be measured at the bedside in a non-invasive way using transcranial Doppler ultrasound or near-infrared spectroscopy (NIRS). Therefore, these two modalities are generally used in *general* studies on neurovascular coupling. Both technologies are safe, relatively cheap and easy to use, however, ultrasound imaging has the disadvantage of being investigator dependent.

Roche-Labarbe et al. (2007) used NIRS in combination with EEG and found a decrease in oxygenation during bursts (high EEG activity in very preterm infants), followed by an important overflow (increase in cerebral oxygenation), suggesting a neurovascular signal that differs from the pattern commonly observed in adults. Tataranno et al. (2015) investigated functional brain activity and found an increase in oxygen extraction in preterm infants with increased electro-cerebral activity. In another study, Mahmoudzadeh et al. (2018) compared the neurovascular coupling in preterm neonates with versus without intraventricular hemorrhage. They reported that in neonates with brain injury the cerebral vascular network was unable to compensate for the increased metabolism resulting from neuronal activation in response to external stimulation. Pfurtscheller et al. (2008) investigated the behavior of the heart rate (HR) variability during the preterm EEG transients and found that EEG burst are associated with increases in HR. In addition, this positive variation of HR was found to disappear over age with the emergence of a continuous EEG trace.

The most recent advancements in signal processing allow an investigation of the neurovascular coupling in a non-invasive way focusing on "resting-state" conditions. In such a setting, the clinical goal is to develop a non-invasive and continuous measurement of the neurovascular coupling, which can be used as bedside monitoring without evoking a potential, i.e., inducing an artificial response via a stimulus. In the past decades, one of the most commonly studied neuronal couplings has been the scalp EEG connectivity, for which a large variety of methodologies has been developed (Pereda et al., 2005). In general, the methods can be classified in *functional methods* on the one hand, if only statistical correlations among the time series are taken into account, versus *effective methods* on the other hand, if the directionality of the coupling is considered as well (Friston, 2011). An overview of these methodologies is presented in **Table 1**.

Based on the EEG connectivity literature, further coupling methodologies have been developed to assess the interaction

TABLE 1 | Overview of the signal processing techniques used to assess neurovascular coupling by integrating NIRS and EEG measurements.

	Linear techniques	Non-linear techniques
Functional methods	► Time domain • Correlation • Time delay stability ► Frequency domain • Coherency ► Time-frequency domain • Wavelet coherency	► Information theory • Mutual information ► Dynamic time warping ► Phase locking value
Effective methods	► Transfer function ► Granger causality	► Information theory • Transfer entropy ► Evolutionary map approach (directionality index)

among signals with different origins or sources. These methodologies can consequently be used to assess regulation mechanisms like neurovascular coupling. Clinical examples on neurovascular coupling are the studies of Pfurtscheller et al. (2008) and Roche-Labarbe et al. (2008). Although these studies investigate the interaction of signals of different nature, these studies do not assess the concise nature of their interaction. The main difficulty to tackle in studies on multimodal integration is the fact that the different time series operate on different time scale, while simultaneously interacting with each other with a certain linear or non-linear degree.

In order to overcome this limitation, the new field of Network Physiology has the scope to describe how the time series of multiple origins interact, either linearly or non-linearly at a specific scale, in a graph or lattice structure (Bashan et al., 2012; Bartsch et al., 2015). Based on a Network Physiology framework, this review will explain new mathematical models to assess the neurovascular coupling, with a specific focus on:

(1) The necessary preprocessing steps that are required to match the temporal scales at which neuronal and vascular activities interact,
(2) The variety of methods to assess the coupling itself, which includes linear and non-linear approaches,
(3) The graph theory approach to describe the multiple simultaneous interactions and manage them in a statistically compact way.

Preprocessing Steps

In order to measure the neurovascular coupling in a correct and robust way, it is very important that the dynamics of the various signals are represented and matched in a careful way. In general, biomedical signals arise from a variety of sources, including movements, breathing, electrical activity of neurons (EEG) and optical absorption of light (NIRS), among others (Clemenson and Lancaster, 2016). Due to the different sources, biomedical time series are typically associated with different time scales of operation. An EEG signal, for example, changes rapidly due to the (de)synchronization of numerous neurons, while a NIRS signal only changes very slowly, due to the fact that the hemodynamic effects that it captures work on a slow time scale. When one wants to compute the interaction between two signals, it is important to define on which time scale the coupling is ought to be computed (the signals are interacting), and the signals have to be matched correspondingly. Although biological systems are extremely complex, it is possible to decompose their macroscopic behavior using mathematical techniques (**Table 1**).

One of the concepts that is frequently used is the notion of frequency, which gives an indication about the dynamics of a signal. By looking at the frequency content of a signal, we can make conclusions on how fast (slow) the signal changes over time. Information about the frequency content of a signal can be deduced from its frequency spectrum, which can be computed using a mathematical transformation (Challis and Kitney, 1991). Some signals change very rapidly, and are therefore mainly associated with high-frequency components. Other signals change very slowly; the frequency spectrum of such signal is mainly comprised of a very narrow band of low frequency components. Examples of the former and the latter include EEG and cerebral hemodynamics, measured by NIRS, respectively. In addition, one can also describe the biological oscillations at different time scales via a time-frequency representation. The extraction of the oscillatory components is not only useful to reduce the complexity of the system, but can also be used to describe the underlying physiology. In the world of neurovascular coupling, the brain heart interaction takes place at long-term scale (low frequency activity), since the NIRS reflects the cardiovascular oscillations which develop in a timeframe of seconds. The speed of fluctuations of NIRS is then much lower compared to EEG oscillations. In addition, however, a NIRS signal can also contain frequency components which are faster, which are caused interference from cardiac pulsations. These frequency components are typically not considered in EEG-NIRS neurovascular coupling studies.

In addition, the recent literature on EEG-NIRS integration also focuses on the low-frequency activity of the EEG, i.e., the delta oscillations. According to Knyazev, the delta oscillation belongs to the "old brain," which phylogenetically traces back to lizards (Knyazev, 2012). This explains why delta brushes dominate the preterm and the neonatal EEG activity. During the development, the scalp electrical activity tends to be discontinuous and is centered around a frequency between 1 and 2 Hz, which defines the periodicity of the bursts in the cortical trace (Andre et al., 2010). Consequently, delta oscillations are of primary importance in the preterm brain. An additional reason is the implication that delta oscillations regulate basic homeostatic needs, such as the blood flow circulation and normotension enforcement. The slow-wave EEG is considered as the expression of the regulation of the brainstem (which is in charge of cerebral hemodynamics) or, at least, as a projection of the subcortical activity to the cortical areas.

Finally, when comparing biomedical signals of different origin, it is very important that the time scales of the various signals are synchronized. In the setting of neurovascular coupling, this means that a rapidly changing neuronal signal (EEG) has to be matched with a very slowly varying cerebral hemodynamics signal (NIRS). In general, there are multiple approaches to overcome

this problem. However, it is common practice to use a running estimate of the power of the slow wave activity, i.e., the delta oscillations. There are mainly three methods to extract such a running measure from the EEG. The most commonly used methodology is the use of the wavelet transform and average the power in the frequency band of interest, as reported by Clemenson and Lancaster (2016). A second option is to extract the delta band from an estimate of the power spectral density that is computed in running windows (Faes et al., 2015). The last option, which is very simple, but also very effective, is the use of the running root mean square (RMS) value, where the window length is chosen large enough to cut off the high frequency components, as reported by Caicedo et al. (2016).

Methodologies to Assess Coupling

Despite the fact that research on bedside markers of neurovascular coupling is a very new domain, there are some studies available that have investigated how different signal processing techniques can be used to quantify the neurovascular coupling based on spontaneous cerebral and vascular activities. As mentioned before, these studies mainly focus on the quantification of interaction between EEG and NIRS as surrogates for brain function and brain hemodynamics, respectively.

In signal processing terms, this interaction can be regarded as a pairwise similarity, which denotes the degree to which a given time series resembles another one. Therefore, pairwise similarity measures can be used to evaluate a regulation mechanism such as neurovascular coupling. As mentioned before, the pairwise similarity belongs to the *functional connectivity* field, which focuses (only) on the statistical correlation among the investigated signals (**Table 1**).

The most commonly used linear similarity methods are the cross-correlation function (CCF) and magnitude squared coherence (MSC), which quantify the linear correlation between two signals at different lags or frequencies, respectively. In the world of neurovascular coupling, the former has been applied by Bari et al. (2011) to study the regulation mechanism in adults during a divided attention cognitive task. In summary, the CCF was able to reveal the presence of a cascade of responses, which was observed to be strongly influenced by the task performed by the patient. Govindan et al. (2016) used the MSC to obtain a measure for the degree of coupling between EEG and NIRS data in premature neonates and identified more signal interaction (higher MSC values) in infants without brain injury compared to other patients that died of brain injury during the course of the study.

A well-known extension of these linear methods is the wavelet coherence, which is a time-variant representation of the neurovascular coupling dynamics, as performed by Musizza et al. (2007) and Chalak et al. (2017). The main advantage of using a wavelet coherence approach is the fact that this technique is able to deal with the non-stationary nature of NIRS and EEG signals. Chalak et al. (2017) performed the time-frequency coherence analysis on the raw data of amplitude-integrated EEG and brain oxygenation signals, while Musizza applied the continuous wavelet transform to extract the oscillation of interest (with frequency below 0.05 Hz). In both studies, the authors emphasize the importance of phase coupling, which defines at which time delay the oscillations lock in.

A further development consists in the usage of the *effective connectivity*, which takes into account the directionality of the neurovascular coupling (**Table 1**). Caicedo et al. (2016) have applied the linear transfer entropy in neonates to quantify the directionality between NIRS signals and the RMS of the EEG (where the window length was chosen large enough to capture only the delta oscillations). In general, transfer entropy assesses whether the current transient of a signal can be explained by the past trajectory of another signal (Lee et al., 2012). More specifically, Caicedo et al. (2016) found that the cortical activity is caused by the hemodynamic and metabolic course of the brain. Remarkably, however, the directionality was found to be reversed when, in addition to the linear interactions, also the non-linear interactions were taken into account (Hendrikx et al., 2018a).

Another mathematical technique to study directionality is the use of parametric transfer function models, as applied by Talukdar et al. (2015). In this study, multiple sets of gamma transfer functions were used. Using such sets, the authors were able to predict NIRS hemodynamics from EEG spectral envelopes, indicating that the gamma transfer function approach can be used to study neurovascular coupling. In addition, the resulting parameters of the model could provide additional insights into the neurovascular coupling mechanism.

To the best of our knowledge, there are currently no studies available that have investigated non-linear signal processing techniques, except for the preliminary results reported by Hendrikx et al. (2018a). In addition to the methods discussed in this section, other methodologies can be considered for the computation of neurovascular coupling markers, based on studies of the brain heart connectivity literature:

(1) The first option is to assess the interaction delays among signals via the cross-correlation landscape [time delay stability (TDS)].
(2) One can extend the transfer entropy to a model-free approach, in order to capture any possible type of interaction, i.e., linear and non-linear interactions.
(3) The combination of the previous two points can be regarded as a general type of phase coupling, which looks only at the phase of the signal and their non-linear interaction.

Graph Theory

Including More Signal Modalities in the Assessment of Neurovascular Coupling

In the previous section, several techniques were introduced to compute parameters for neurovascular coupling, based on multimodal processing of NIRS and EEG signals. From a broader perspective, however, there are in fact multiple regulation mechanisms that are working simultaneously in order to provide and maintain an adequate brain perfusion (Banaji et al., 2005). On a very general level, two major regulation mechanisms can be distinguished in addition to the neurovascular coupling: cerebral autoregulation and cerebral oxygen balance (Smits et al., 2017).

The former ensures that cerebral blood flow is kept more or less constant, despite variations in cerebral perfusion pressure, while the latter determines the relation between oxygen delivery and metabolic demand of the brain.

A concise study on brain perfusion thus has to take into account that multiple regulation mechanisms are working at the same time. Therefore, such an analysis requires the incorporation of other signal modalities besides EEG and NIRS, such as arterial blood pressure (ABP) and fractional tissue oxygen extraction (FTOE). The former is needed to compute a marker for cerebral autoregulation, while the latter can be used to get insight into the cerebral oxygen balance (Naulaers et al., 2007). A simultaneous analysis of EEG, NIRS, ABP, and FTOE measurements leads to the definition of three markers, each indicating and assessing the status of one of the regulation mechanisms that are essential in providing the brain with a proper amount of energy in order to support its function.

Once all of these signals are available, recent studies indicate that it is also useful to compare the interaction between the remaining pairs of signals. Indeed, Semenova et al. (2017) compared the dynamics of EEG and ABP signals in preterm neonates and found that a higher degree of EEG-ABP interaction is associated with a worse clinical outcome, assessed using clinical risk index for babies II scores. Furthermore, if we add other signals like HR, which is known to have a key influence on cerebral hemodynamics in preterm infants, simultaneous markers on HR passivity can be obtained (Mitra et al., 2014). Such markers were found to be of diagnostic value in identifying impaired cerebrovascular reactivity, leading to adverse clinical outcome. In summary, it is important to incorporate a wide variety of signal modalities in order to enable an adequate study on brain perfusion, since the pairwise interactions between the different modalities can be used as physiological markers.

One of the first studies that investigated the simultaneous interactions in a multimodal dataset, is a study by Pfurtscheller et al. (2008). More specifically, in this study, NIRS, EEG, ABP, respiration and HR were combined with cross spectral and sliding cross correlation calculations, which are both linear, functional methods (**Table 1**). Results indicate that slow precentral (de)oxyhemoglobin concentration oscillations during awake rest can be temporarily coupled with EEG fluctuations in sensorimotor areas and modulate the excitability level in the brains' motor areas, respectively. Therefore, this study provides support for the idea that resting state networks fluctuate with frequencies between 0.01 and 0.1 Hz (Mantini et al., 2007).

Graph Theory for Functional Connectivity

The main limitation of the methodology outlined above is the combinatorial number of couplings based on the variables involved. If one starts with five variables (e.g., HR, ABP, SpO_2, NIRS, and EEG), one gets 10 couples of interaction. The complexity of the interaction problem thus increases exponentially, which is difficult to manage statistically (Pavlopoulos et al., 2011). Furthermore, it is often of interest to study the interaction among systems, rather than the interaction between single signals (e.g., the interaction between the cerebral system and the cardiovascular system). A possible solution is the description of the couplings among variables as a graph of a network, whose activity can be compactly represented using graph theory.

Formally, a graph is defined by a non-zero number of nodes (vertices) and a number of links (edges) between these nodes. In general, graphs can be used to describe a great variety of real-world situations, which explains the extensive use of these mathematical objects in many different fields. A good introduction on graph theory mathematics and an overview of the numerous applications of graph theory is presented in Pavlopoulos et al. (2011). Using graph structures has some advantages:

(1) It is a straightforward methodology to deal with the exponentially increasing number of interaction pairs when adding signals to the analysis.
(2) It allows for a clear and visual representation.
(3) It allows to study clusters of signals in a straightforward way. This is especially useful when the interaction between different subsystems is of interest, rather than the interaction between the signals themselves.
(4) The behavior of the network as a whole can easily be studied, using the extensive methods on connectivity that are available in literature.

Despite the fact that graph theory was only recently introduced in the field of Network Physiology, it has been used extensively in studies on functional connectivity of the brain, as outlined by Fallani et al. (2010). Naturally, the brain is modeled by a graph in these studies, which allows to study the interactions between different brain regions. Depending on the number of nodes, it can even be possible to study the interactions in one particular brain region. However, in such case, the number of nodes has to be large enough, which is mainly limited by the data at hand, i.e., the number of channels in the multichannel EEG recording. In general, studies on functional connectivity of the brain build graph models using one signal or imaging modality, mostly EEG (Hata et al., 2016; Tokariev et al., 2016) or fMRI (Wang et al., 2010; Smitha et al., 2017).

Gao et al. (2011) constructed graph models from fMRI BOLD data recorded in a large cohort of normal pediatric subjects. Using sequential measurements, they observed a remarkable improvement in whole brain wiring from 3 weeks up to 2 years after birth, which is a critical time period for brain development. Lavanga et al. (2018) investigated the use of graph models constructed from multichannel EEG measurements in order to construct a brain-age model for preterm neonates (**Figure 3**). Using 8-channel EEG measurements, they observed a decrease in functional connectivity with post-menstrual age (PMA). In addition, the functional connectivity was found to be an accurate predictor for the age of neonates.

Graph Theory in Network Physiology

In the field of Network Physiology, graph models are constructed from a multimodal dataset (Bashan et al., 2012; Bartsch et al., 2015). Before constructing such graphs, one has to make sure that the time scales of the signals are synchronized, as

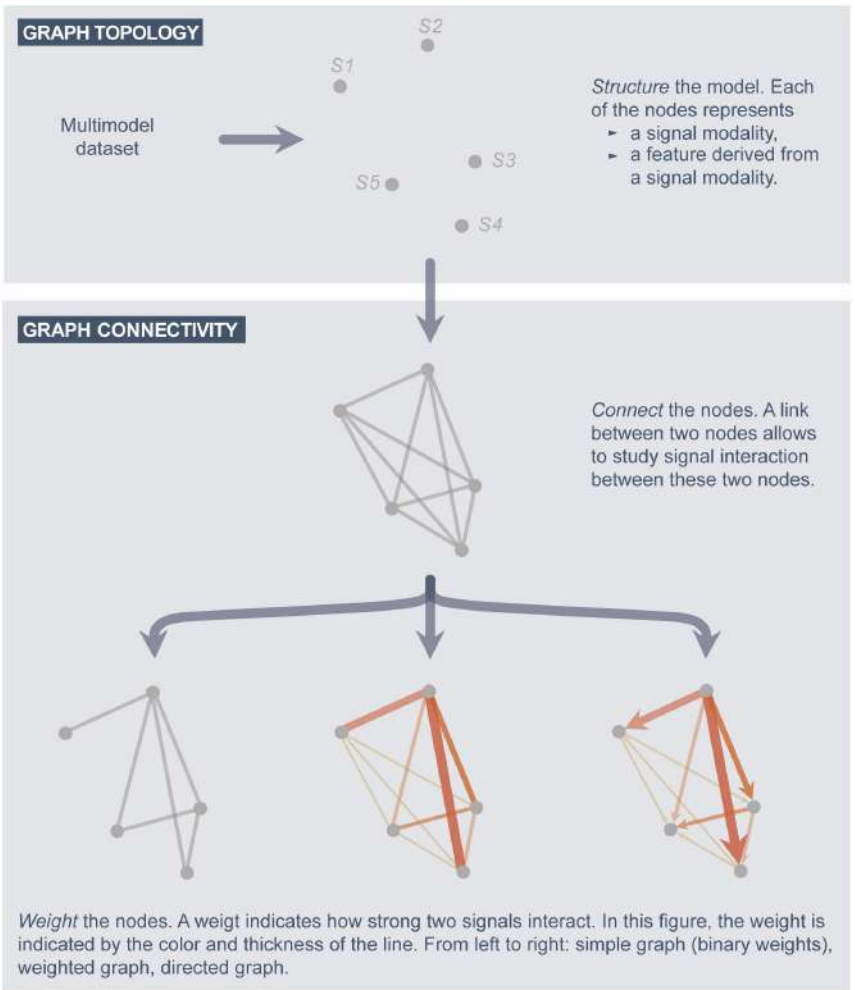


FIGURE 2 | A systematic approach to construct graph models.

explained in subsection G1. Afterward, a signal interaction graph is constructed as follows (**Figure 2**).

Graph topology

The structure of the graph is defined by the nodes. In a signal interaction graph, a node corresponds to one particular signal modality of the analysis, or a feature extracted from a signal (e.g., the power in the delta frequency band of the EEG).

Graph connectivity

(a) Define a link between each two nodes for which the signal coupling is of interest.
(b) Associate each link with a weight value, which indicates the strength of the signal interaction (coupling) and/or the directionality.

Regarding the *graph topology*, there is an endless amount of possibilities to define the structure of signal interaction graphs. Some networks to model physiological function can be quite simple (Bashan et al., 2012), while other networks are more detailed, and thus, more complex (Bartsch et al., 2015). To illustrate the variety of applications in the field of biomedical signal processing, some examples are presented in **Figure 3**, based on studies by Hendrikx et al. (2018b) and Lavanga et al. (2018). For each application, a graph with a specific topology can be constructed, based on the research question(s) of the analysis. The structure of the graph is limited by the signal modalities available in the dataset and/or the features derived from these signals.

Regarding the *graph connectivity*, there is again a wide variety of design choices on how to connect the nodes and how to define the weights associated with the links. First of all, one has to define which nodes are connected and which nodes lack a connection. Second, one has to specify how the weights associated with the links are computed. This is equivalent to the choice of one particular signal processing technique, since the weights denote signal interaction (coupling). Which signal processing technique to use has to be defined by the researcher, based on the characteristics of the research. In particular, one has to think about the nature of the interactions that needs to be captured: *linear* versus *non-linear* interactions and similarity (*functional*

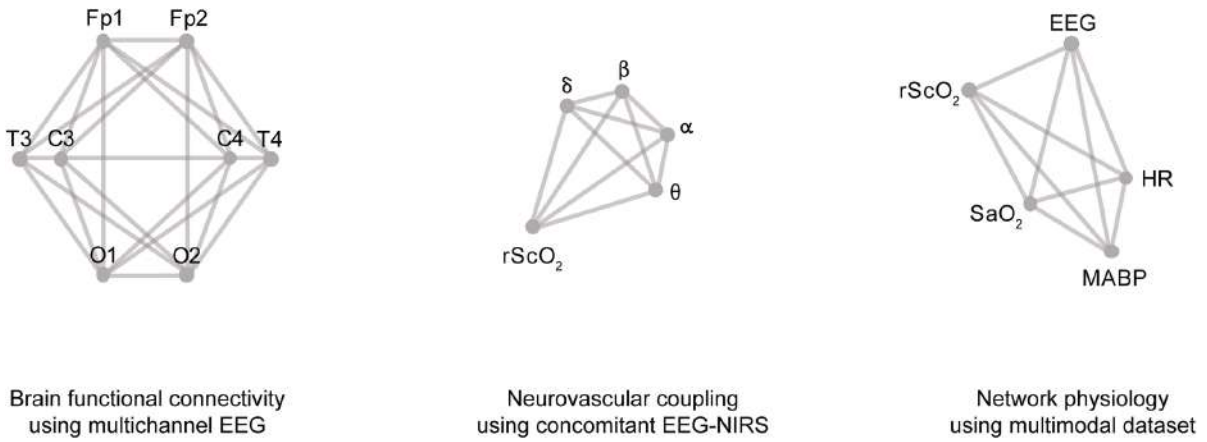


FIGURE 3 | Examples of graph models in biomedical data processing, from left to right: a graph model used for functional connectivity using 8-channel EEG; a graph model to quantify neurovascular coupling by incorporating different frequency bands of the EEG and a NIRS signal; and a graph model to study network physiology, which includes five different signal modalities.

methods) versus directionality (*effective* methods). The most commonly used signal processing techniques are summarized in **Table 1**. In the setting of graph connectivity, two additional remarks need to be taken into account.

As a first remark, it is important to note that the graphs depicted in **Figure 3** are characterized by the presence of a link between every pair of nodes. Formally, this type of graph is referred to as a *complete* graph. Such graphs are the most general type of graphs, since they allow to investigate the interaction between every possible pair of signals. It is important to note that in general it is always a good strategy to consider a link between every pair of signals, even when the signals are not expected to interact. Indeed, the weights of such links could be used as a validation for signal processing technique used to define the signal interaction: can the method detect that these signals are not interacting?

As a second remark, it is also possible to determine the presence (or absence) of a link automatically. In such an analysis, the weights are generally chosen binary. Graphs which only include the presence and absence of links (and no specific weight value), are formally referred to as *simple* graphs. An example: one could assign a '1' (presence of a link) if there is a significant correlation between two signals, while a '0' (absence of a link) denotes the lack of a significant correlation. In this setting, it is thus not necessary to manually define the presence of the links, since this is automatically detected by the signal processing technique.

The first study on the use of graph models in the field of Network Physiology is a study by Bashan et al. (2012). This study demonstrated that each physiological state is characterized by a specific network structure, demonstrating a robust interplay between network topology and function. Furthermore, high network flexibility in response to perturbations was observed, since the network was observed to be able to reorganize on time scales of a few minutes. A follow-up study on graph models in Network Physiology, is a study by Bartsch et al. (2015), who used the TDS framework in order to define the strength of the signal interactions. In short, TDS is based on linear correlations and is therefore only able of capturing linear signal interactions. The results of this study again demonstrated a direct association between network topology and physiologic function. In addition, the graph models were found to be useful in understanding how health and distinct physiologic states emerge from networked interactions among non-linear multi-component complex systems.

A second study on graph models in the field of Network Physiology is a study by our own group (Hendrikx et al., 2018b). In this study, graph models were used to investigate the interaction between cerebral hemodynamics, brain function and systemic variables. The graph models were constructed using the RBF kernel function, which is a non-linear similarity measure. Therefore, the methodological framework is able to capture non-linear signal interactions. The study showed that graph theory can be used to capture changes in signal interaction accurately and that the resulting graph models can be used to study the difference between distinct physiological states (Hendrikx et al., 2018b).

Graph models do not only provide a compact, efficient, and statistically well-defined framework to study simultaneous signal interaction, but they are in fact an entirely new way of thinking, which has been shown to produce an added value in multiple studies already (Bashan et al., 2012; Bartsch et al., 2015; Hendrikx et al., 2018b). The usage of interaction and coupling techniques provides an in-depth view of the interaction among physiological systems, moving from sectorial or univariate medicine to a more holistic approach. The latter is well-suited in case of continuous monitoring of critical states (e.g., in cases where it's likely to have multiple organ failures), and the well-being and mental status of a person, which is known to rely on the interaction between multiple systems (Chaparro-Vargas et al., 2016). Moreover, the necessity to improve the sleep quality and the developmental and cognitive abilities of neonates in the near future (which interact differently in neonates compared to adults) will require the

summarized view of a graph approach instead of long recordings of multiple apparently unrelated variables. In this perspective, Network Physiology gives a broader view on the body activity (Bashan et al., 2012), comparable to the broader view on the cortical status given by the brain connectivity at its discovery (Friston, 1994).

In general, the overall purpose of the signal processing techniques (such as computational graph models) is to obtain physiological markers starting from raw measurements that are routinely obtained in neonatal intensive care units. These physiological markers could in turn be used in clinical practice to make neonatal intensive care more patient-specific. In addition, signal processing advancements are also of high importance in neonatal pharmacological studies, since the effect of medication on the neurovascular coupling in neonates is yet unclear (Smits et al., 2017). Neonates are exposed to a large number of medications, most of which are used off-label in infants because clinical trials for safety, dosing, and efficacy of drugs are lacking in this population (Hsieh et al., 2014; Ku and Smith, 2015). In this setting, signal processing techniques could thus be used to identify neonates at risk for neurodevelopmental disabilities and could assist clinicians in making a timely diagnosis, which enables to start appropriate personalized therapies early. Therefore, this patient-centered neonatal intensive care can help to reduce the occurrence of neurodevelopmental disabilities in the preterm population (Molteno et al., 1999; Cioni et al., 2016).

CONCLUSION

We suggest that further research in the field of physiology is required in order to gain more insight into the exact working principle of the neurovascular coupling in neonates (*spatiotemporal* studies). In addition, further studies on the use of signal processing in EEG-NIRS integration are also required (*general* studies). Such studies enable to construct new software, based on algorithms that describe the relation between cerebral metabolism and oxygen delivery. Graph models in particular can be of special interest in future studies, since these models allow to study the simultaneous action of different regulation mechanisms, which is essential in studies on brain perfusion. These new measurements will provide a better understanding of the coupling mechanisms in the neonatal brain, and will eventually lead to an improvement of the neonatal intensive care in general.

AUTHOR CONTRIBUTIONS

DH, ML, and GN wrote the review together. AS, ODW, LT, KJ, AC, and SVH reviewed and corrected the text.

FUNDING

The authors acknowledge the financial support of Bijzonder Onderzoeksfonds KU Leuven (BOF): SPARKLE – Sensor-based Platform for the Accurate and Remote monitoring of Kinematics Linked to E-health #: IDO-13-0358, The effect of perinatal stress on the later outcome in preterm babies #: C24/15/036, and TARGID – Development of a novel diagnostic medical device to assess gastric motility #: C32-16-00364; Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (FWO): Hercules Foundation (AKUL 043) 'Flanders BCI Lab - High-End, Modular EEG Equipment for Brain Computer Interfacing'; Agentschap Innoveren en Ondernemen (VLAIO): 150466: OSA+, and O&O HBC 2016 0184 eWatch; imec funds 2017; imec ICON projects: ICON HBC.2016.0167, 'SeizeIT'; Belgian Foreign Affairs-Development Cooperation: VLIR UOS programs (2013–2019); EU: European Union's Seventh Framework Program (FP7/2007–2013) The HIP Trial: #260777, EU H2020 FETOPEN 'AMPHORA' #766456, EU EFRO Interreg: Nano4Sports, EU H2020 MSCA-ITN-2018: 'INtegrating Magnetic Resonance SPectroscopy and Multimodal Imaging for Research and Education in MEDicine (INSPiRE-MED)', funded by the European Commission under Grant Agreement #813120, and EU H2020 MSCA-ITN-2018: 'INtegrating Functional Assessment measures for Neonatal Safeguard (INFANS)', funded by the European Commission under Grant Agreement #813483. ERASMUS+: INGDIVS 2016-1-SE01-KA203-022114; European Research Council: The research leading to these results has received funding from the European Research Council under the European Union's Seventh Framework Program (FP7/2007–2013)/ERC Advanced Grant: BIOTENSORS (n° 339804). This paper reflects only the authors' views and the Union is not liable for any use that may be made of the contained information. Dries Hendrikx and Mario Lavanga are SB Ph.D. fellows at Fonds voor Wetenschappelijk Onderzoek (FWO), Vlaanderen, supported by the Flemish government. The research activities of AS are supported by the Clinical Research and Education Council of the University Hospitals Leuven.

REFERENCES

Abrue, R., Leal, A., and Figueirdo, P. (2018). EEG-informed fMRI: a review of data analysis methods. *Front. Hum. Neurosci.* 12:29. doi: 10.3389/fnhum.2018.00029

Andre, M., Lamblin, M. D., d'Allest, A. M., Curzi-Dascalova, L., Moussalli-Salefranque, F., Tich, S. N. T., et al. (2010). Electroencephalography in premature and full-term infants. developmental features and glossary. *Neurophysiol. Clin.* 40, 59–124. doi: 10.1016/j.neucli.2010.02.002

Arichi, T., Fagiolo, G., Varela, M., Melendez-Calderon, A., Allievi, A., Merchant, N., et al. (2012). Development of BOLD signal hemodynamic responses in the human brain. *Neuroimage* 63, 663–673. doi: 10.1016/j.neuroimage.2012.06.054

Arichi, T., Moraux, A., Melendez, A., Doria, V., Groppo, M., Mechant, N., et al. (2010). Somatosensory cortical activation identified by functional MRI in preterm and term infants. *NeuroImage* 49, 2063–2071. doi: 10.1016/j.neuroimage.2009.10.038

Azevedo, E., Castro, P., Santos, R., Freitas, J., Coelho, T., Rosengarten, B., et al. (2011). Autonomic dysfunction affects cerebral neurovascular coupling. *Clin. Auton. Res.* 21, 395–403. doi: 10.1007/s10286-011-0129-3

Banaji, M., Tachtsidis, I., Delpy, D., and Baigent, S. (2005). A physiological model of cerebral blood flow control. *Math. Biosci.* 194, 125–173. doi: 10.1016/j.mbs.2004.10.005

Bari, V., Calcagnile, P., Molteni, E., Re, R., Contini, D., Spinelli, L., et al. (2011). Study of neurovascular and autonomic response in a divided attention test by

means of EEG, ECG and NIRS signals. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 2011, 1403–1406. doi: 10.1109/IEMBS.2011.6090330

Bartsch, R. P., Liu, K. K., Bashan, A., and Ivanov, P. (2015). Network physiology: how organ systems dynamically interact. *PLoS One* 10:e0142143. doi: 10.1371/journal.pone.0142143

Bashan, A., Bartsch, R. P., Kantelhardt, J. W., Havlin, S., and Ch Ivanov, P. (2012). Netwrok physiology reveals relations between network topology and physiological function. *Nat. Commun.* 28:702. doi: 10.1038/ncomms1705

Boms, N., Yonai, Y., Molnar, S., Rosengarten, B., Bornstein, N. M., Csiba, L., et al. (2010). Effect of smoking cessation on visually evoked cerebral blood flow response in healthy volunteers. *J. Vasc. Res.* 47, 214–220. doi: 10.1159/000255964

Born, P., Leth, H., Miranda, M. J., Rostrup, E., Stensgaard, A., Peitersen, B., et al. (1998). Visual activation in infants and young children studied by functional magnetic resonance imaging. *Pediatr. Res.* 44, 578–583. doi: 10.1203/00006450-199810000-00018

Brew, N., Walker, D., and Wong, F. (2014). Cerebral vascular regulation and brain injury in preterm infants. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 206, R773–R786. doi: 10.1152/ajpregu.00487.2013

Caicedo, A., Thewissen, L., Smits, A., Naulaers, G., Allegaert, K., and Van Huffel, S. (2016). Changes in oxygenation levels precede changes in amplitude of the EEG in premature infants. *Adv. Exp. Med. Biol.* 923, 143–149. doi: 10.1007/978-3-319-38810-6_19

Chalak, L. F., Tian, F., Adams-Huet, B., Vasil, D., Laptook, A., Tarumi, T., et al. (2017). Novel wavelet real time analysis of neurovascular coupling in neonatal encephalopathy. *Sci. Rep.* 7:45958. doi: 10.1038/srep45958

Challis, R. E., and Kitney, R. I. (1991). Biomedical signal processing (in four parts). Part 3. The power spectrum and coherence function. *Med. Biol. Eng. Comput.* 29, 225–242. doi: 10.1007/BF02446704

Chaparro-Vargas, R., Schilling, C., Schredl, M., and Cvetkovic, D. (2016). Sleep electroencephalography and heart rate variability interdependence among health subjects and insomnia/schizophrenia patients. *Med. Biol. Eng. Comput.* 54, 77–91. doi: 10.1007/s11517-015-1297-4

Cioni, G., Inguaggiato, E., and Sgandurra, G. (2016). Early intervention in neurodevelopmental disorders: underlying neural mechanisms. *Dev. Med. Child Neurol.* 58, 61–66. doi: 10.1111/dmcn.13050

Clemenson, P., and Lancaster, G. (2016). Reconstructing time-dependent dynamics. *Proc. IEEE* 104:18.

Demene, C., Baranger, J., Bernal, M., Delanoe, C., Auvin, S., Biran, V., et al. (2017). Functional ultrasound imaging of brain activity in human newborns. *Sci. Transl. Med.* 9:411. doi: 10.1126/scitranslmed.aah6756

Dettmers, C., Connelly, A., Stephan, K. M., Turner, R., Friston, K. J., Frackowiak, R. S., et al. (1996). Quantitative comparison of functional magnetic resonance imaging with positron emission tomography using a force-related paradigm. *Neuroimage* 4(3 Pt 1), 201–209. doi: 10.1006/nimg.1996.0071

Donders, F. C. (1851). Bewegungen des gehirns und die veränderungen der gefässfüllung der pia mater. *Schmid's Fahrbucher* 69:5.

Duschek, S., and Schandry, R. (2004). Cognitive performance and cerebral blood flow in essential hypotension. *Psychophysiology* 41, 905–913. doi: 10.1111/j.0048-5772.2004.00249.x

Dyson, R. M., Palliser, H. K., Lakkundi, A., de Waal, K., Latter, J. L., Clifton, V. L., et al. (2014). Early miscrovascular changes in the preterm neonate: a comparative study of the human and guinea pig. *Physiol. Rep.* 2:e12145. doi: 10.14814/phy2.12145

Erberich, S. G., Panigraphy, A., Friedlich, P., Seri, I., Nelson, M. D., and Gilles, F. (2006). Somatosensory lateralization in the newborn brain. *NeuroImage* 29, 155–161. doi: 10.1016/j.neuroimage.2005.07.024

Faes, L., Marinazzo, D., Jurysta, F., and Nollo, G. (2015). Linear and non-linear brain-heart and brain-brain interactions during sleep. *Physiol. Meas.* 36, 683–698. doi: 10.1088/0967-3334/36/4/683

Fallani, F. D. V., da Fontoura Costa, L., Rodriguez, F. A., Astolfi, L., Vecchiato, G., Toppi, J., et al. (2010). A graph-theoretical approach in brain functional networks. Possible implications in EEG studies. *Nonlin. Biomed. Phys.* 4:S8. doi: 10.1186/1753-4631-4-S1-S8

Farraro, R., Fathi, O., and Choi, B. (2016). Handheld, point-of-care laser speckle imaging. *J. Biomed. Opt.* 21:094001. doi: 10.1117/1/JBO.21.9.094001

Franceschini, M. A., Radhakrishnan, H., Thakur, K., Wu, W., Ruvinskaya, S., Carp, S., et al. (2010). The effect of different anesthetics on neurovascular coupling. *Neuroimage* 51, 1367–1377. doi: 10.1016/j.neuroimage.2010.03.060

Friston, K. J. (1994). Functional and effective connectivity in neuroimaging: a synthesis. *Hum. Brain Mapp.* 2, 56–78. doi: 10.1002/hbm.460020107

Friston, K. J. (2011). Functional and effective connectivity: a review. *Brain Connect* 1, 13–36. doi: 10.1089/brain.2011.0008

Galderisi, A., Brigadoi, S., Cutini, S., Moro, S. B., Lolli, E., Meconi, F., et al. (2016). Long-term continuous monitoring of the preterm brain with diffuse optical tomography and electroencephalography: a technical note on cap manufacturing. *Neurophoton* 3:045009. doi: 10.1117/1.NPh.3.4.045009

Gao, W., Gilmore, J. H., Giovanello, K. S., Smith, J. K., Shen, D., Zhu, H., et al. (2011). Temporal and spatial evolution of brain network topology during the first two years of life. *PLoS One* 6:e25278. doi: 10.1371/journal.pone.0025278

Girouard, H., and Iadecola, C. (2006). Neurovascular coupling in the normal brain and in hypertension, stroke, and Alzheimer disease. *J. Appl. Physiol.* 100, 328–335. doi: 10.1152/japplphysiol.00966.2005

Govindan, R. B., Massaro, A., Chang, T., Gilbert, V., and du Plessis, A. (2016). A novel technique for quantitative bedside monitoring of neurovascular coupling. *J. Neurosci. Meth.* 259:7. doi: 10.1016/j.jneumeth.2015.11.025

Hata, M., Kazui, H., Tanaka, T., Ishii, R., Canuet, L., Pascual-Marqui, R. D., et al. (2016). Functional connectivity assessed by resting state EEG correlates with cognitive decline of Alzheimer's disease – An eLORETA study. *Clin. Neurophys.* 127, 1269–1278. doi: 10.1016/j.clinph.2015.10.030

Heekeren, H. R., Obrig, H., Wenzel, R., Eberle, K., Ruben, J., Villringer, K., et al. (1997). Cerebral haemoglobin oxygenation during sustained visual stimulation – a near-infrared spectroscopy study. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 352, 743–750. doi: 10.1098/rstb.1997.0057

Heep, A., Scheef, L., Jankowski, J., Born, M., Zimmermann, N., Sival, D., et al. (2009). Functional magnetic resonance imaging of the sensorimotor system in preterm infants. *Pediatrics* 123, 294–300. doi: 10.1542/peds.2007-3475

Hendrikx, D., Thewissen, L., Smits, A., Naulaers, G., Allegaert, K., Van Huffel, S., et al. (2018a). Nonlinear transfer entropy to assess the neurovascular coupling in premature infants. *Adv. Exp. Med. Biol.*

Hendrikx, D., Thewissen, L., Smits, A., Naulaers, G., Allegaert, K., Van Huffel, S., et al. (2018b). Using graph theory to assess the interaction between cerebral function, brain hemodynamics, and systemic variables in premature infants. *Complexity* 2018:6504039. doi: 10.1155/2018/6504039

Hillman, E. M. C. (2014). Coupling mechanism and significance of the BOLD signal: a status report. *Annu. Rev. Neurosci.* 37, 161–181. doi: 10.1146/annurev-neuro-071013-014111

Hsieh, E. M., Hornik, C. P., Clark, R. H., Laughon, M. M., Benjamin, D. K., Smith, P. B., et al. (2014). Medication use in the neonatal intensive care unit. *Am. J. Perinatol.* 31, 811–822. doi: 10.1055/s-0033-1361933

Huneau, C., Benali, H., and Chabriat, H. (2015). Investigating human neurovascular coupling using functional neuroimaging: a critical review of dynamic models. *Front. Neurosci.* 9:467. doi: 10.3389/fnins.2015.00467

Hyder, F. (2009). Dynamic imaging of brain function. *Methods Mol. Biol.* 489, 3–21. doi: 10.1007/978-1-59745-543-5_1

Jang, H., Huang, S., Hammer, D. X., Wang, L., Rafi, H., Ye, M., et al. (2017). Alterations in neurovascular coupling following acute traumatic brain injury. *Neurophotonics* 4:045007. doi: 10.1117/1.NPh.4.4.045007

Juhász, C., Chugani, D. C., Muzik, O., Watson, C., Shah, J., Shah, A., et al. (2000). Relationship between EEG and positron emission tomography abnormalities in clinical epilepsy. *J. Clin. Neurophysiol.* 17, 29–42. doi: 10.1097/00004691-200001000-00004

Karen, T., Morren, G., Haensse, E., Bauschatz, A. S., Bucher, H. U., and Wolf, M. (2008). Hemodynamic response to visual stimulation in newborn infants using functional near-infrared spectroscopy. *Hum. Brain Mapp.* 29, 453–460. doi: 10.1002/hbm.20411

Kissack, C. M., Garr, R., Wardle, S. P., and Weindling, A. M. (2005). Cerebral fractional oxygen extraction is inversely correlated with oxygen delivery in the sick, newborn, preterm infant. *J. Cereb. Blood Flow Metab.* 25, 545–553. doi: 10.1038/sj.jcbfm.9600046

Knyazev, G. G. (2012). EEG delta oscillations as a correlate of basic homeostatic and motivational processes. *Neurosci. Biobehav. Rev.* 36, 677–695. doi: 10.1016/j.neubiorev.2011.10.002

Kozberg, M., and Hillman, E. (2016a). Neurovascular coupling and energy metabolism in the developing brain. *Prog. Brain Res.* 225, 213–242. doi: 10.1016/bs.pbr.2016.02.002

Kozberg, M. G., Chen, B. R., DeLeo, S. E., Bouchard, M. B., and Hillman, E. M. (2013). Resolving the transition from negative to positive blood oxygen level-dependent responses in the developing brain. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4380–4385. doi: 10.1073/pnas.1212785110

Kozberg, M. G., and Hillman, E. M. (2016b). Neurovascular coupling develops alongside neural circuits in the postnatal brain. *Neurogenesis* 3:e1244439. doi: 10.1080/23262133.2016.1244439

Kozberg, M. G., Ma, Y., Shaik, M. A., Kim, S. H., and Hillman, E. M. (2016). Rapid postnatal expansion of neural networks occurs in an environment of altered neurovascular and neurometabolic coupling. *J. Neurosci.* 36, 6704–6717. doi: 10.1523/JNEUROSCI.2363-15.2016

Ku, L. C., and Smith, B. (2015). Dosing in neonates: special considerations in physiology and trial design. *Pediatr. Res.* 77, 2–9. doi: 10.1038/pr.2014.143

Kusaka, T., Kawada, K., Okubo, K., Namba, M., Okada, H., Imai, T., et al. (2004). Noninvasice optical imaging in the visual cortex in young infants. *Hum. Brain Mapp.* 22, 122–132. doi: 10.1002/hbm.20020

Lavanga, M., De Wel, O., Caicedo, A., Jansen, K., Dereymaeker, A., Naulaers, G., et al. (2018). A brain-age model for preterm infants based on functional connectivity. *Physiol. Meas.* 39:044006. doi: 10.1088/1361-6579/aabac4

Lee, J., Nemati, S., Silva, I., Edwards, B. A., Butler, J. P., and Malhotra, A. (2012). Transfer entropy estimation and directional coupling change detection in biomedical time series. *Biomed. Eng. Online* 11:19. doi: 10.1186/1475-925X-11-19

Liao, S. M., Gregg, N. M., White, B. R., Zeff, B. W., Bjerkaas, K. A., Inder, T. E., et al. (2010). Neonatal hemodynamic response to visual cortex activity: high-density near-infrared spectroscopy study. *J. Biomed. Opt.* 15:026010. doi: 10.1117/1.3369809

Lin, P.-Y., Hagan, K., Fenoglio, A., Grant, P. E., and Franceschini, M. A. (2016). Reduced cerebral blood flow and oxygen metabolism in extremely preterm neonates with low-grade germinal matrix- intraventricular hemorrhage. *Sci. Rep.* 6:25903. doi: 10.1038/srep25903

Lindauer, U., Leithner, C., Kaasch, H., Rohrer, B., Foddis, M., Füchtemeier, M., et al. (2010). Neurovascular coupling in rat brain operates independent of hemoglobin deoxygenation. *J. Cereb. Blood Flow Metab.* 30, 757–768. doi: 10.1038/jcbfm.2009.259

Mahmoudzadeh, M., Dehaene-Lamberts, G., Kongolo, G., Fournier, M., Goudjil, S., and Wallois, F. (2018). Conseuqnece of intraventricular hemorrhage on neurovascular coupling evoked by speech syllables in preterm neonates. *Dev. Cogn. Neurosci.* 30, 60–69. doi: 10.1016/j.dcn.2018.01.001

Mantini, D., Perrucci, M. G., Del Gratta, C., Romani, G. L., and Corbetta, M. (2007). Electrophysiological signatures of resting state networks in the human brain. *Proc. Natl. Acad. Sci. U.S.A.* 104, 13170–13175. doi: 10.1073/pnas.0700668104

Meek, J. H., Firbank, M., Elwell, C. E., Atkinson, J., Braddick, O., and Wyatt, J. S. (1998). Regional hemodynamic reponses to visual stimulation in awake infants. *Pediatr. Res.* 43, 840–843. doi: 10.1203/00006450-199806000-00019

Mitra, S., Czosnyka, M., Smielewski, P., O'Reilly, H., Brady, K., and Austin, T. (2014). Heart rate passivity of cerebral tissue oxygenation is associated with predictors of poor outcome in preterm infants. *Acta Paediatr.* 103, e374–e382. doi: 10.1111/apa.12696

Molteno, C. D., Thompson, M. C., Buccimazza, S. S., Magasiner, V., and Hann, F. M. (1999). Evaluation of the infant at risk for neurovelopmental disability. *S. Afr. Med. J.* 89, 1084–1087.

Mullinger, K., and Bowtell, R. (2011). Combining EEG and fMRI. *Methods Mol. Biol.* 711, 303–326. doi: 10.1007/978-1-61737-992-5_15

Muramoto, S., Yamada, H., Sadato, N., Kimura, H., Konishi, Y., Kimura, K., et al. (2002). Age-dependent change in metabolic response to photic stimulation of the primary visual cortex in infants: functional magnetic resonance imaging study. *J. Comput. Assist. Tomogr.* 26, 894–901. doi: 10.1097/00004728-200211000-00007

Musizza, B., Stefanovska, A., McClintock, P. V., Palus, M., Petrovcic, J., Ribaric, S., et al. (2007). Interactions between cardiac, respiratory and EEG-delta oscillations in rats during anaesthesia. *J. Physiol.* 580(Pt 1), 315–326. doi: 10.1113/jphysiol.2006.126748

Nakamura, S., Walker, D. W., and Wong, F. Y. (2017). Cerebral haemodynamic response to somatosensory stimulation in near-term fetal sheep. *J. Physiol.* 595, 1289–1303. doi: 10.1113/JP273163

Naulaers, G., Meyns, B., Miserez, M., Leunens, V., Van Huffel, S., Casaer, P., et al. (2007). Use of tissue oxygenation index and fractional tissue oxygen extraction as non-invasive parameters for cerebral oxygenation. A validation study in piglets. *Neonatology* 92, 120–126. doi: 10.1159/000101063

Nowak-Fluck, D., Ainslie, P. N., Bain, A. R., Ahmed, A., Wildfong, K. W., Morris, L. E., et al. (2018). Effect of healthy ageing on cerebral blood flow, CO2 reactivity and neurovascular coupling during exercise. *J. Appl. Physiol.* 125, 1917–1930. doi: 10.1152/japplphysiol.00050.2018

Pavlopoulos, G. A., Secrier, M., Moschopoulos, C. N., Soldator, T. G., Kossida, S., Aerts, J., et al. (2011). Using graph theory to analyze biological networks. *Biodata Min.* 4:10. doi: 10.1186/1756-0381-4-10

Pereda, E., Quiroga, R. Q., and Bhattacharya, J. (2005). Nonlinear multivariate analysis of neurophysiological signals. *Prog. Neurobiol.* 77, 1–37. doi: 10.1016/j.pneurobio.2005.10.003

Pfurtscheller, K., Bauernfeind, G., Muller-Putz, G. R., Urlesberger, B., Muller, W., and Pfurtscheller, G. (2008). Correlation between EEG burst-to-burst intervals and HR acceleration in preterm infants. *Neurosci. Lett.* 437, 103–106. doi: 10.1016/j.neulet.2008.03.079

Phillips, A. A., Chan, F. H., Zheng, M. M., Krassioukov, A. V., and Ainslie, P. N. (2016). Neurovascular coupling in humans: physiology, methodological advances and clinical implications. *J. Cereb. Blood Flow Metab.* 36, 647–664. doi: 10.1177/0271678X15617954

Phillips, A. A., Warburton, D. E., Ainslie, P. N., and Krassioukov, A. V. (2014). Regional neurovascular coupling and cognitive performance in those with low blood pressure secondary to high-level spinal cord injury: improved by alpha-1 agonist midodrine hydrochloride. *J. Cereb. Blood Flow Metab.* 34, 794–801. doi: 10.1038/jcbfm.2014.3

Powers, W. J., Hirsch, I. B., and Cryer, P. E. (1996). Effect of stepped hypoglycemia on regional cerebral blood flow response to physioloigcal brain activation. *Am. J. Physiol.* 270, H554–H559. doi: 10.1152/ajpheart.1996.270.2.H554

Quinn, R. (2005). Comparing rat's to human's age: how old is my rat in people years? *Nutrition* 21, 775–777. doi: 10.1016/j.nut.2005.04.002

Roche-Labarbe, N., Wallois, F., Ponchel, E., Kongolo, G., and Grebe, R. (2007). Coupled oxygenation oscillation measured by NIRS and intermittent cerebral activation on EEG in premature infants. *Neuroimage* 36, 718–727. doi: 10.1016/j.neuroimage.2007.04.002

Roche-Labarbe, N., Zaaimi, B., Berquin, P., Nehlig, A., Grebe, R., and Wallois, F. (2008). NIRS-measured oxy- and deoxyhemoglobin changes associated with EEG spike-and-wave discharges in children. *Epilepsia* 49, 1871–1880. doi: 10.1111/j.1528-1167.2008.01711.x

Rosengarten, B., Schermuly, R. T., Voswinckel, R., Kohstall, M. G., Olschewski, H., Weissmann, N., et al. (2006). Sildenafil improves dynamic vascular function in the brain: studies in patients with pulmonary hypertension. *Cerebrovasc. Dis.* 21, 194–200. doi: 10.1159/000090555

Sakatani, K., Chen, S., Lichty, W., Zuo, H., and Wang, Y. P. (1999). Cerebral blood oxygenation changes induced by auditory stimulation in newbord infants measured by near infrared spectroscopy. *Early Hum. Dev.* 55, 229–236. doi: 10.1016/S0378-3782(99)00019-5

Schwartz, T. H. (2007). Neurovascular coupling and epilepsy: hemodynamic markers for localizing and predicting seizure onset. *Epilepsy. Curr.* 7, 91–94. doi: 10.1111/j.1535-7511.2007.00183.x

Semenova, O., Lightbody, G., O'Toole, J. M., Boylan, G., Dempsey, E., and Temko, A. (2017). Modelling interactions between blood pressure and brain activity in preterm neonates. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 2017:3972. doi: 10.1109/EMBC.2017.8037725

Shin, J., van Lühmann, A., Kim, D.-W., Mehnert, J., Hwang, H.-J., and Müller, K.-R. (2018). Simultaneous acquisition of EEG and NIRS during cognitive tasks for an open accedd dataset. *Sci. Data* 5:180003. doi: 10.1038/sdata.2018.3

Singh, H., Cooper, R. J., Lee, C. W., Dempsey, L., Edwards, A., Brigadoi, S., et al. (2014). Mapping cortical haemodynamics during neonatal seizures using diffuse optical tomography: a case study. *NeuroImage Clin.* 5, 256–265. doi: 10.1016/j.nicl.2014.06.012

Smitha, K. A., Raja, K. A., and Arun, K. M. (2017). Resting state fMRI: a review on methods in resting state connectivity analysis and resting state networks. *Neuroradiol. J.* 30, 305–317. doi: 10.1177/1971400917697342

Smits, A., Thewissen, L., Dereymaeker, A., Dempsey, E., Caicedo, A., and Naulaers, G. (2017). The use of hemodynamic and cerebral monitoring to study pharmacodynamics in neonates. *Curr. Pharm. Des.* 23, 5955–5963. doi: 10.2174/1381612823666170918124419

Talukdar, M. T., Frost, H. R., and Diamong, S. G. (2015). Modeling neurovascular coupling from clustered parameter sets for multimodal EEG-NIRS. *Comp. Math. Meth. Med.* 2015:830849. doi: 10.1155/2015/830849

Tarantini, S., Tran, C. H. T., Gordon, G. R., Ungvari, Z., and Csiszar, A. (2017). Impaired neurovascular coupling in aging and Alzheimer's disease: contribution of astrocyte dysfunction and endothelial impairment to cognitive decline. *Exp. Gerontol.* 94, 52–58. doi: 10.1016/j.exger.2016.11.004

Tataranno, M. L., Alderliesten, T., de Vries, L., Groenendaal, F., Toet, M. C., Lemmers, P. M. A., et al. (2015). Early oxygen-utilization and brain activity in preterm infants. *PLoS One* 10:e0124623. doi: 10.1371/journal.pone.0124623

Tokariev, A., Videman, M., Palva, J. M., and Vanhatalo, S. (2016). Functional brain connectivity develops rapidly around term age and changes between vigilance states in the human newborn. *Cereb. Cortex* 26, 4540–4550. doi: 10.1093/cercor/bhv219

Tortora, D., Mattei, P. A., Navarra, R., Panara, V., Salomone, R., Rossi, A., et al. (2017). Prematurity and brain perfusion: arterial spin labeling MRI. *NeuroImage Clin.* 15, 401–407. doi: 10.1016/j.nicl.2017.05.023

Vanhatalo, S., Alnaijar, A., Nguyen, V. T., Colditz, P., and Fransson, P. (2014). Safety of EEG-fMRI recodrings in newborn infants at 3T: a study using baby-size phantom. *Clin. Neurophysiol.* 125, 941–946. doi: 10.1016/j.clinph.2013.09.041

Verriotis, M., Fabrizi, L., Lee, A., Cooper, R. J., Fitzgerald, M., and Meek, J. (2016). Mapping cortical responses to somatosensory stimuli in human infants with simultaneous near-infrared spectroscopy and event-related potential recording. *eNeuro* 3:ENEURO.0026-16.2016. doi: 10.1523/ENEURO.0026-16.2016

Vetri, F., Xu, H., Paisansathan, C., and Pelligrino, D. A. (2012). Impairment of neurovascular coupling in type 1 diabetes mellitus in rats is linked to PKC modulation of BK(Ca) and Kir channels. *Am. J. Physiol. Heart Circ. Physiol.* 302, H1274–H1284. doi: 10.1152/ajpheart.01067.2011

Wang, J., Zuo, X., and He, Y. (2010). Graph-based network analysis of resting-state functional MRI. *Front. Syst. Neurosci.* 4:16. doi: 10.3389/fnsys.2010.00016

Wolf, T., Lindauer, U., Villringer, A., and Dirnagl, U. (1997). Excessive oxygen or glucose supply does not alter the blood flow response to somatosensory stimulation or spreading depression in rats. *Brain Res.* 761, 290–299. doi: 10.1016/S0006-8993(97)00354-5

Wong, F. Y., Barfield, C. P., Horne, R. S., and Walker, A. M. (2009). Dopamine theray promotes cerebral flow-metabolism coupling in preterm infants. *Intens. Care Med.* 35, 1777–1782. doi: 10.1007/s00134-009-1602-5

Yamada, H., Sadato, N., Konishi, Y., Kimura, K., Tanaka, M., Yonekura, Y., et al. (1997). A rapid brain metabolic change in infants detected by fMRI. *Neuroreport* 8, 3775–3778. doi: 10.1097/00001756-199712010-00024

Zaramella, P., Freato, F., Amigoni, A., Salvadori, S., Marangoni, P., Suppjei, A., et al. (2001). Brain auditory activation measured by near-infrared spectroscopy (NIRS) in neonates. *Pediatr. Res.* 49, 213–219. doi: 10.1203/00006450-200102000-00014

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Hendrikx, Smits, Lavanga, De Wel, Thewissen, Jansen, Caicedo, Van Huffel and Naulaers. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 27 February 2019
doi: 10.3389/fphys.2019.00141

The Role of Connexin and Pannexin Channels in Perinatal Brain Injury and Inflammation

*Kelly Q. Zhou[1], Colin R. Green[2], Laura Bennet[1], Alistair J. Gunn[1] and Joanne O. Davidson[1]**

[1] Department of Physiology, The University of Auckland, Auckland, New Zealand, [2] Department of Ophthalmology, The University of Auckland, Auckland, New Zealand

Perinatal brain injury remains a major cause of death and life-long disability. Perinatal brain injury is typically associated with hypoxia-ischemia and/or infection/inflammation. Both hypoxia-ischemia and infection trigger an inflammatory response in the brain. The inflammatory response can contribute to brain cell loss and chronic neuroinflammation leading to neurological impairments. It is now well-established that brain injury evolves over time, and shows a striking spread from injured to previously uninjured regions of the brain. There is increasing evidence that this spread is related to opening of connexin hemichannels and pannexin channels, both of which are large conductance membrane channels found in almost all cell types in the brain. Blocking connexin hemichannels within the first 3 h after hypoxia-ischemia has been shown to improve outcomes in term equivalent fetal sheep but it is important to also understand the downstream pathways linking membrane channel opening with the development of injury in order to identify new therapeutic targets. Open membrane channels release adenosine triphosphate (ATP), and other neuroactive molecules, into the extracellular space. ATP has an important physiological role, but has also been reported to act as a damage-associated molecular pattern (DAMP) signal mediated through specific purinergic receptors and so act as a primary signal 1 in the innate immune system inflammasome pathway. More crucially, extracellular ATP is a key inflammasome signal 2 activator, with purinergic receptor binding triggering the assembly of the multi-protein inflammasome complex. The inflammasome pathway and complex formation contribute to activation of inflammatory caspases, and the release of inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-18, and vascular endothelial growth factor (VEGF). We propose that the NOD-like receptor protein-3 (NLRP3) inflammasome, which has been linked to inflammatory responses in models of ischemic stroke and various inflammatory diseases, may be one mechanism by which connexin hemichannel opening especially mediates perinatal brain injury.

Keywords: connexin, pannexin, hemichannel, ischemia, inflammation, inflammasome, ATP

OPEN ACCESS

Edited by:
Mary Tolcos,
RMIT University, Australia

Reviewed by:
Julie Wixey,
University of Queensland, Australia
Meredith Anne Kelleher,
Oregon Health & Science University, United States

***Correspondence:**
Joanne O. Davidson
joanne.davidson@auckland.ac.nz

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *12 November 2018*
Accepted: *07 February 2019*
Published: *27 February 2019*

Citation:
Zhou KQ, Green CR, Bennet L, Gunn AJ and Davidson JO (2019) The Role of Connexin and Pannexin Channels in Perinatal Brain Injury and Inflammation. Front. Physiol. 10:141. doi: 10.3389/fphys.2019.00141

INTRODUCTION

Perinatal brain injury is associated with death or significant long-term neurodevelopmental impairment, affecting 1.15 million infants in the world in 2010 (Lee et al., 2013). It affects both term and preterm infants (born <37 weeks of completed gestation), with the greatest incidence in the most preterm infants (Larroque et al., 2008). In term born infants, perinatal brain injury

is most often linked to hypoxia-ischemia (HI) (Vannucci, 2000; Shankaran et al., 2005), where the incidence of moderate to severe hypoxic-ischemic encephalopathy (HIE) is ∼1–3 in 1,000 live births (Edwards et al., 2010). By contrast, in preterm infants, brain injury arises from the complex interaction of HI, infection/inflammation and preterm birth itself (Galinsky et al., 2018b). Strikingly, ∼50% of extremely preterm infants (born <26 weeks of gestation) develop moderate to severe disability (Marlow et al., 2005).

Currently, there is only one established neuroprotective treatment for term babies born with HIE, which is therapeutic hypothermia. Clinically, this treatment is only partially effective, with a number needed to treat of ∼8, meaning of 8 treated infants with HIE, one additional infant will survive without moderate to severe disability (Gunn et al., 2017; Natarajan et al., 2018). However, there are currently no proven treatments to reduce brain damage for preterm infants with HIE or perinatal infection/inflammation. Therefore, there is significant interest in developing complementary or novel treatments for perinatal brain injury after HI or infection/inflammation. For term infants, it is important to develop interventions with additive benefit to therapeutic hypothermia, as it is now standard therapy. Novel treatments are needed for term and preterm infection/inflammation.

After HI, there is a striking evolution of injury which progresses over time from injured to previously uninjured regions of the brain (Azzopardi et al., 1989; Thornton et al., 1998). The mechanisms behind this spread are not well-understood. Increasing evidence supports the opening of cell membrane channels after HI as one of the mediators of this propagation of injury, particularly connexin hemichannels, which are the unopposed half of a gap junction, forming large conductance channels in the cell membrane (Davidson et al., 2012, 2014; Wang et al., 2014; Li et al., 2015). Pannexin channels may also open under pathological conditions, although they appear to be tightly regulated (Qiu and Dahl, 2009) and their role in the developing brain remains unclear. In this review, we examine the hypothesis that prolonged opening of connexin hemichannels leads to a cascade of injurious mechanism, potentially through inflammation by activation, amplification and perpetuation of the inflammasome pathway.

An inflammatory response is seen in perinatal brain injury after HI as well as infection/inflammation, and if chronic inflammation is established can have deleterious effects (Hagberg et al., 2015; Bennet et al., 2018). Hemichannels release adenosine triphosphate (ATP) (Kang et al., 2008; Chekeni et al., 2010; Orellana et al., 2011; Bennett et al., 2012), which may act as a damage associated molecular pattern (DAMP), but is also a key inflammasome activator signal. The release of ATP through connexin hemichannels can perpetuate inflammation through purinergic signaling (Pedata et al., 2016). Purinergic signaling has been associated with the activation of microglia (Li et al., 2011; Kaiser et al., 2016) and the inflammasome complex—a multi-protein complex involved in initiating an innate immune system inflammatory response (Feng et al., 2015). This review will dissect the evidence for these pathways downstream of connexin hemichannel and pannexin channel opening and how they contribute to inflammation. One other membrane pore forming protein, Gasdermin D, has been implicated in the inflammasome pathway (Groslambert and Py, 2018; Kerur et al., 2018), but that channel is very small in comparison with the pannexin and connexin channels and appears to be primarily associated with apoptosis. As will become evident in this review, both *in vitro* and animal studies using connexin hemichannel or pannexin channel blockers, would suggest that other channels are likely to play minor roles in relation to ATP release, and inflammasome activation in particular.

EVOLUTION OF INJURY

Perinatal brain injury after HI is an evolving process that can be characterized into four phases (Bennet et al., 2010; Davidson et al., 2015b). The primary phase of injury occurs during the HI insult itself, when the failure of oxidative metabolism results in anoxic depolarization, edema and necrosis (Wassink et al., 2018). After restoration of blood flow and oxygen supply, there is a period of apparent transient recovery when oxidative metabolism is at least partially restored, known as the latent phase (Davidson et al., 2018a). However, the latent phase is the key time when the deleterious mechanisms leading to the spread of brain injury may be initiated; for example, the opening of connexin hemichannels, which will be discussed further below (Davidson et al., 2012).

Following the latent phase, there is a delayed ("secondary") deterioration of oxidative metabolism starting ∼6–15 h after the insult (Azzopardi et al., 1989; Williams et al., 1991; Gunn et al., 1997). This phase is characterized by delayed cerebral energy failure followed by seizures and secondary cell swelling (Bennet et al., 2006; Davidson et al., 2015c). There is marked neuronal injury after HI at term, with the majority of neuronal loss occurring during the secondary phase, through a continuum of necrosis-apoptosis and autophagy (Northington et al., 2007, 2011).

The tertiary phase is a period of repair and reorganization, persisting for weeks to years after the initial insult (Fleiss and Gressens, 2012). During this period, surviving cells in the brain can rewire, but there may be a low level of ongoing cell death due to the loss of trophic support and problems with connectivity (Ness et al., 2001; Romanko et al., 2004). Long-term impairment in perinatal brain injury may also be associated with epigenetic changes (Fleiss and Gressens, 2012), but also persistent inflammation (Bennet et al., 2018) as reported for other types of brain injury and degenerative diseases (Patterson and Holahan, 2012; Freeman and Ting, 2016).

INFLAMMATION IN PERINATAL BRAIN INJURY

Perinatal brain injury associated with HI or infection/inflammation can trigger an inflammatory response. The innate immune response is the body's first line of defense against pathogens, reacting rapidly following exposure to invading organisms (Medzhitov, 2007). As part of the innate immune response, pattern recognition receptors

(PRRs) expressed on immune cells recognize both the conserved molecular structures found on the pathogen known as pathogen-associated molecular patterns (PAMPs), and the endogenous signals released by damaged tissues known as danger-associated molecular patterns (DAMPs) (Medzhitov, 2007; Takeuchi and Akira, 2010).

The activation of PRRs initiates the inflammatory response leading to release of inflammatory cytokines, such as interleukin (IL)-1β (Turner et al., 2014). This early phase of inflammation targets the invading pathogens and/or clears injured tissue, which is beneficial to the host. However, this inflammatory response also leads to the death of uninjured neural cells in a process known as "bystander cell loss" (Hagberg et al., 2015). It is believed that the initial pro-inflammatory response is followed by anti-inflammatory and reparative processes, and either eventual resolution of inflammation or chronic inflammation (Gilroy and De Maeyer, 2015; Hagberg et al., 2015).

Elevated levels of inflammatory markers after birth are associated with adverse neurodevelopmental outcomes. A prospective cohort study of 73 term infants exposed to perinatal asphyxia showed that those who died or were diagnosed with cerebral palsy at a 1 year follow up, were associated with higher levels of IL-1, IL-6 and tumor necrosis factor (TNF)-α in heel-stick blood samples collected on the first or second day of birth (Foster-Barber et al., 2001). In the same cohort, followed up at 30 months of age, those with higher serum cytokine levels of IL-1β, IL-6, and IL-8 at birth were associated with abnormal cognitive and motor outcomes (Bartha et al., 2004). The extremely low gestational age newborns (born before 28 weeks of gestation) (ELGANs) study is a large multi-center observational study. In this cohort, elevated blood concentrations of inflammatory proteins, measured in the first few weeks of life was associated with mental and motor impairments at 2 years old (O'Shea et al., 2012) and adverse cognitive outcome at 10 years of age (Kuban et al., 2017), as well as increased risk of behavioral problems, including autism (Korzeniewski et al., 2018). Cytokine levels were not measured at later time points to determine if inflammation was sustained. However, a study by Lin and colleagues has suggested that perinatal brain injury is associated with long-lasting alterations to the inflammatory response (Lin et al., 2010). Peripheral blood mononuclear cells from a small cohort of preterm born children with periventricular leukomalacia induced cerebral palsy, were shown to have higher mRNA levels of inflammatory molecules, both before and after lipopolysaccharide (LPS) stimulation (Lin et al., 2010). Non-resolving inflammation could be the result of a prolonged or excessive inflammatory response, which may lead to the disruption of pathways that normally induce inflammatory resolution (Nathan and Ding, 2010; Bennet et al., 2018).

CONNEXIN HEMICHANNELS

Connexin gap junctions connect the intracellular space of two adjacent cells allowing for the exchange of ions and molecules (Kumar and Gilula, 1996; Alexander and Goldberg, 2003; Davidson et al., 2013a). A connexin hemichannel is comprised of six connexin subunits. Connexin hemichannels from adjacent cells dock together to form a gap junction (Unger et al., 1999; Davidson et al., 2013a; Leybaert et al., 2017) (**Figure 1**). Humans have 21 connexin genes, and 11 of these are expressed in the brain (Theis et al., 2005). Connexin 43 (Cx43) has been of particular interest in the brain as it is abundantly expressed, especially in astrocytes, microglia and microvascular endothelium (Dermietzel et al., 2000; Nagy et al., 2003; Davidson et al., 2013a). Under physiological conditions, prior to forming a gap junction, hemichannels at the cell surface have a low probability of opening, shown by low membrane permeability during resting conditions in cultured cells (Decrock et al., 2009). It is not known whether this is the case *in vivo*, as cells may be exposed to a wide range of stimuli influencing hemichannel opening (Sáez et al., 2005). When hemichannels do open though, they form a large non-selective membrane channel capable of passing molecules up to about 1 kDa in size (Evans et al., 2006). There is increasing evidence that dysregulated hemichannel opening can be detrimental and they are often referred to as "pathological pores" (Paul et al., 1991; Decrock et al., 2015; Willebrords et al., 2016).

Connexin hemichannels are involved in the spread of brain injury after HI or infection in the adult and immature brain (Davidson et al., 2012, 2013a) (studies are summarized in **Table 1**). They open in response to stimuli mimicking exposure to ischemia and inflammation *in vitro*. For example, in cultured cells, oxygen-glucose deprivation (OGD), metabolic inhibition, low extracellular Ca^{2+}, and strong depolarization have all been proven to stimulate hemichannel opening (Contreras et al., 2002; Decrock et al., 2009; Orellana et al., 2010). Conditioned medium from LPS-activated microglia or TNF-α and IL-1β increased hemichannel activity in cultured astrocytes (Retamal et al., 2007). In addition, neuroinflammation induced by S. aureus intracerebral inoculation increased Cx43 hemichannel activity in astrocytes surrounding the abscess (Karpuk et al., 2011). The opening of connexin hemichannels allows for the influx of Na^+, Cl^-, and Ca^{2+} and the efflux of K^+ ions. This increases cell permeability, leading to the depolarization of the cell, and cell lysis (Paul et al., 1991; Gómez-Hernández et al., 2003). Molecules, such as ATP, glutamate and aspartate can be released; all of these molecules are associated with injury (Ye et al., 2003; Gomes et al., 2005; Zhao et al., 2005; Kang et al., 2008). Additionally, Cx43 hemichannels are likely involved in the propagation of seizures following severe HI in near-term fetal sheep (Davidson et al., 2012).

An upregulation of Cx43 has been shown to occur in adult and perinatal animals after HI and in adult human post-mortem studies of cerebral ischemia (Nakase et al., 2006, 2009; Davidson et al., 2012; Wang et al., 2014; Li et al., 2015). The timing of this Cx43 increase varies between studies, with Cx43 mRNA levels significantly increased at 6 h after carotid artery occlusion in near-term fetal sheep (Davidson et al., 2012). Protein levels progressively increased in the ischemic region from 8 h to 7 days after common carotid artery ligation and hypoxia (commonly referred to as Rice-Vannucci model of HI) in post-natal day (P)7 rats (equivalent to 33–34 weeks of human brain maturation) (Li et al., 2015). In the subventricular zone, however,

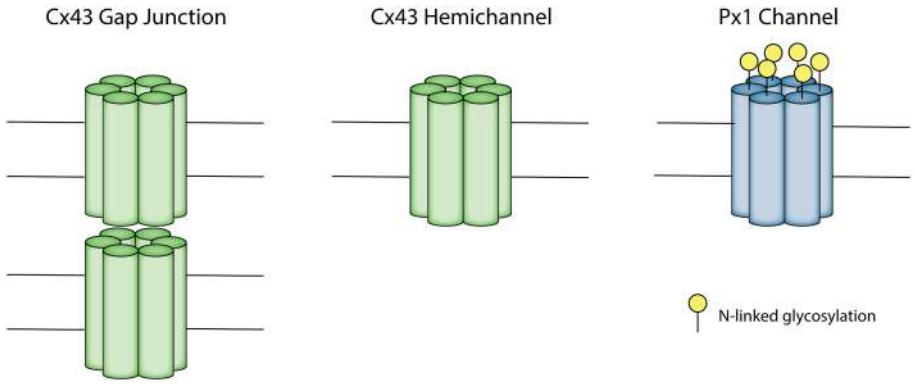


FIGURE 1 | Schematic depicting the Connexin 43 (Cx43) gap junction and hemichannel and pannexin 1 (Px1) channel. A connexon consists of six connexin subunits. A Cx43 gap junction is made up of two opposing connexons, each contributed from two adjacent cells. A Cx43 hemichannel is an unopposed connexon. A Px1 channel is formed by six pannexin subunits. Px1 does not form gap junctions due to N-linked glycosylation on the extracellular loop.

Wang and colleagues reported Cx43 protein expression was only significantly increased from 24 h after the same insult in P7 rats (Wang et al., 2014). However, there was no change in hippocampal Cx43 protein expression at all time-points between 10 min and 35 days after acute hypoxia in P10 rats (equivalent to human term brain maturation) (Zeinieh et al., 2010). Thus, the changes in Cx43 expression may be dependent on species, brain region, type and/or severity of insult. It should be noted that, the Rice-Vannucci model of HI in rodents produces a unilateral infarction in the brain but only moderate systemic hypoxia (Rice et al., 1981), whereas bilateral carotid artery occlusion in near-term fetal sheep produces a global cerebral insult resulting in a watershed pattern of brain injury, without systemic hypoxia (Williams et al., 1992). The reader should note that protein assays and immunohistochemical labeling do not allow ready discrimination between gap junctions and hemichannels, or channel function.

The blockade of Cx43 hemichannels, however, has been neuroprotective in a variety of different animal models of perinatal HI (Davidson et al., 2012, 2014; Li et al., 2015). Intracerebroventricular (i.c.v.) infusion (50 μmol/kg over 1 h, then 50 μmol/kg over 24 h) of the Cx43 mimetic peptide—Peptide5, started at 90 min after the end of carotid artery occlusion in near-term fetal sheep, was associated with improved neuronal and oligodendrocyte survival and, electroencephalogram (EEG) recovery and reduced seizure burden (Davidson et al., 2012). Similarly, Peptide5 infusion following the same protocol as above in preterm fetal sheep after umbilical cord occlusion improved EEG recovery and reduced neuronal and oligodendrocyte loss (Davidson et al., 2014). Reduced infarct size and improved motor and memory scores were observed in Gap 26 treated P7 rats [a connexin mimetic peptide which blocks hemichannels and gap junctions (Wang et al., 2013)], given i.p., 50 μg/kg, 1 h before HI (Li et al., 2015).

Cx43 hemichannel opening *in vivo* has been shown to occur after ischemia (in the latent phase), but not during ischemia. Pre-administration of Peptide5 was not neuroprotective when given 1 h before and during ischemia, but was neuroprotective when given after ischemia (Davidson et al., 2012, 2013b). Supporting this, hemichannels in astrocyte cultures subjected to OGD have been shown to open 1 h after OGD (Orellana et al., 2010). In addition, carbenoxolone (100 μmol/L) inhibition of connexin hemichannels did not attenuate ATP release during OGD in hippocampal slices (Frenguelli et al., 2007). Both Cx43 hemichannel and pannexin channel opening was reported to occur during hypoxia in endothelial cells with two thirds of ATP release connexin hemichannel mediated, but upon reperfusion, only Cx43 hemichannel opening occurred (Kim and Green, 2016). These studies indicate that Cx43 hemichannels likely do open after ischemia and may have an ongoing role in the evolution of injury after the insult. This is supported by the demonstration that prolonged infusion of Peptide5 (i.c.v., starting at 90 min) at a dose of 50 μmol/kg over 1 h, then 50 μmol/kg over 24 h of Peptide5 provided greater neuroprotection compared to a 1 h infusion only (50 μmol/kg) (Davidson et al., 2012). Furthermore, delayed administration of another known hemichannel blocker, Gap 26 (i.p., 50 μmol/kg) at 24 h after HI still had neuroprotective effects (Li et al., 2015). Interestingly, when Peptide5 administration (i.c.v., 50 μmol/kg for 1 h followed by 50 μmol/kg for 24 h) was delayed until 3 h after the end of ischemia in near-term fetal sheep, the neuroprotective effects were reduced when compared to earlier administration (Davidson et al., 2012, 2015a). The delayed administration reduced the seizure burden, but there was no clear effect on cell survival or EEG recovery (Davidson et al., 2012, 2015a).

The mechanisms behind how Cx43 hemichannels lead to neuronal and oligodendrocyte loss in HI is not well-understood. During and/or after HI there is impaired intracellular Ca^{2+} handling. This has been shown to contribute to mitochondrial dysfunction and necrotic and apoptotic cell death (Puka-Sundvall et al., 2000; Mallard et al., 2014). Potentially, the opening of hemichannels could lead to an influx of excessive intracellular Ca^{2+} accumulation leading directly to the neuronal and/or oligodendrocyte death (Galinsky et al.,

TABLE 1 | Summary of studies of Cx43 hemichannels and Px1 channels and purinergic receptors P2X4 and P2X7.

	Species	Age	Paradigm	Hemichannel activity/expression	Drug/knockout	Drug/knockout effect	References
Connexin 43 (Cx43)	Rat primary astrocyte culture	Embryonic day (E) 9	Metabolic inhibition	↑ Cx43 hemichannel opening during metabolic inhibition			Contreras et al., 2002
	Rat primary astrocyte culture	P1/2	Conditioned medium from LPS-activated microglia or TNF-α and IL-1β	↑ Cx43 hemichannel activity 24 h after treatment			Retamal et al., 2007
	Rat primary astrocyte culture	P1/2	Hypoxia and artificial cerebrospinal fluid medium mimicking ischemic conditions	↑ Cx43 hemichannel opening 1 h after reoxygenation			Orellana et al., 2010*
	Human microvascular endothelial cells		Hypoxic acidic ion-shifted ringer solution	↑ ATP release from Cx43 hemichannels during treatment and after reperfusion			Kim and Green, 2016*
	C6 glioma cells transfected with Cx43		Voltage activation of Cx43 hemichannels	↑ ATP release from Cx43 hemichannels			Kang et al., 2008
	Human adult retinal pigment epithelial cells		High glucose, TNF-α and IL-1β	↑ ATP release from Cx43 hemichannels	Peptide 5 (5–50 μM), administered at the same time as high glucose and cytokine treatment	↓ NLRP3 oligomerization ↓ Cytokine release	Mugisho et al., 2018
	Mouse acute brain slice	8–12 weeks	*S. aureus in vivo* intracerebral inoculation	↑ Cx43 hemichannel activity			Karpuk et al., 2011*
	Sheep	Near-term	Carotid artery occlusion	↑ mRNA expression (6 h after end of occlusion)	Peptide5 i.c.v. (50 μmol/kg over 1 h, then 50 μmol/kg over 24 h) started 90 min after end of occlusion	↑ Neuronal and oligodendrocyte survival Improved electroencephalogram recovery ↓ Seizure burden	Davidson et al., 2012; Galinsky et al., 2017
	Sheep	Near-term	Carotid artery occlusion		Peptide5 i.c.v. (50 μmol/kg over 1 h, then 50 μmol/kg over 24 h) started 3 h after end of occlusion	↓ Seizure burden	Davidson et al., 2015a
	Sheep	Near-term	Carotid artery occlusion		Peptide5 i.c.v. (50 μmol/kg/h) given 1 h before, and during occlusion only	No neuroprotection	Davidson et al., 2013b
	Sheep	Preterm	Umbilical cord occlusion		Peptide5 i.c.v. (50 μmol/kg over 1 h, then 50 μmol/kg over 24 h) started 90 min after end of occlusion	↑ Neuronal and oligodendrocyte Improved electroencephalogram recovery	Davidson et al., 2013a
	Rat	P7	Common carotid artery ligation and hypoxia	↑ Protein expression (24–48 h after HI)			Wang et al., 2014
	Rat	P7	Common carotid artery ligation and hypoxia	↑ Protein expression (8 h to 7 d after HI)	Gap 26 i.p. (50 μg/kg, 1 h) before HI	↓ Infarct size ↑ Motor and memory scores	Li et al., 2015

(Continued)

TABLE 1 | Continued

	Species	Age	Paradigm	Hemichannel activity/expression	Drug/knockout	Drug/knockout effect	References
	Rat	P10	Acute hypoxia	No change (10 min to 35 d after HI)			Zeinieh et al., 2010
Pannexin 1 (Px1)	Rat primary neurons	P20-25	N-methyl-D-aspartate receptor (NMDAR) activation	↑ Px1 channel opening			Thompson et al., 2008
	Rat primary neurons	P15-20	OGD	↑ Px1 channel opening during OGD			Thompson et al., 2006
	Mouse hippocampal slice	P13-14	High K^+ medium	↑ Px1 channel opening			Santiago et al., 2011
	Mouse primary astrocytes		OGD		Probenecid (10 μM)	↓ IL-1β release ↓ NLRP3 protein expression	Jian et al., 2016
	Mouse acute brain slice	8–12 weeks	*S. aureus in vivo* intracerebral inoculation	↑ Px1 channel activity			Karpuk et al., 2011*
	Xenopus oocytes expressing Px1		High K^+ medium	↑ ATP release during treatment from Px1 channels			Bao et al., 2004
	Rat primary astrocyte culture	P1/2	Hypoxia and artificial cerebrospinal fluid medium mimicking ischemic conditions	No Px1 channel opening at 1 h after reoxygenation			Orellana et al., 2010*
	Human microvascular endothelial cells		Hypoxic acidic ion-shifted ringer solution	↑ ATP release from Px1 channels during treatment but not after reperfusion			Kim and Green, 2016*
	Mice	Adult	MCAO		Mefloquine i.p. (1 mg/kg/d) given during the start of MCAO	↓ Infarction size ↑ Motor scores * No additive effect with P2X7R blockade	Cisneros-Mejorado et al., 2015a*
	Rat	Adult	MCAO		Probencid (i.v., 2 mg/kg) given before reperfusion	↑ Neuronal survival	Wei et al., 2015
	Mice	Adult	MCAO	↑ Px1 protein expression in females vs. male on ischemic and non-ischemic hemispheres	Probenicid i.p. (250 mg/kg) at 1.5 and 5 h	↓ Infarct volume (female only)	Freitas-Andrade et al., 2017
Purinergic receptor (P2X4R)	Rat hippocampal slice	P8-10	OGD	↑ Protein expression at 24 h			Cavaliere et al., 2003
	Mice	Adult	MCAO	↑ Protein expression at 6, 24, and 72 h	Global P2X4R knockout Myeloid cell P2X4R knockout	↓ Infarct volume ↓ IL-1β and TNF-α release ↓ Infarct volume (females only)	Verma et al., 2017

(Continued)

TABLE 1 | Continued

	Species	Age	Paradigm	Hemichannel activity/expression	Drug/knockout	Drug/knockout effect	References
	Rat primary microglia	P2-3			TNP-ATP (20 μM)	↓ Change of microglia to rounded morphology ↓ IL-1β and TNF-α protein expression	Li et al., 2011
	Rat	P0	Hypoxia	↑ Protein expression from 4 h to 14 d			Li et al., 2011
	Rat	P3	Common carotid artery occlusion and hypoxia	↑ Protein expression 5 and 7 d after HI			Wixey et al., 2009
Puringeric receptor (P2X7R)	Neuronal culture		OGD		Brilliant Blue G (50 nM or 5 μM) during OGD	↓ Cell death	Arbeloa et al., 2012
	Rat	Adult	MCAO		Brilliant Blue G i.p. (30 mg/kg) during MCAO	↓ Neuronal loss ↓ Infarct volume	Arbeloa et al., 2012
	Rat	P0	Intrauterine asphyxia	↑ Protein expression immediately after asphyxia			Frizzo et al., 2010
	Rat	Adult	MCAO	↑ Protein expression 4 d after MCAO			Franke et al., 2004
	Rat	Adult	Common carotid and vertebral arteries occlusion	↑ Protein expression from 6 h to 7 d after insult	Brilliant Blue G treatment i.v. (50 mg/kg) administered daily, for 3 d immediately after occlusion	↓ Neuronal loss	Yu et al., 2013
	Rat	Adult	MCAO		A-438079 (3 μg, i.c.v.) or Brilliant Blue G (10 μg, i.c.v.), or Oxidized ATP (1 μg, i.c.v.), prior to HI	↓ Neuronal loss ↑ Motor performance	Chu et al., 2012
	Rat	Adult	MCAO	*De novo* expression of P2X7R on microglia			Melani et al., 2006
	Rat	Adult	Intracerebral hemorrhage		P2X7R siRNA i.c.v. (1,000 pmol)	↓ NLRP3 inflammasome activation ↓ IL-1β and IL-18 release	Feng et al., 2015
	Neuronal culture		OGD	↑ Protein expression after 3, 6, and 12 h of OGD			Ye et al., 2017
	Mice	Adult	Photothrombotic cerebral ischemia	↑ Protein expression from 1 to 5 d after insult	Brilliant Blue G (45.5 mg/kg, i.p.) given at 1, 3, and 5 d	↓ Infarct volume ↓ Protein expression of NLRP3, ASC, and caspase 1 p20	Ye et al., 2017
	Mice	Adult	MCAO		Brilliant Blue G (30 mg/kg twice per day, i.p.) during MCAO	↓ Infarction size ↑ Motor scores * No additive effect with Px1 blockade	Cisneros-Mejorado et al., 2015a*
	Mice	Adult	MCAO		P2X7R knockout	↓ Microglia activation	Kaiser et al., 2016

Studies that report interactions between connexin hemichannels, pannexin channels, and/or purinergic receptors.

2018a). In support, GABAergic striatal neurons expressing intracellular calcium binding proteins are shown to be highly susceptible to HI injury in near-term fetal sheep (Galinsky et al., 2017).

ATP has been widely associated with both inflammasome signal 1 priming which results in transcriptional upregulation of pro-IL-1β and pro-IL-18 and molecules in the inflammasome pathway itself, and inflammasome signal 2 activation that results in assembly of the NLRP3-ASC-pro-caspase1 inflammasome complex within the cell cytoplasm, and activation of caspase 1. Cx43 hemichannel mediated ATP release in particular has been associated with NOD-like receptor protein-3 (NLRP3) inflammasome complex assembly as shown in retinal pigment epithelial cells (Mugisho et al., 2018), and with inflammasome activation *in vivo* in a model of chronic pain (Tonkin et al., 2018). Neuron and oligodendrocyte damage after perinatal brain injury may also be due to this innate immune system inflammatory response, and is discussed further in section Activation of the Inflammasome below.

PANNEXIN CHANNELS

Pannexin channels may have similar functions to connexin hemichannels, but their role in perinatal brain injury is not well-understood. Pannexins share homology with the gap junction proteins in invertebrates called innexins (Panchin, 2005), and have a similar topological structure to connexins, despite not sharing sequence homology (Panchin, 2005) (**Figure 1**). Unlike connexins, pannexins have N-glycosylation on the extracellular loop, which appears to prevent the formation of cell-cell junctions (Boassa et al., 2007) although there is some evidence for pannexin junction formation in C2C12 cells in culture (Ishikawa et al., 2011). There are three subtypes of pannexin, of which pannexin 1 (Px1) and pannexin 2 (Px2) are found to be expressed in the adult and developing brain (Bruzzone et al., 2003; Vogt et al., 2005). Px1 is predominantly expressed in the plasma membrane, whereas Px2 is mainly expressed in intracellular membranes (Boassa et al., 2007).

Opening of pannexin channels can be induced by a range of stimuli *in vitro*—including OGD, high extracellular K^+ concentration, hypoglycemia, cell swelling and the stimulation of NMDA receptors (Thompson et al., 2006, 2008; Kawamura et al., 2010; Santiago et al., 2011). Px1 channels are permeable to ions, and molecules, such as ATP and glucose (Bruzzone et al., 2003; Bao et al., 2004; Riquelme et al., 2013) and the opening of Px1 channels contributes to anoxic depolarization, which can lead to cell death (Thompson et al., 2008; Weilinger et al., 2012). In astrocytes on the border of abscess regions caused by S. aureus intracerebral inoculation in mice, there is increased Cx43 hemichannel and Px1 channel activity (Karpuk et al., 2011). Increased Px1 channel activity in apoptotic lymphocytes *in vitro* has been associated with providing "find me" signals through ATP release to attract macrophages (Chekeni et al., 2010). In addition, Px1 channel opening has been linked to the activation of the NLRP3 inflammasome and IL-1β release in astrocytes subjected to OGD (Jian et al., 2016).

In adult rodent models of stroke, blocking Px1 has neuroprotective effects (Cisneros-Mejorado et al., 2015a; Wei et al., 2015; Freitas-Andrade et al., 2017). However, to the best of our knowledge, the effect of Px1 blockade after HI has not been examined in the developing brain. Mefloquine (Px1 blocker) injections given during the start of middle cerebral artery occlusion (MCAO) (i.p., 1 mg/kg/day) in adult mice reduced infarction size and improved motor scores (Cisneros-Mejorado et al., 2015a). It should be noted, however, that mefloquine may also inhibit connexin hemichannel opening (Cruikshank et al., 2004). The effectiveness of Px1 blockade is time dependent, as the administration of probenecid, another non-specific Px1 blocker, was most protective against the death of hippocampal CA1 neurons when given before reperfusion (i.v., 2 mg/kg) in adult rats subjected to MCAO (Wei et al., 2015). There was partial protection when administered at 2 h, but no effect when delayed until 6 h after the insult (Wei et al., 2015). Probenecid administration for 7 days starting at 6 h, however, was more protective than a single dose at 6 h. There was also reduced inflammation, shown by reduced astrocytic and microglial immunoreactivity (Wei et al., 2015). The role of Px1 may also be sex dependent, as Px1 protein levels were higher in female than male mice before and after MCAO (Freitas-Andrade et al., 2017). Both Px1 knockout mice and probenecid treated mice (at 1.5 and 5 h, 250 mg/kg, i.p.), had reduced infarct volumes after MCAO compared to control, but the effect was not seen in males (Freitas-Andrade et al., 2017). It is unclear what mechanisms are involved in producing this sex difference, but it may be related to differences in caspase-dependent cell death pathways and estrogen receptor β signaling in females (Freitas-Andrade et al., 2017).

The interaction of connexin hemichannels and pannexin cell channels has been examined in astrocyte cultures exposed to hypoxia and artificial cerebral spinal fluid medium that mimics ischemic conditions in the brain (Orellana et al., 2010). After reoxygenation, there was increased hemichannel activity, indicated by increased uptake of ethidium bromide, which peaked at 1 h after rexoygenation. This hemichannel activity was mediated by Cx43 hemichannels and not pannexin channels, as dye uptake was reduced by Cx43 hemichannel blockade or Cx43 knockout, but not pannexin blockade (Orellana et al., 2010). This is consistent with the endothelial cell hypoxia-reperfusion study referred to above, where pannexin channel opening was reported during hypoxia, but not reperfusion (Kim and Green, 2016). Furthermore, connexin hemichannels, but not pannexin channels, have been implicated in the inflammasome pathway in muscular dystrophy (Cea et al., 2016), and inflammatory cytokine release normally associated with the inflammasome pathway in rodent models of Parkinson's disease (Maatouk et al., 2018) and Alzheimer's disease (Yi et al., 2016). Finally, pannexin channels are self-regulated, and whilst they may release ATP, they are also closed by the presence of ATP in the extracellular milieu (Qiu and Dahl, 2009). Taken overall, it is possible that pannexin channel opening may play a role in inflammasome pathway initiation, but amplification and perpetuation in chronic disease conditions may be primarily connexin hemichannel mediated. This is likely to apply to

perinatal brain injury and inflammation too, but remains to be proven.

ASTROCYTES AND INFLAMMATION

Astrocytes play an important physiological role in maintaining the homeostasis of the microenvironment in the brain. Astrocytes are the most abundant cell type and account for half of the cells in the central nervous system (Markiewicz and Lukomska, 2006). They are electrically non-excitable cells, but have a role in propagating Ca^{2+} waves through gap junctions between astrocytes-astrocytes and astrocytes-neurons (Nedergaard, 1994), and extracellularly between isolated cells (Hassinger et al., 1996). Astrocytes maintain K^{+} ionic balance (Wallraff et al., 2006), and have an important role in the reuptake and recycling of glutamate (Rothstein et al., 1994). Additionally, astrocytes provide metabolic support for neurons by producing lactate, which is taken up by neurons to produce ATP (Mächler et al., 2016).

Astrocytes can also contribute to the inflammatory response after perinatal brain injury. Reactive astrogliosis can occur where astrocytes undergo hypertrophy, increase expression of glial fibrillary protein and form a glial scar around a focal injury (Romero et al., 2014). However, astrogliosis responses have varied between different experimental paradigms. GFAP expression was increased but numbers of GFAP positive cells did not change in P7 rats at 24 h after HI (Odorcyk et al., 2017). There were increased GFAP positive cells and area fraction in preterm fetal sheep at 3 days after umbilical cord occlusion (Wassink et al., 2017). In contrast, there were no changes in the number and area fraction of GFAP positive cells in near-term fetal sheep at 7 days after carotid artery occlusion (Davidson et al., 2016). Moreover, there was a reduction in area fraction and the size of astrocytes in neonatal pigs at 3 days after hypoxia (Sullivan et al., 2010).

Astrocytes predominantly express Cx43, with some expression of Cx26, Cx30, Cx40, and Cx45 (Nagy et al., 1997, 2003; Dermietzel et al., 2000). The opening of astrocytic Cx43 hemichannels after HI may compromise the ability of astrocytes to maintain homeostasis and neuronal support. This in turn, can have detrimental effects on neuronal and oligodendrocyte survival, the propagation of seizures and secondary energy failure (Davidson et al., 2013a). Additionally, astrocytes cultures subjected to OGD/reperfusion increased ATP release through Cx43 hemichannels. Further, treating OGD/reperfusion microglial cultures with ATP or medium from astrocytes subjected to OGD/reperfusion induced microglial activation (Yin et al., 2018b). The potential mechanisms of microglial activation through ATP signaling is discussed further below.

MICROGLIA AND INFLAMMATION

Microglia are the innate immune cells of the brain; having an important role in immune surveillance under normal conditions (Nimmerjahn et al., 2005). Aside from their immune function, they are important for apoptosis and pruning of excessive neurons and synapses, and the phagocytosis of debris during brain development (Baburamani et al., 2014). After brain injury, however, microglia undergo proliferation and activation during the inflammatory response (Baburamani et al., 2014). Resting microglia commonly have ramified morphology and become amoeboid when activated. However, during development, microglia migrate in the brain in amoeboid form and change into a ramified morphology (Pierre et al., 2017).

Activated microglia have various roles, where the classically activated, or M1-like polarization facilitates the progression of inflammation and the alternatively activated, or M2-like polarization is involved in the resolution of inflammation (Bonestroo et al., 2013; Jaworska et al., 2017). The M1-like microglia express cluster of differentiation (CD)86, CD16, CD32, produce reactive oxygen species and nitric oxide synthase, proteases, a range of interleukins and inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Czeh et al., 2011; Barakat and Redzic, 2015; Fumagalli et al., 2015). The M2-like polarization express CD206 and arginase 1 and produce anti-inflammatory IL-10 and growth factors, favoring repair (Czeh et al., 2011; Barakat and Redzic, 2015; Fumagalli et al., 2015). However, the view that there is a clear polarization of the microglia phenotypes has been challenged in recent years, and currently it is hypothesized that microglial polarization is a continuum with M1 on one end, and M2 on the other (Cherry et al., 2014; Hellström et al., 2016).

In P7 rats, there was a marked increase in CD45/CD11b positive cells in injured areas at 24 h after transient MCAO. These cells were predominantly microglia and not infiltrated blood monocytes (Denker et al., 2007). Supporting this, microglia/macrophage counts were significantly elevated in the hippocampus from 1 day after common carotid artery ligation and hypoxia in P9 mice (human term brain maturation equivalent) (Ferrazzano et al., 2013). There may be a biphasic response of microglial upregulation, with an initial increase in microglial number in the hippocampus at 2 days, followed by a delayed increased in the striatum and cortex at 9 days, after common carotid artery ligation and hypoxia in P9 mice (Cikla et al., 2016). Similarly, classically activated CD11b/CD86 positive microglia from whole brain homogenates were upregulated at 24 h, followed by a secondary peak at 1 week, which was resolved to control levels by 2 weeks after HI (Rice-Vannucci model) in P10 mice (Winerdal et al., 2012). However, the alternative microglial activation markers were not assessed in that study. Significantly elevated counts of ionized calcium-binding adapter molecule 1 (Iba1) positive microglia in the intragyral white matter of the first and second parasagittal gyrus, and the periventricular white matter were present after 1 week of recovery from global cerebral ischemia in near-term fetal sheep (Davidson et al., 2015c, 2016). Moreover, Iba1 positive microglial number was still elevated in the same white matter regions as above at 21 days after severe HI induced by acute umbilical cord occlusion in preterm fetal sheep (van den Heuij et al., 2019). This longer term recovery highlights the chronic nature of inflammation after perinatal HI insults.

The functions of activated microglia appear to change over time after HI. The activation characterization of microglia and infiltrated macrophages has been assessed in P9 mice

after common carotid artery occlusion followed by hypoxia (Hellström et al., 2016). There was an increase in mRNA levels of both pro and anti-inflammatory genes at 24 h after the insult. Furthermore, there was a significant increase in classically activated CD86 positive microglia. In contrast, although absolute numbers of alternatively activated CD206 positive microglia increased, their relative proportion was reduced. Surprisingly, there was a population of microglia which did not express CD86 or CD206, highlighting the complexity of microglia activation polarization (Hellström et al., 2016). The early rise of pro-inflammatory markers is supported by an increase of IL-1β and TNF-α mRNA expression between 1 and 24 h after HI in P7 rats (Rice-Vannucci model) (Bona et al., 1999). In another study, using the same paradigm to induce HI in P7 rats, both pro-inflammatory (IL-1β, TNF-α) and anti-inflammatory (IL-10) mRNA expression increased at 3 h after the insult. At 24 h, TNF-α expression was still elevated, but was lower than at 3 h. In addition, anti-inflammatory cytokine TGF-β expression also increased at 24 h along with an increase of CD206 and Iba1 positive microglia (Bonestroo et al., 2013). However, in P7 rats, the majority of CD86 positive microglia were co-localized with IL-1β and only a small number were co-localized with arginase-1, at 6 days after carotid artery ligation and hypoxia (Jaworska et al., 2017). Although some studies indicate a trend toward resolution of inflammation over time, microglia remain upregulated even 3 weeks after HI in preterm fetal sheep (van den Heuij et al., 2019), and so further experimental studies are needed to determine the function of microglia at these later time points.

The increase in inflammatory cytokine release highlighted in the studies above is consistent with NLRP3 inflammasome activation (Shao et al., 2018). Both astrocytes and microglia are known to play key roles in the inflammasome pathway (for review see Song et al., 2017).

ATP RELEASE FROM MEMBRANE CHANNELS

Both connexin hemichannels and pannexin channels can contribute to ATP release into the extracellular space (Kang et al., 2008; Chekeni et al., 2010; Orellana et al., 2011; Bennett et al., 2012), although, as discussed above, pannexin channels may also be regulated (closed) by extracellular ATP (Qiu and Dahl, 2009). ATP is a high energy molecule, essential for driving many cellular processes in the body. It also has a crucial neurotransmitter and neuromodulatory role in the brain (Melani et al., 2005; Pedata et al., 2016). ATP can be co-released with other neurotransmitters or function as an extracellular signaling molecule on its own (Burnstock, 2009; Suurväli et al., 2017). However, under pathological conditions, it may have injurious effects acting as a DAMP (Melani et al., 2005; Pedata et al., 2016) but more crucially as an inflammasome signal 2 activator. Extracellular ATP can activate purinergic receptors, which can perpetuate inflammasome activity in a number of brain cell types, including microglia (Bours et al., 2011) (**Figure 2**).

The bulk of ATP production occurs in mitochondria through oxidative metabolism, but a small amount is produced through anaerobic glycolysis. The disruption of oxygen and glucose delivery during HI affects oxidative metabolism, attenuating the production of ATP (Bainbridge et al., 2014; Wassink et al., 2014). ATP is critical for the function of Na^+/K^+-ATPase, which maintain ionic gradients. When ATP is depleted, the failure to maintain cellular ionic homeostasis causes cytotoxic edema, which is indicated by a rise in cortical impedance starting soon after the onset of ischemia in near-term fetal sheep (Williams et al., 1993; Davidson et al., 2018b). We have previously shown that during profound asphyxia in near-term fetal sheep, there was a rapid increase in oxidized cytochrome oxidase at 2–6 min from the start of asphyxia (Drury et al., 2012). The increase in oxidized cytochrome oxidase indicate that there was a decreased availability of reducing equivalents in the mitochondrial electron transport chain, impairing ATP production. Additionally, a phosphorus magnetic resonance spectroscopy study showed that cerebral phosphocreatine and total nucleotide triphosphate decreased, whereas inorganic phosphate increased during HI in neonatal piglets (Bainbridge et al., 2014). During secondary energy failure at 24 and 48 h after HI in neonatal piglets, the cerebral phosphocreatine/inorganic phosphate and nucleotide triphosphate/exchangeable phosphate pool also decreased (Lorek et al., 1994).

Paradoxically, at a time when ATP production is impaired, there is augmented ATP release into the extracellular space after permanent MCAO in the adult rat brain (Melani et al., 2005, 2012). Microdialysis samples collected from the striatum showed that ATP concentrations were an average of 30 nmol/L before ischemia, which increased to an average of 50 nmol/L after permanent MCAO (Melani et al., 2012). When an ecto-ATPase inhibitor—hexapotassium dihydrogen monotitanoundecatungstocobaltate (II) tridecahydrate (PV4, 100 μmol/L) was used, the extracellular ATP concentrations were between 320 and 450 nmol/L (Melani et al., 2012). This is within the range of what was detected in hippocampal slices during reoxygenation after OGD (700 nmol/L), using a biosensor measured in real time (Frenguelli et al., 2007). However, extracellular ATP release has not been examined after HI in the developing brain.

The increase in extracellular ATP can lead to a subsequent increase in adenosine, as ATP is catabolized into adenosine, through the ATP → ADP → AMP → adenosine pathway, mediated by ecto-nucleotidase enzymes (Pedata et al., 2016). Adenosine concentrations increased by 90% during a 1 h period of fetal hypoxia induced by reduction of maternal fraction of inspired oxygen, compared to levels during normoxia in 0.8 gestation fetal sheep (Koos et al., 1997). The rise in adenosine concentrations was attributed to the degradation of AMP, as blocking the degradation of AMP to adenosine with adenosine 5′-(α,β-methylene) diphosphate (AOPCP), an ecto-5′-nucleotidase inhibitor, attenuated the hypoxia-induced rise in adenosine (Koos et al., 1997). In support, in the first hour after permanent MCAO in the adult rat brain, extracellular adenosine was mainly derived from ATP hydrolysis (Melani et al., 2012). This is in contrast to physiological conditions, when adenosine can be directly released from intracellular stores (Melani et al., 2012). Adenosine is very important to the initial metabolic suppression

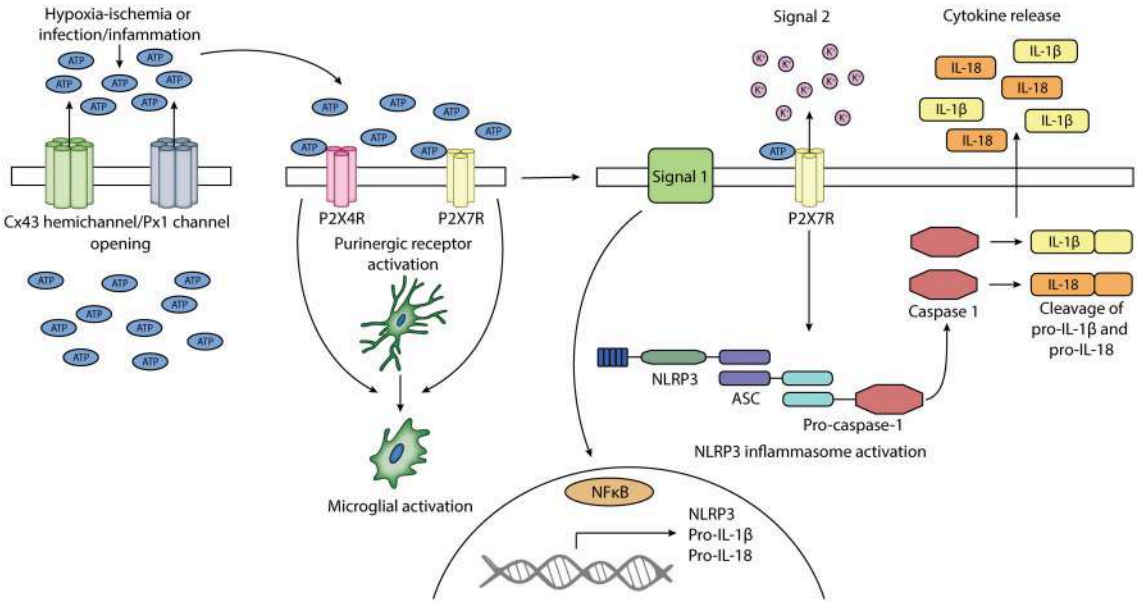


FIGURE 2 | Schematic diagram of the potential involvement of connexin 43 hemichannels and pannexin 1 channels and purinergic receptors P2X4 and P2X7 in the activation of microglia and NLRP3 inflammasome. Cx43 hemichannels and Px1 channels may open after exposure to HI or infection/inflammation. The opening of these channels leads to the release of ATP into the extracellular space. Extracellular ATP can activate purinergic receptors P2X4R and P2X7R. The activation of P2X4R and P2X7R can lead to the activation of microglia. Signal 1 of NLRP3 inflammasome activation is provided by PAMPs or DAMPs, which leads to the upregulation of NLRP3, pro-IL-1β, and pro-IL-18. Signal 2 is mediated by ATP activation of P2X7R and can lead to the assembly of NLRP3, ASC, and pro-caspase 1, forming the NLRP3 inflammasome. Pro-caspase 1 is activated into caspase 1, which cleaves pro-IL-1β and pro-IL-18 into their mature forms. Mature IL-1β and IL-18 is secreted.

that occurs at the beginning of an HI insult (Hunter et al., 2003). Blocking this response with the adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (2.5 mg/mL) in near-term fetal sheep before and during profound asphyxia, was associated with increased neuronal loss compared to the vehicle group (Hunter et al., 2003). Further studies are needed to examine the interaction of ATP and adenosine *in vivo*, in the developing brain after HI, and to investigate inflammasome complex assembly under those conditions. In parallel, it is necessary to consider the purinergic receptors themselves (see section Purinergic Receptors).

PURINERGIC RECEPTORS

Purinergic P1 and P2 receptors are activated by adenosine and ATP, respectively. Within the group of P2 receptors, there are P2X receptors, which are ionotropic and P2Y, which are metabotropic (Pedata et al., 2016). In this review, we will focus on the P2X receptors, which form trimeric cationic channels in the cell membrane (Habermacher et al., 2016). Of the seven P2X subtypes (P2X1-7), P2X4 receptor (P2X4R), and P2X7 receptor (P2X7R) have been more widely studied in the brain (Pedata et al., 2016). These channels open in response to ATP binding and are permeable to Na^+, K^+, and especially Ca^{2+} (Hattori and Gouaux, 2012). The activation of purinergic receptors can lead to the opening of more connexin hemichannels (and at least potentially pannexin channels), triggering further release of ATP in a process known as "ATP-induced ATP release" in a positive feedback loop (Stout et al., 2002; Baroja-Mazo et al., 2013).

P2X4 Receptor

P2X4R is one of the most sensitive P2X receptors, as it binds ATP in the nanomolar range, whereas P2X7R binds at a micromolar range (Suurväli et al., 2017). It also has the highest permeability to Ca^{2+}, and is the most abundantly expressed P2X subtype in the brain (Egan and Khakh, 2004; Cheng et al., 2014), found on neurons and glial cells, in various brain regions (Bo et al., 2003; Stokes et al., 2017; Suurväli et al., 2017). Under physiological conditions, P2X4R may have an important role in neurotransmission pathways (Suurväli et al., 2017). P2X4 knockout mice show deficits in sensorimotor tasks and social interactions, which was associated with altered subunit expression of glutamate and gamma-aminobutyric acid (GABA) receptors (Wyatt et al., 2013). As P2X4R is expressed abundantly in cells of myeloid origin, such as microglia and monocytes, they may play important roles in pro-inflammatory cytokine release following injury (Cavaliere et al., 2003).

P2X4R has been targeted for neuroprotection after HI. Global P2X4R knockout mice have a reduction in infarct volume and IL-1β and TNF-α release after MCAO compared to wild type mice (Verma et al., 2017). However, when P2X4R was knocked out in myeloid cells only, the neuroprotective effect was only seen in females. The role of P2X4R is complex, as the P2X4R knockout was associated with increased depressive-like behavior compared to wild type mice measured at 30 days after MCAO (Verma

et al., 2017). In addition, P2X4R on vascular endothelial cells was involved in neuroprotection via ischemic preconditioning after MCAO in adult mice, as the inhibition of P2X4R abolished this effect (Ozaki et al., 2016).

P2X4 Receptor and Activation of Microglia

Microglia and peripheral monocytes have been shown to express P2X4R, and electrical currents can be induced by ATP activation of P2X4R (Wang et al., 2004; Cheng et al., 2014). The expression of P2X4R is upregulated in organotypic hippocampal culture at 24 h after OGD (Cavaliere et al., 2003) In P0 rats, P2X4R protein expression increased from 4 h after hypoxia until 14 days (Li et al., 2011). However, in P3 rats, there was a significant increase in P2X4R protein expression only at 5 days after common carotid artery occlusion and hypoxia (Wixey et al., 2009). In both neonatal rat studies, P2X4R expression was predominantly colocalized with microglia (Wixey et al., 2009; Li et al., 2011). Although the increase in P2X4R at 5 days was not temporally correlated with the early increase in Iba1 protein expression seen at 2 days after HI, it correlated with the delayed increase in Iba1 protein expression from 6 days after HI. It was postulated that these P2X4R positive microglia may be a distinct population of microglia involved in neuroinflammation (Wixey et al., 2009). Furthermore, P2X4R blockade with trinitrophenyl (TNP)-ATP (20 μM) prevented the change of primary microglial cells to a more rounded morphology after hypoxia, and attenuated the increased IL-1β and TNF-α protein expression (Li et al., 2011).

P2X7 Receptor

Although, P2X7R has a lower affinity of 0.1–1 mM for ATP, when compared to P2X4R, the increased extracellular ATP levels after ischemia may be sufficient to activate it (Surprenant and North, 2009; Melani et al., 2012). The deleterious roles of the P2X7R include increasing intracellular Ca^{2+} concentrations, glutamate release and inflammasome activation (Di Virgilio, 2007; Matute et al., 2007; Rossi and Volterra, 2009; Ye et al., 2017). Additionally, prolonged ATP activation of P2X7R can transform the cationic channel into a large membrane pore, permeable to molecules 900 Da in size (Yan et al., 2010).

Animal studies have shown that P2X7R is upregulated after ischemia in the immature and adult brain. In a fetal rat model of intrauterine asphyxia, P2X7R expression in the hippocampus was upregulated 3-fold immediately after asphyxia, but returned to control levels at 60 min (Frizzo et al., 2010). In adult rats, however, cortical P2X7R expression was increased 5-fold compared to controls at 4 days after permanent MCAO (Franke et al., 2004). Whereas, in adult mice subjected to photothrombotic cerebral ischemia, cortical P2X7R expression was upregulated from 1 day post-ischemia until 5 days (Ye et al., 2017). In cortical neuronal culture, there was a significant upregulation of P2X7R after exposure to OGD (Ye et al., 2017). P2X7R expression was increased in the CA1 region of the hippocampus from 6 h and maintained until 7 days after global cerebral ischemia in adult rats (Yu et al., 2013). The differences in the timing of P2X7R upregulation could be due age, species, brain region and type of insult.

Blocking P2X7R has been shown to be beneficial for ischemic brain injury *in vitro* and in adult animals. In neuronal culture, Brilliant Blue G—a P2X7R blocker (50 nM or 5 μM), applied during OGD, reduced cell death measured at 24 h after OGD (Arbeloa et al., 2012). This was replicated *in vivo*; Brilliant Blue G (30 mg/kg, i.p.) administered at 30 min during a 90 min MCAO and continued daily in adult rats reduced infarct volume and neuronal loss when measured at 3 days after the insult (Arbeloa et al., 2012). In support, Brilliant Blue G treatment (50 mg/kg, i.v.) administered daily, for 3 days, starting immediately after occluding bilateral common carotid and vertebral arteries in adult rats, partially attenuated CA1 neuronal loss in the hippocampus at 7 days after injury (Yu et al., 2013). Although Brilliant Blue G has higher affinity for P2X7R, it can also bind to other P2X receptors at higher concentrations (Chu et al., 2012). However, the administration of a more selective P2X7R antagonist—A-438079 (3 μg, i.c.v.), prior to transient common carotid artery occlusion in adult rats, had similar neuroprotective effects to Brilliant Blue G (10 μg, i.c.v.) and Oxidized ATP (1 μg, i.c.v.), in terms of neuronal survival and motor performance (Chu et al., 2012). Additionally, in an adult rat model of intracerebral hemorrhage, silencing the P2X7R gene reduced NLRP3 inflammasome activation and release of IL-1β and IL-18 at 24 h after the insult (Feng et al., 2015). It is difficult to disentangle the timing of when P2X7R is involved in injury, as most of these studies above started blocking P2X7R either during or immediately after ischemia.

Several studies have shown that connexin hemichannels and pannexin channels are involved in purinergic signaling. For example, IL-1β release induced by P2X7R activation was associated with Px1 channels in macrophages (Pelegrin and Surprenant, 2006). When a Px1 blocker—mefloquine (1 mg/kg, i.p.) was co-administered with Brilliant Blue G (30 mg/kg twice per day, i.p.) to block P2X7R, starting at 30 min during 60 min of MCAO, there was no additive benefit when compared to administering either drug alone in adult mice after MCAO (Cisneros-Mejorado et al., 2015a). However, as stated earlier, mefloquine can also target connexin hemichannels (Cruikshank et al., 2004). A potential mechanism of injury may be ATP release, as both the prevention of ATP release, or inhibition of P2X7R may have beneficial effects (Cisneros-Mejorado et al., 2015b). For example, targeting Cx43 using Peptide5 at 0 h (10 mg/kg), 2 h (5 mg/kg), and 4 h (2.5 mg/kg, i.p.), after spinal cord injury in rats was associated with a reduction is tissue damage and improved functional recovery (Mao et al., 2017). Similarly, the administration of Brilliant Blue G to inhibit P2X7R (10 or 50 mg/kg daily for 3 days) starting 10–15 min after spinal cord injury in rats, reduced tissue damage and improved motor performance (Peng et al., 2009).

P2X7 Receptor and Activation of Microglia

In adult rats after MCAO, the expression of P2X7R was colocalized with microglia at 1 and 4 days, neurons at 4 and 7 days, and astrocytes at 4 days after the insult (Franke et al., 2004). *De novo* expression of P2X7R was observed on activated microglia at 24 h after MCAO in adult rats, with no expression seen in control animals (Melani et al., 2006). It has been debated

as to whether the overexpression of P2X7R drives the activation of microglia, or whether the overexpression of P2X7R is a result of microglial activation (Bai and Li, 2013). In support of the role of P2X7R in microglia activation, the overexpression of P2X7R by transfection of microglia in culture resulted in activation and proliferation of microglia (Monif et al., 2009). Additionally, P2X7R knockout mice showed significantly attenuated microglial activation at 72 h following MCAO compared to wild type mice (Kaiser et al., 2016). In the developing brain, both P2X7R and P2X4R were constitutively expressed in microglia in P3 rats, but P2X4R immunofluorescence was more intense than P2X7R. However, only the changes in P2X4R expression was examined after exposure to hypoxia in P0 rats (Li et al., 2011).

ACTIVATION OF THE INFLAMMASOME

The family of PRRs include Toll-like receptors (TRLs), NOD-like receptors (NLRs), and retinoic acid-inducible gene-I-like receptors (RLRs) which recognize PAMPs or DAMPs (Hagberg et al., 2015; Mallard et al., 2018). There is growing interest in the role of NLRs in inflammatory diseases, but relatively little is known about their role in perinatal brain injury. Inflammasomes are a multimeric complex of proteins which oligomerize after sensing PAMP or DAMP signals (Jo et al., 2016). Typically, NLR inflammsomes are formed by the assembly of the NLR family protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase 1. The 22 members of the NLR family are distinguished by their N-terminal effector domains, and grouped into NLRA, NLRB, NLRC and NLRP (Ting et al., 2008). One of the better characterized inflammasomes in the NLR family is the NOD-like receptor protein-3 (NLRP3) inflammasome. The activation of the NLRP3 inflammasome leads to the activation of caspase 1, the cleavage and release of mature IL-1β and IL-18 and subsequent release of other cytokines (Jo et al., 2016; Mugisho et al., 2018).

There are two steps to NLRP3 inflammasome activation. Signal 1 is a priming step, induced by PAMPS, such as microbial toxins and surface proteins, TLR ligands and viral RNA (Martinon et al., 2009; Jun et al., 2012; Chakrabarti et al., 2015) or DAMPs, such as amyloid-β, hyaluronan, monosodium urate, calcium pyrophosphate dehydrate and extracellular ATP (Martinon et al., 2009; Gong et al., 2018). Endogenous inflammatory cytokines including TNF-α and IL-1β can also act as priming signals (Franchi et al., 2009; He et al., 2016; Gong et al., 2018). The priming signal leads to the upregulation of NLRP3 and pro-IL-1β and pro-IL-18 expression through the nuclear factor kappa-light-chain-(NF-κB) translocation to the nucleus to trigger their transcription (He et al., 2016). Signal 2 of the NLRP3 inflammasome is the activation signal. This leads to assembly of the inflammasome complex. A clear model for activation remains unclear; much of the literature suggests that many of the same molecules involved in priming may have a secondary role in activation (Shao et al., 2018). That seems unlikely. Thus, is remains a significant question to define which molecular mechanism(s) trigger activation of the NLRP3 inflammasome (He et al., 2016; Jo et al., 2016). More recently, the list of potential activator signals has been narrowed down. Key players are now suggested to include: ATP, calcium signaling, and reactive oxygen species (He et al., 2016; Jo et al., 2016; Groslambert and Py, 2018). Mitochondrial dysfunction, lysosomal rupture and K^+ efflux are also reported to be activators (He et al., 2016; Gao et al., 2017), although it is unclear to what extent some of these may be effects of inflammation rather than initiators *per se*.

Potassium efflux can be mediated through the opening of endogenous ion channels or through bacterial pore forming toxins (Gao et al., 2017). Purinergic receptors have been associated with the efflux of K^+ (Yan et al., 2008) as have connexin hemichannels (Schalper et al., 2010; Leybaert et al., 2017). It is unclear how the reduction in intracellular K^+ leads to the activation of the inflammasome although studies have shown that it is a necessary step for the kinase Never In Mitosis A-Related Kinase 7 to bind to NLRP3, required for the assembly of the inflammasome (He et al., 2016; Shi et al., 2016). Additionally, K^+ efflux can lead to the disruption of mitochondrial function and production of mitochondrial reactive oxygen species (Tang et al., 2017). It is also of note that potassium efflux is induced by ATP suggesting that ATP release may therefore be the primary or upstream activator not only for potassium efflux, but also mitochondrial dysfunction and reactive oxygen species. Our work has shown that reducing ATP release on its own alone (by blocking connexin hemichannels alone) is sufficient to shut down inflammasome complex assembly, while the addition of exogenous ATP sufficient to reestablish assembly (Mugisho et al., 2018), consistent with the evidence that purinergic receptors play a key role, as outlined above and in **Table 1**.

Inflammasome Activation in Brain Injury

A large body of evidence in the adult brain shows that inflammation is a least in part mediated by the NLRP3 inflammasome in animal models of stroke, traumatic brain injury and subarachnoid hemorrhage with upregulation of mRNA or protein expression of NLRP3, ASC, caspase-1 and associated inflammatory cytokines, such as TNF-α, IL-18 and IL-6 (Gao et al., 2017; Ye et al., 2017; Ismael et al., 2018; Lee et al., 2018; Wang et al., 2018; Xu et al., 2018; Yin et al., 2018a). Additionally, clinical data show that children with severe traumatic brain injury (open/closed head injury not specified) had elevated levels of NLRP3 in cerebrospinal fluid collected between 0 to ∼72 h after the injury (Wallisch et al., 2017). NLRP3 and IL-1β mRNA expression was increased in the hippocampus, striatum and the thalamus at 24 h after common carotid artery ligation followed by hypoxia in P9 neonatal mice (Ystgaard et al., 2015). Supporting this, NLRP3 immunofluorescence increased at 24 h after common carotid artery ligation and hypoxia in P7 rats, which was predominantly colocalized with microglia (Chen et al., 2018). Additionally, there was an increase in IL-1β, caspase 1 and NLRP3 protein expression (Chen et al., 2018).

Targeting the NLRP3 inflammasome has neuroprotective effects in ischemic brain injury either through selective inhibition of NLRP3, or through anti-inflammatory drugs which affect the NLRP3 pathway (Ye et al., 2017; Chen et al., 2018; Ismael et al., 2018). The selective inhibition of NLRP3 using MCC950 (50 mg/kg, i.p. *in vivo* and 1 μM *in vitro*) reduced neuronal apoptosis

when delivered after photothrombotic cerebral ischemia in adult mice, and before OGD in neuronal cultures (Ye et al., 2017). In support, MCC950 (50 mg/kg, i.p.) administration at 1 and 3 h was associated with a reduction in infarction size and cerebral edema, and improved neurological deficit scores, after MCAO in adult mice. These findings were in turn associated with a decrease in protein levels of NLRP3, caspase 1 and ASC at 24 h after the insult (Ismael et al., 2018). Ginkgolide B—a component of Ginkgo biloba extracts (5–10 mg/kg, i.p.), which has anti-inflammatory effects, was administered to P7 rats 30 min prior to common carotid artery ligation and hypoxia and was associated with the attenuation of the increased expression of NLRP3, caspase 1 and IL-1β at 24 h after injury. These changes were accompanied by reduced infarct size and cerebral edema measured at 72 h (Chen et al., 2018). In contrast, NLRP3 knockout was not associated with neuroprotection in P9 mice, as infarction volume was not significantly different compared to wild type mice at 24 h after common carotid artery ligation and hypoxia (Ystgaard et al., 2015) and in some cases other NLRs or other pathways may be implicated.

Inflammasome Activation in Perinatal Infection/Inflammation

The studies discussed in this review predominantly focused on the role of connexin hemichannels and pannexin channels and purinergic receptors in experimental models of HI. Given that HI and infection/inflammation both lead to neuroinflammation, it is possible that both may be perpetuated through inflammasome pathway activation. In this section, we discuss the evidence for inflammasome activation in infection/inflammation in clinical and experimental studies.

Inflammasome activation, at least in adults, is an important inflammatory response to combat infection. For example, adult mice deficient in NLRP3, ASC, or caspase 1 have reduced survival rates compared to wild type mice after Group B Streptococcus infection (Costa et al., 2012). Group B Streptococcus is a Gram-positive bacterium that is common cause of life-threatening sepsis and meningitis in neonates and pregnant women (Henneke and Berner, 2006). Preterm and very-low-birthweight neonates are susceptible to neonatal sepsis likely due to increased risk of exposure to microbes (for example via mechanical ventilation, intravenous catheters and parenteral feeding). Secondly, they may have deficits in their innate and adaptive immune responses leading to insufficient inflammatory mediators (Wynn and Wong, 2010; Strunk et al., 2011). In view of the latter, a study of 21 preterm infants born 24–32 weeks showed that preterm umbilical cord blood monocytes, had a reduction in IL-1β secretion and an induction of NLRP3 expression, compared to term infants and adult peripheral monocytes after ATP/LPS stimulation (Sharma et al., 2015). However, IL-1β secretion from preterm peripheral blood monocytes (24–29 weeks of gestation) collected at an average of 15 post-natal days was comparable to adult monocytes, suggesting that the impairment in IL-1β secretion is restored shortly after birth (Sharma et al., 2015). A study of 72 neonates showed that IL-1β secretion from peripheral blood mononuclear cells did not differ between extremely preterm (born <28 weeks) and very preterm infants (28–32 weeks) on the fifth day of life. However, the peripheral blood mononuclear cells (collected within 24 h of late onset sepsis) from the extremely preterm group secreted higher levels of IL-1β, than the very preterm neonates (Zasada et al., 2018). It is unclear whether this gestational age dependent response is related to the higher counts of circulating mononuclear cells found in the extremely preterm group.

Conversely, the excessive activation of the inflammasome and release of pro-inflammatory cytokines in neonatal sepsis, can lead to multi-organ failure and death (Gentile et al., 2015). Caspase-1/11 knockout mice (P5-7) exposed to cecal slurry to induce polymicrobial intra-abdominal neonatal sepsis had greater survival rates compared to wild type mice (Gentile et al., 2015). Additionally, there was reduced IL-1β and IL-18 release at 2, 6, 18, and 24 h after the induction of sepsis. Surprisingly, genetic ablation of ASC or NLRP3 was not associated with a protective effect (Gentile et al., 2015). The knockout of ASC or NLRP3 may be insufficient for the complete elimination of caspase 1 activity as it is not solely activated by the NLRP3 inflammasome (Gentile et al., 2015). Further adverse effects are evidenced from infants who have rare genetic mutations leading to over activation of the NLRP3 inflammasome which manifests as neonatal-onset multi-system inflammatory disease (Aróstegui et al., 2010).

Recent evidence has demonstrated that increased inflammasome activity is associated with chorioamnionitis (Gomez-Lopez et al., 2017). A study of 70 pregnant women who had undergone preterm labor showed that the chorioamniotic membranes from those with acute histological chorioamnionitis compared to those without, had greater mRNA levels of inflammasome components, including NLRP3, and other NLR proteins, caspase 1, IL-1β, IL-18, and increased ASC and caspase 1 complex formation (Gomez-Lopez et al., 2017). Additionally, a study of amniotic fluid from 143 women showed that those who had undergone spontaneous preterm labor, with intra-amniotic infection/inflammation (positive culture for microorganisms in the amniotic fluid, or a white blood cell count of >100 cells/mm^3) had significantly higher caspase 1 levels compared to those without intra-amniotic infection/inflammation, delivering at term or preterm (Gotsch et al., 2008). Furthermore, umbilical cord blood monocytes from preterm infants with histological chorioamnionitis showed reduced caspase 1 activity compared to those without histological chorioamnionitis (Sharma et al., 2015).

Although NLRP3 mediated inflammation may be implicated in many diseases, further study of its involvement in perinatal brain injury after HI and infection/inflammation is necessary. Disentangling the temporal effects of inflammation is particularly important, as discussed earlier the role of inflammation can be both beneficial and deleterious.

CONCLUSION

Connexin and pannexin membrane channels can contribute to the evolution of perinatal brain injury. Connexin hemichannel, and to a lesser extent pannexin channel, blockade, has been

associated with neuroprotection in a number of models of brain injury. A potential mechanism of injury perpetuated by connexin hemichannels could be release of what may be a key inflammasome signal 2 activator, ATP. Extracellular ATP activates purinergic receptors, which in turn have been shown to be involved with the activation of microglia and the inflammasome complex. Blockade of purinergic receptors P2X4 and P2X7 has been protective in adult animal models of stroke but further studies are required to investigate the involvement of purinergic receptors in the propagation of perinatal brain injury, and in particular the timing of their contribution to spreading injury. However, although P2X4R and P2X7R have been implicated in microglial activation following HI insults, and could be targets for modulating microglial responses after perinatal brain injury, connexin hemichannel perpetuation of the inflammasome would be upstream of both. Increasing evidence suggests that modulating the cascade upstream of inflammasome activation may attenuate brain injury mediated through inflammation. Inflammasome activation should be investigated further in perinatal brain injury, as it may be an important mediator of deleterious inflammatory responses.

AUTHOR CONTRIBUTIONS

JD and KZ conceptualized this topical review. KZ, JD, AG, LB, and CG undertook manuscript writing and preparation of figures. All authors reviewed and edited this manuscript.

FUNDING

The authors' studies discussed in this review was funded by The Health Research Council of New Zealand, grant numbers: 16/003, 12/613, 14/216, 17/601 and the Marsden Fund, grant number 17-UOA232. JD holds a Sir Charles Hercus Fellowship from the Health Research Council of New Zealand (16/003). CG acknowledges support from Wendy and Bruce Hadden. KZ was supported by a University of Auckland Doctoral Scholarship.

REFERENCES

Alexander, D. B., and Goldberg, G. S. (2003). Transfer of biologically important molecules between cells through gap junction channels. *Curr. Med. Chem.* 10, 2045–2058. doi: 10.2174/0929867033456927

Arbeloa, J., Pérez-Samartín, A., Gottlieb, M., and Matute, C. (2012). P2X7 receptor blockade prevents ATP excitotoxicity in neurons and reduces brain damage after ischemia. *Neurobiol. Dis.* 45, 954–961. doi: 10.1016/j.nbd.2011.12.014

Aróstegui, J. I., Lopez Saldana, M. D., Pascal, M., Clemente, D., Aymerich, M., Balaguer, F., et al. (2010). A somatic NLRP3 mutation as a cause of a sporadic case of chronic infantile neurologic, cutaneous, articular syndrome/neonatal-onset multisystem inflammatory disease: novel evidence of the role of low-level mosaicism as the pathophysiologic mechanism underlying mendelian inherited diseases. *Arthritis Rheum.* 62, 1158–1166. doi: 10.1002/art.27342

Azzopardi, D., Wyatt, J. S., Cady, E. B., Delpy, D. T., Baudin, J., Stewart, A. L., et al. (1989). Prognosis of newborn infants with hypoxic-ischemic brain injury assessed by phosphorus magnetic resonance spectroscopy. *Pediatr. Res.* 25, 445–451. doi: 10.1203/00006450-198905000-00004

Baburamani, A. A., Supramaniam, V. G., Hagberg, H., and Mallard, C. (2014). Microglia toxicity in preterm brain injury. *Reprod. Toxicol.* 48, 106–112. doi: 10.1016/j.reprotox.2014.04.002

Bai, H. Y., and Li, A. P. (2013). P2X(7) receptors in cerebral ischemia. *Neurosci. Bull.* 29, 390–398. doi: 10.1007/s12264-013-1338-7

Bainbridge, A., Tachtsidis, I., Faulkner, S. D., Price, D., Zhu, T., Baer, E., et al. (2014). Brain mitochondrial oxidative metabolism during and after cerebral hypoxia-ischemia studied by simultaneous phosphorus magnetic-resonance and broadband near-infrared spectroscopy. *Neuroimage* 102, 173–183. doi: 10.1016/j.neuroimage.2013.08.016

Bao, L., Locovei, S., and Dahl, G. (2004). Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett.* 572, 65–68. doi: 10.1016/j.febslet.2004.07.009

Barakat, R., and Redzic, Z. (2015). Differential cytokine expression by brain microglia/macrophages in primary culture after oxygen glucose deprivation and their protective effects on astrocytes during anoxia. *Fluids Barriers CNS* 12:6. doi: 10.1186/s12987-015-0002-1

Baroja-Mazo, A., Barberà-Cremades, M., and Pelegrin, P. (2013). The participation of plasma membrane hemichannels to purinergic signaling. *Biochim. Biophys. Acta* 1828, 79–93. doi: 10.1016/j.bbamem.2012.01.002

Bartha, A. I., Foster-Barber, A., Miller, S. P., Vigneron, D. B., Glidden, D. V., Barkovich, A. J., et al. (2004). Neonatal encephalopathy: association of cytokines with MR spectroscopy and outcome. *Pediatr. Res.* 56, 960–966. doi: 10.1203/01.PDR.0000144819.45689.BB

Bennet, L., Booth, L., and Gunn, A. J. (2010). Potential biomarkers for hypoxic-ischemic encephalopathy. *Semin. Fetal Neonatal Med.* 15, 253–260. doi: 10.1016/j.siny.2010.05.007

Bennet, L., Dhillon, S., Lear, C. A., Van Den Heuij, L., King, V., Dean, J. M., et al. (2018). Chronic inflammation and impaired development of the preterm brain. *J. Reprod. Immunol.* 125, 45–55. doi: 10.1016/j.jri.2017.11.003

Bennet, L., Roelfsema, V., Pathipati, P., Quaedackers, J., and Gunn, A. J. (2006). Relationship between evolving epileptiform activity and delayed loss of mitochondrial activity after asphyxia measured by near-infrared spectroscopy in preterm fetal sheep. *J. Physiol.* 572, 141–154. doi: 10.1113/jphysiol.2006.105197

Bennett, M. V., Garré, J. M., Orellana, J. A., Bukauskas, F. F., Nedergaard, M., and Saez, J. C. (2012). Connexin and pannexin hemichannels in inflammatory responses of glia and neurons. *Brain Res.* 1487, 3–15. doi: 10.1016/j.brainres.2012.08.042

Bo, X., Kim, M., Nori, S. L., Schoepfer, R., Burnstock, G., and North, R. A. (2003). Tissue distribution of P2X4 receptors studied with an ectodomain antibody. *Cell Tissue Res.* 313, 159–165. doi: 10.1007/s00441-003-0758-5

Boassa, D., Ambrosi, C., Qiu, F., Dahl, G., Gaietta, G., and Sosinsky, G. (2007). Pannexin1 channels contain a glycosylation site that targets the hexamer to the plasma membrane. *J. Biol. Chem.* 282, 31733–31743. doi: 10.1074/jbc.M702422200

Bona, E., Andersson, A. L., Blomgren, K., Gilland, E., Puka-Sundvall, M., Gustafson, K., et al. (1999). Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. *Pediatr. Res.* 45, 500–509. doi: 10.1203/00006450-199904010-00008

Bonestroo, H. J., Nijboer, C. H., van Velthoven, C. T., Kavelaars, A., Hack, C. E., Van Bel, F., et al. (2013). Cerebral and hepatic inflammatory response after neonatal hypoxia-ischemia in newborn rats. *Dev. Neurosci.* 35, 197–211. doi: 10.1159/000346685

Bours, M. J., Dagnelie, P. C., Giuliani, A. L., Wesselius, A., and Di Virgilio, F. (2011). P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. *Front. Biosci. (Schol. Ed.)* 3, 1443–1456. doi: 10.2741/s235.

Bruzzone, R., Hormuzdi, S. G., Barbe, M. T., Herb, A., and Monyer, H. (2003). Pannexins, a family of gap junction proteins expressed in brain. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13644–13649. doi: 10.1073/pnas.2233464100

Burnstock, G. (2009). Purinergic cotransmission. *F1000 Biol. Rep.* 1:46. doi: 10.3410/B1-46

Cavaliere, F., Florenzano, F., Amadio, S., Fusco, F. R., Viscomi, M. T., D'ambrosi, N., et al. (2003). Up-regulation of P2X2, P2X4 receptor and ischemic cell death: prevention by P2 antagonists. *Neuroscience* 120, 85–98. doi: 10.1016/S0306-4522(03)00228-8

Cea, L. A., Balboa, E., Puebla, C., Vargas, A. A., Cisterna, B. A., Escamilla, R., et al. (2016). Dexamethasone-induced muscular atrophy is mediated by functional expression of connexin-based hemichannels. *Biochim. Biophys. Acta* 1862, 1891–1899. doi: 10.1016/j.bbadis.2016.07.003

Chakrabarti, A., Banerjee, S., Franchi, L., Loo, Y. M., Gale, M. Jr., Nunez, G., et al. (2015). RNase L activates the NLRP3 inflammasome during viral infections. *Cell Host Microbe* 17, 466–477. doi: 10.1016/j.chom.2015.02.010

Chekeni, F. B., Elliott, M. R., Sandilos, J. K., Walk, S. F., Kinchen, J. M., Lazarowski, E. R., et al. (2010). Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature* 467, 863–867. doi: 10.1038/nature09413

Chen, A., Xu, Y., and Yuan, J. (2018). Ginkgolide B ameliorates NLRP3 inflammasome activation after hypoxic-ischemic brain injury in the neonatal male rat. *Int. J. Dev. Neurosci.* 69, 106–111. doi: 10.1016/j.ijdevneu.2018.07.004

Cheng, R. D., Ren, J. J., Zhang, Y. Y., and Ye, X. M. (2014). P2X4 receptors expressed on microglial cells in post-ischemic inflammation of brain ischemic injury. *Neurochem. Int.* 67, 9–13. doi: 10.1016/j.neuint.2014.01.011

Cherry, J. D., Olschowka, J. A., and O'banion, M. K. (2014). Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J. Neuroinflammation* 11:98. doi: 10.1186/1742-2094-11-98

Chu, K., Yin, B., Wang, J., Peng, G., Liang, H., Xu, Z., et al. (2012). Inhibition of P2X7 receptor ameliorates transient global cerebral ischemia/reperfusion injury via modulating inflammatory responses in the rat hippocampus. *J. Neuroinflammation* 9:69. doi: 10.1186/1742-2094-9-69

Cikla, U., Chanana, V., Kintner, D. B., Covert, L., Dewall, T., Waldman, A., et al. (2016). Suppression of microglia activation after hypoxia-ischemia results in age-dependent improvements in neurologic injury. *J. Neuroimmunol.* 291, 18–27. doi: 10.1016/j.jneuroim.2015.12.004

Cisneros-Mejorado, A., Gottlieb, M., Cavaliere, F., Magnus, T., Koch-Nolte, F., Scemes, E., et al. (2015a). Blockade of P2X7 receptors or pannexin-1 channels similarly attenuates postischemic damage. *J. Cereb. Blood Flow Metab.* 35, 843–850. doi: 10.1038/jcbfm.2014.262

Cisneros-Mejorado, A., Pérez-Samartín, A., Gottlieb, M., and Matute, C. (2015b). ATP signaling in brain: release, excitotoxicity and potential therapeutic targets. *Cell. Mol. Neurobiol.* 35, 1–6. doi: 10.1007/s10571-014-0092-3

Contreras, J. E., Sánchez, H. A., Eugenin, E. A., Speidel, D., Theis, M., Willecke, K., et al. (2002). Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. *Proc. Natl. Acad. Sci. U.S.A.* 99, 495–500. doi: 10.1073/pnas.012589799

Costa, A., Gupta, R., Signorino, G., Malara, A., Cardile, F., Biondo, C., et al. (2012). Activation of the NLRP3 inflammasome by group B streptococci. *J. Immunol.* 188, 1953–1960. doi: 10.4049/jimmunol.1102543

Cruikshank, S. J., Hopperstad, M., Younger, M., Connors, B. W., Spray, D. C., and Srinivas, M. (2004). Potent block of Cx36 and Cx50 gap junction channels by mefloquine. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12364–12369. doi: 10.1073/pnas.0402044101

Czeh, M., Gressens, P., and Kaindl, A. M. (2011). The yin and yang of microglia. *Dev. Neurosci.* 33, 199–209. doi: 10.1159/000328989

Davidson, J. O., Dean, J. M., Fraser, M., Wassink, G., Andelius, T. C., Dhillon, S. K., et al. (2018a). Perinatal brain injury: mechanisms and therapeutic approaches. *Front. Biosci. (Landmark Ed.)* 23, 2204–2226. doi: 10.2741/4700

Davidson, J. O., Draghi, V., Whitham, S., Dhillon, S. K., Wassink, G., Bennet, L., et al. (2018b). How long is sufficient for optimal neuroprotection with cerebral cooling after ischemia in fetal sheep? *J. Cereb. Blood Flow Metab.* 38, 1047–1059. doi: 10.1177/0271678X17707671

Davidson, J. O., Drury, P. P., Green, C. R., Nicholson, L. F., Bennet, L., and Gunn, A. J. (2014). Connexin hemichannel blockade is neuroprotective after asphyxia in preterm fetal sheep. *PLoS ONE* 9:e96558. doi: 10.1371/journal.pone.0096558

Davidson, J. O., Green, C. R., Bennet, L., Nicholson, L. F., Danesh-Meyer, H., Carroll, S. J., et al. (2013a). A key role for connexin hemichannels in spreading ischemic brain injury. *Curr. Drug Targets* 14, 36–46. doi: 10.2174/138945013804806479

Davidson, J. O., Green, C. R., Nicholson, L. F., Bennet, L., and Gunn, A. J. (2013b). Connexin hemichannel blockade is neuroprotective after, but not during, global cerebral ischemia in near-term fetal sheep. *Exp. Neurol.* 248, 301–308. doi: 10.1016/j.expneurol.2013.06.026

Davidson, J. O., Green, C. R., Nicholson, L. F., O'carroll, S. J., Fraser, M., Bennet, L., et al. (2012). Connexin hemichannel blockade improves outcomes in a model of fetal ischemia. *Ann. Neurol.* 71, 121–132. doi: 10.1002/ana.22654

Davidson, J. O., Rout, A. L., Wassink, G., Yuill, C. A., Zhang, F. G., Green, C. R., et al. (2015a). Non-additive effects of delayed connexin hemichannel blockade and hypothermia after cerebral ischemia in near-term fetal sheep. *J. Cereb. Blood Flow Metab.* 35, 2052–2061. doi: 10.1038/jcbfm.2015.171

Davidson, J. O., Wassink, G., Van Den Heuij, L. G., Bennet, L., and Gunn, A. J. (2015b). Therapeutic hypothermia for neonatal hypoxic-ischemic encephalopathy–where to from here? *Front. Neurol.* 6:198. doi: 10.3389/fneur.2015.00198

Davidson, J. O., Wassink, G., Yuill, C. A., Zhang, F. G., Bennet, L., and Gunn, A. J. (2015c). How long is too long for cerebral cooling after ischemia in fetal sheep? *J. Cereb. Blood Flow Metab.* 35, 751–758. doi: 10.1038/jcbfm.2014.259

Davidson, J. O., Yuill, C. A., Zhang, F. G., Wassink, G., Bennet, L., and Gunn, A. J. (2016). Extending the duration of hypothermia does not further improve white matter protection after ischemia in term-equivalent fetal sheep. *Sci. Rep.* 6:25178. doi: 10.1038/srep25178

Decrock, E., De Bock, M., Wang, N., Bultynck, G., Giaume, C., Naus, C. C., et al. (2015). Connexin and pannexin signaling pathways, an architectural blueprint for CNS physiology and pathology? *Cell. Mol. Life Sci.* 72, 2823–2851. doi: 10.1007/s00018-015-1962-7

Decrock, E., De Vuyst, E., Vinken, M., Van Moorhem, M., Vranckx, K., Wang, N., et al. (2009). Connexin 43 hemichannels contribute to the propagation of apoptotic cell death in a rat C6 glioma cell model. *Cell Death Differ.* 16, 151–163. doi: 10.1038/cdd.2008.138

Denker, S. P., Ji, S., Dingman, A., Lee, S. Y., Derugin, N., Wendland, M. F., et al. (2007). Macrophages are comprised of resident brain microglia not infiltrating peripheral monocytes acutely after neonatal stroke. *J. Neurochem.* 100, 893–904. doi: 10.1111/j.1471-4159.2006.04162.x

Dermietzel, R., Gao, Y., Scemes, E., Vieira, D., Urban, M., Kremer, M., et al. (2000). Connexin43 null mice reveal that astrocytes express multiple connexins. *Brain Res. Brain Res. Rev.* 32, 45–56. doi: 10.1016/S0165-0173(99)00067-3

Di Virgilio, F. (2007). Liaisons dangereuses: P2X(7) and the inflammasome. *Trends Pharmacol. Sci.* 28, 465–472. doi: 10.1016/j.tips.2007.07.002

Drury, P. P., Bennet, L., Booth, L. C., Davidson, J. O., Wassink, G., and Gunn, A. J. (2012). Maturation of the mitochondrial redox response to profound asphyxia in fetal sheep. *PLOS ONE* 7:e39273. doi: 10.1371/journal.pone.0039273

Edwards, A. D., Brocklehurst, P., Gunn, A. J., Halliday, H., Juszczak, E., Levene, M., et al. (2010). Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. *BMJ* 340:c363. doi: 10.1136/bmj.c363

Egan, T. M., and Khakh, B. S. (2004). Contribution of calcium ions to P2X channel responses. *J. Neurosci.* 24, 3413–3420. doi: 10.1523/JNEUROSCI.5429-03.2004

Evans, W. H., De Vuyst, E., and Leybaert, L. (2006). The gap junction cellular internet: connexin hemichannels enter the signalling limelight. *Biochem. J.* 397, 1–14. doi: 10.1042/BJ20060175

Feng, L., Chen, Y., Ding, R., Fu, Z., Yang, S., Deng, X., et al. (2015). P2X7R blockade prevents NLRP3 inflammasome activation and brain injury in a rat model of intracerebral hemorrhage: involvement of peroxynitrite. *J. Neuroinflammation* 12:190. doi: 10.1186/s12974-015-0409-2

Ferrazzano, P., Chanana, V., Uluc, K., Fidan, E., Akture, E., Kintner, D. B., et al. (2013). Age-dependent microglial activation in immature brains after hypoxia- ischemia. *CNS Neurol. Disord. Drug Targets* 12, 338–349. doi: 10.2174/18715272131312030007

Fleiss, B., and Gressens, P. (2012). Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? *Lancet Neurol.* 11, 556–566. doi: 10.1016/S1474-4422(12)70058-3

Foster-Barber, A., Dickens, B., and Ferriero, D. M. (2001). Human perinatal asphyxia: correlation of neonatal cytokines with MRI and outcome. *Dev. Neurosci.* 23, 213–218. doi: 10.1159/000046146

Franchi, L., Eigenbrod, T., and Núñez, G. (2009). Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J. Immunol.* 183, 792–796. doi: 10.4049/jimmunol.0900173

Franke, H., Günther, A., Grosche, J., Schmidt, R., Rossner, S., Reinhardt, R., et al. (2004). P2X7 receptor expression after ischemia in the cerebral cortex of rats. *J. Neuropathol. Exp. Neurol.* 63, 686–699. doi: 10.1093/jnen/63.7.686

Freeman, L. C., and Ting, J. P. (2016). The pathogenic role of the inflammasome in neurodegenerative diseases. *J. Neurochem.* 136, 29–38. doi: 10.1111/jnc.13217

Freitas-Andrade, M., Bechberger, J. F., Macvicar, B. A., Viau, V., and Naus, C. C. (2017). Pannexin1 knockout and blockade reduces ischemic stroke injury in female, but not in male mice. *Oncotarget* 8, 36973–36983. doi: 10.18632/oncotarget.16937

Frenguelli, B. G., Wigmore, G., Llaudet, E., and Dale, N. (2007). Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. *J. Neurochem.* 101, 1400–1413. doi: 10.1111/j.1471-4159.2006.04425.x

Frizzo, J. K., Cardoso, M. P., De Assis, A. M., Perry, M. L., Volonté, C., and Frizzo, M. E. (2010). Effects of acute perinatal asphyxia in the rat hippocampus. *Cell. Mol. Neurobiol.* 30, 683–692. doi: 10.1007/s10571-009-9492-1

Fumagalli, S., Perego, C., Pischiutta, F., Zanier, E. R., and De Simoni, M. G. (2015). The ischemic environment drives microglia and macrophage function. *Front. Neurol.* 6:81. doi: 10.3389/fneur.2015.00081

Galinsky, R., Davidson, J. O., Dean, J. M., Green, C. R., Bennet, L., and Gunn, A. J. (2018a). Glia and hemichannels: key mediators of perinatal encephalopathy. *Neural Regen. Res.* 13, 181–189. doi: 10.4103/1673-5374.226378

Galinsky, R., Davidson, J. O., Lear, C. A., Bennet, L., Green, C. R., and Gunn, A. J. (2017). Connexin hemichannel blockade improves survival of striatal GABA-ergic neurons after global cerebral ischaemia in term-equivalent fetal sheep. *Sci. Rep.* 7:6304. doi: 10.1038/s41598-017-06683-1

Galinsky, R., Lear, C. A., Dean, J. M., Wassink, G., Dhillon, S. K., Fraser, M., et al. (2018b). Complex interactions between hypoxia-ischemia and inflammation in preterm brain injury. *Dev. Med. Child Neurol.* 60, 126–133. doi: 10.1111/dmcn.13629

Gao, L., Dong, Q., Song, Z., Shen, F., Shi, J., and Li, Y. (2017). NLRP3 inflammasome: a promising target in ischemic stroke. *Inflamm. Res.* 66, 17–24. doi: 10.1007/s00011-016-0981-7

Gentile, L. F., Cuenca, A. L., Cuenca, A. G., Nacionales, D. C., Ungaro, R., Efron, P. A., et al. (2015). Improved emergency myelopoiesis and survival in neonatal sepsis by caspase-1/11 ablation. *Immunology* 145, 300–311. doi: 10.1111/imm.12450

Gilroy, D., and De Maeyer, R. (2015). New insights into the resolution of inflammation. *Semin. Immunol.* 27, 161–168. doi: 10.1016/j.smim.2015.05.003

Gomes, P., Srinivas, S. P., Van Driessche, W., Vereecke, J., and Himpens, B. (2005). ATP release through connexin hemichannels in corneal endothelial cells. *Invest. Ophthalmol. Vis. Sci.* 46, 1208–1218. doi: 10.1167/iovs.04-1181

Gómez-Hernández, J. M., De Miguel, M., Larrosa, B., Gonzalez, D., and Barrio, L. C. (2003). Molecular basis of calcium regulation in connexin-32 hemichannels. *Proc. Natl. Acad. Sci. U.S.A.* 100, 16030–16035. doi: 10.1073/pnas.2530348100

Gomez-Lopez, N., Romero, R., Xu, Y., Garcia-Flores, V., Leng, Y., Panaitescu, B., et al. (2017). Inflammasome assembly in the chorioamniotic membranes during spontaneous labor at term. *Am. J. Reprod. Immunol.* 77:e12648. doi: 10.1111/aji.12648

Gong, T., Yang, Y., Jin, T., Jiang, W., and Zhou, R. (2018). Orchestration of NLRP3 inflammasome activation by ion fluxes. *Trends Immunol.* 39, 393–406. doi: 10.1016/j.it.2018.01.009

Gotsch, F., Romero, R., Chaiworapongsa, T., Erez, O., Vaisbuch, E., Espinoza, J., et al. (2008). Evidence of the involvement of caspase-1 under physiologic and pathologic cellular stress during human pregnancy: a link between the inflammasome and parturition. *J. Matern. Fetal Neonatal Med.* 21, 605–616. doi: 10.1080/14767050802212109

Groslambert, M., and Py, B. F. (2018). Spotlight on the NLRP3 inflammasome pathway. *J. Inflamm. Res.* 11, 359–374. doi: 10.2147/JIR.S141220

Gunn, A. J., Gunn, T. R., De Haan, H. H., Williams, C. E., and Gluckman, P. D. (1997). Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. *J. Clin. Invest.* 99, 248–256. doi: 10.1172/JCI119153

Gunn, A. J., Laptook, A. R., Robertson, N. J., Barks, J. D., Thoresen, M., Wassink, G., et al. (2017). Therapeutic hypothermia translates from ancient history in to practice. *Pediatr. Res.* 81, 202–209. doi: 10.1038/pr.2016.198

Habermacher, C., Dunning, K., Chataigneau, T., and Grutter, T. (2016). Molecular structure and function of P2X receptors. *Neuropharmacology* 104, 18–30. doi: 10.1016/j.neuropharm.2015.07.032

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Hassinger, T. D., Guthrie, P. B., Atkinson, P. B., Bennett, M. V., and Kater, S. B. (1996). An extracellular signaling component in propagation of astrocytic calcium waves. *Proc. Natl. Acad. Sci. U.S.A.* 93, 13268–13273. doi: 10.1073/pnas.93.23.13268

Hattori, M., and Gouaux, E. (2012). Molecular mechanism of ATP binding and ion channel activation in P2X receptors. *Nature* 485, 207–212. doi: 10.1038/nature11010

He, Y., Hara, H., and Núñez, G. (2016). Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem. Sci.* 41, 1012–1021. doi: 10.1016/j.tibs.2016.09.002

Hellström, E. N., Smith, P. L., Fleiss, B., Nair, S., Svedin, P., Wang, W., et al. (2016). Temporal characterization of microglia/macrophage phenotypes in a mouse model of neonatal hypoxic-ischemic brain injury. *Front. Cell. Neurosci.* 10:286. doi: 10.3389/fncel.2016.00286

Henneke, P., and Berner, R. (2006). Interaction of neonatal phagocytes with group B streptococcus: recognition and response. *Infect. Immun.* 74, 3085–3095. doi: 10.1128/IAI.01551-05

Hunter, C. J., Bennet, L., Power, G. G., Roelfsema, V., Blood, A. B., Quaedackers, J. S., et al. (2003). Key neuroprotective role for endogenous adenosine A1 receptor activation during asphyxia in the fetal sheep. *Stroke* 34, 2240–2245. doi: 10.1161/01.STR.0000083623.77327.CE

Ishikawa, M., Iwamoto, T., Nakamura, T., Doyle, A., Fukumoto, S., and Yamada, Y. (2011). Pannexin 3 functions as an ER Ca(2+) channel, hemichannel, and gap junction to promote osteoblast differentiation. *J. Cell Biol.* 193, 1257–1274. doi: 10.1083/jcb.201101050

Ismael, S., Zhao, L., Nasoohi, S., and Ishrat, T. (2018). Inhibition of the NLRP3-inflammasome as a potential approach for neuroprotection after stroke. *Sci. Rep.* 8:5971. doi: 10.1038/s41598-018-24350-x

Jaworska, J., Ziemka-Nalecz, M., Sypecka, J., and Zalewska, T. (2017). The potential neuroprotective role of a histone deacetylase inhibitor, sodium butyrate, after neonatal hypoxia-ischemia. *J. Neuroinflammation* 14:34. doi: 10.1186/s12974-017-0807-8

Jian, Z., Ding, S., Deng, H., Wang, J., Yi, W., Wang, L., et al. (2016). Probenecid protects against oxygen-glucose deprivation injury in primary astrocytes by regulating inflammasome activity. *Brain Res.* 1643, 123–129. doi: 10.1016/j.brainres.2016.05.002

Jo, E. K., Kim, J. K., Shin, D. M., and Sasakawa, C. (2016). Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol. Immunol.* 13, 148–159. doi: 10.1038/cmi.2015.95

Jun, H. K., Lee, S. H., Lee, H. R., and Choi, B. K. (2012). Integrin alpha5beta1 activates the NLRP3 inflammasome by direct interaction with a bacterial surface protein. *Immunity* 36, 755–768. doi: 10.1016/j.immuni.2012.05.002

Kaiser, M., Penk, A., Franke, H., Krugel, U., Norenberg, W., Huster, D., et al. (2016). Lack of functional P2X7 receptor aggravates brain edema development after middle cerebral artery occlusion. *Purinergic Signal.* 12, 453–463. doi: 10.1007/s11302-016-9511-x

Kang, J., Kang, N., Lovatt, D., Torres, A., Zhao, Z., Lin, J., et al. (2008). Connexin 43 hemichannels are permeable to ATP. *J. Neurosci.* 28, 4702–4711. doi: 10.1523/JNEUROSCI.5048-07.2008

Karpuk, N., Burkovetskaya, M., Fritz, T., Angle, A., and Kielian, T. (2011). Neuroinflammation leads to region-dependent alterations in astrocyte gap junction communication and hemichannel activity. *J. Neurosci.* 31, 414–425. doi: 10.1523/JNEUROSCI.5247-10.2011

Kawamura, M. Jr., Ruskin, D. N., and Masino, S. A. (2010). Metabolic autocrine regulation of neurons involves cooperation among pannexin hemichannels, adenosine receptors, and KATP channels. *J. Neurosci.* 30, 3886–3895. doi: 10.1523/JNEUROSCI.0055-10.2010

Kerur, N., Fukuda, S., Banerjee, D., Kim, Y., Fu, D., Apicella, I., et al. (2018). cGAS drives noncanonical-inflammasome activation in age-related macular degeneration. *Nat. Med.* 24, 50–61. doi: 10.1038/nm.4450

Kim, Y., and Green, C. R. (2016). "Assessing connexin hemichannel function during ischemic injury and reperfusion," in *Gap Junction Channels and Hemichannels*, eds D. Bai and J. C. Saez (Boca Raton, FL: CRC Press; Taylor & Francis Group), 169–188.

Koos, B. J., Kruger, L., and Murray, T. F. (1997). Source of extracellular brain adenosine during hypoxia in fetal sheep. *Brain Res.* 778, 439–442. doi: 10.1016/S0006-8993(97)01207-9

Korzeniewski, S. J., Allred, E. N., O'shea, T. M., Leviton, A., and Kuban, K. C. K. (2018). Elevated protein concentrations in newborn blood and the risks of autism spectrum disorder, and of social impairment, at age 10 years among infants born before the 28th week of gestation. *Transl. Psychiatry* 8:115. doi: 10.1038/s41398-018-0156-0

Kuban, K. C., Joseph, R. M., O'shea, T. M., Heeren, T., Fichorova, R. N., Douglass, L., et al. (2017). Circulating inflammatory-associated proteins in the first month of life and cognitive impairment at age 10 years in children born extremely preterm. *J. Pediatr.* 180, 116–123 e111. doi: 10.1016/j.jpeds.2016.09.054

Kumar, N. M., and Gilula, N. B. (1996). The gap junction communication channel. *Cell* 84, 381–388. doi: 10.1016/S0092-8674(00)81282-9

Larroque, B., Ancel, P. Y., Marret, S., Marchand, L., Andre, M., Arnaud, C., et al. (2008). Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study. *Lancet* 371, 813–820. doi: 10.1016/S0140-6736(08)60380-3

Lee, A. C., Kozuki, N., Blencowe, H., Vos, T., Bahalim, A., Darmstadt, G. L., et al. (2013). Intrapartum-related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990. *Pediatr. Res.* 74, 50–72. doi: 10.1038/pr.2013.206

Lee, S. W., Gajavelli, S., Spurlock, M. S., Andreoni, C., De Rivero Vaccari, J. P., Bullock, M. R., et al. (2018). Microglial inflammasome activation in penetrating ballistic-like brain injury. *J. Neurotrauma* 35, 1681–1693. doi: 10.1089/neu.2017.5530

Leybaert, L., Lampe, P. D., Dhein, S., Kwak, B. R., Ferdinandy, P., Beyer, E. C., et al. (2017). Connexins in cardiovascular and neurovascular health and disease: pharmacological implications. *Pharmacol. Rev.* 69, 396–478. doi: 10.1124/pr.115.012062

Li, F., Wang, L., Li, J. W., Gong, M., He, L., Feng, R., et al. (2011). Hypoxia induced amoeboid microglial cell activation in postnatal rat brain is mediated by ATP receptor P2X4. *BMC Neurosci.* 12:111. doi: 10.1186/1471-2202-12-111

Li, X., Zhao, H., Tan, X., Kostrzewa, R. M., Du, G., Chen, Y., et al. (2015). Inhibition of connexin43 improves functional recovery after ischemic brain injury in neonatal rats. *Glia* 63, 1553–1567. doi: 10.1002/glia.22826

Lin, C. Y., Chang, Y. C., Wang, S. T., Lee, T. Y., Lin, C. F., and Huang, C. C. (2010). Altered inflammatory responses in preterm children with cerebral palsy. *Ann. Neurol.* 68, 204–212. doi: 10.1002/ana.22049

Lorek, A., Takei, Y., Cady, E. B., Wyatt, J. S., Penrice, J., Edwards, A. D., et al. (1994). Delayed ("secondary") cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. *Pediatr. Res.* 36, 699–706. doi: 10.1203/00006450-199412000-00003

Maatouk, L., Yi, C., Carrillo-De Sauvage, M. A., Compagnion, A. C., Hunot, S., Ezan, P., et al. (2018). Glucocorticoid receptor in astrocytes regulates midbrain dopamine neurodegeneration through connexin hemichannel activity. *Cell Death Differ.* 26, 580–596. doi: 10.1038/s41418-018-0150-3

Mächler, P., Wyss, M. T., Elsayed, M., Stobart, J., Gutierrez, R., Von Faber-Castell, A., et al. (2016). *In vivo* evidence for a lactate gradient from astrocytes to neurons. *Cell Metab.* 23, 94–102. doi: 10.1016/j.cmet.2015.10.010

Mallard, C., Davidson, J. O., Tan, S., Green, C. R., Bennet, L., Robertson, N. J., et al. (2014). Astrocytes and microglia in acute cerebral injury underlying cerebral palsy associated with preterm birth. *Pediatr. Res.* 75, 234–240. doi: 10.1038/pr.2013.188

Mallard, C., Tremblay, M. E., and Vexler, Z. S. (2018). Microglia and neonatal brain injury. *Neuroscience*. doi: 10.1016/j.neuroscience.2018.01.023. [Epub ahead of print].

Mao, Y., Nguyen, T., Tonkin, R. S., Lees, J. G., Warren, C., O'carroll, S. J., et al. (2017). Characterisation of Peptide5 systemic administration for treating traumatic spinal cord injured rats. *Exp. Brain Res.* 235, 3033–3048. doi: 10.1007/s00221-017-5023-3

Markiewicz, I., and Lukomska, B. (2006). The role of astrocytes in the physiology and pathology of the central nervous system. *Acta Neurobiol. Exp. (Warsz).* 66, 343–358.

Marlow, N., Wolke, D., Bracewell, M. A., and Samara, M. (2005). Neurologic and developmental disability at six years of age after extremely preterm birth. *N. Engl. J. Med.* 352, 9–19. doi: 10.1056/NEJMoa041367

Martinon, F., Mayor, A., and Tschopp, J. (2009). The inflammasomes: guardians of the body. *Annu. Rev. Immunol.* 27, 229–265. doi: 10.1146/annurev.immunol.021908.132715

Matute, C., Torre, I., Perez-Cerda, F., Pérez-Samartín, A., Alberdi, E., Etxebarria, E., et al. (2007). P2X(7) receptor blockade prevents ATP excitotoxicity in oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis. *J. Neurosci.* 27, 9525–9533. doi: 10.1523/JNEUROSCI.0579-07.2007

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature* 449, 819–826. doi: 10.1038/nature06246

Melani, A., Amadio, S., Gianfriddo, M., Vannucchi, M. G., Volontè, C., Bernardi, G., et al. (2006). P2X7 receptor modulation on microglial cells and reduction of brain infarct caused by middle cerebral artery occlusion in rat. *J. Cereb. Blood Flow Metab.* 26, 974–982. doi: 10.1038/sj.jcbfm.9600250

Melani, A., Corti, F., Stephan, H., Müller, C. E., Donati, C., Bruni, P., et al. (2012). Ecto-ATPase inhibition: ATP and adenosine release under physiological and ischemic *in vivo* conditions in the rat striatum. *Exp. Neurol.* 233, 193–204. doi: 10.1016/j.expneurol.2011.09.036

Melani, A., Turchi, D., Vannucchi, M. G., Cipriani, S., Gianfriddo, M., and Pedata, F. (2005). ATP extracellular concentrations are increased in the rat striatum during *in vivo* ischemia. *Neurochem. Int.* 47, 442–448. doi: 10.1016/j.neuint.2005.05.014

Monif, M., Reid, C. A., Powell, K. L., Smart, M. L., and Williams, D. A. (2009). The P2X7 receptor drives microglial activation and proliferation: a trophic role for P2X7R pore. *J. Neurosci.* 29, 3781–3791. doi: 10.1523/JNEUROSCI.5512-08.2009

Mugisho, O. O., Green, C. R., Kho, D. T., Zhang, J., Graham, E. S., Acosta, M. L., et al. (2018). The inflammasome pathway is amplified and perpetuated in an autocrine manner through connexin43 hemichannel mediated ATP release. *Biochim. Biophys. Acta* 1862, 385–393. doi: 10.1016/j.bbagen.2017.11.015

Nagy, J. I., Ionescu, A. V., Lynn, B. D., and Rash, J. E. (2003). Coupling of astrocyte connexins Cx26, Cx30, Cx43 to oligodendrocyte Cx29, Cx32, Cx47: implications from normal and connexin32 knockout mice. *Glia* 44, 205–218. doi: 10.1002/glia.10278

Nagy, J. I., Ochalski, P. A., Li, J., and Hertzberg, E. L. (1997). Evidence for the co-localization of another connexin with connexin-43 at astrocytic gap junctions in rat brain. *Neuroscience* 78, 533–548. doi: 10.1016/S0306-4522(96)00584-2

Nakase, T., Maeda, T., Yoshida, Y., and Nagata, K. (2009). Ischemia alters the expression of connexins in the aged human brain. *J. Biomed. Biotechnol.* 2009:147946. doi: 10.1155/2009/147946

Nakase, T., Yoshida, Y., and Nagata, K. (2006). Enhanced connexin 43 immunoreactivity in penumbral areas in the human brain following ischemia. *Glia* 54, 369–375. doi: 10.1002/glia.20399

Natarajan, G., Laptook, A., and Shankaran, S. (2018). Therapeutic hypothermia: how can we optimize this therapy to further improve outcomes? *Clin. Perinatol.* 45, 241–255. doi: 10.1016/j.clp.2018.01.010

Nathan, C., and Ding, A. (2010). Nonresolving inflammation. *Cell* 140, 871–882. doi: 10.1016/j.cell.2010.02.029

Nedergaard, M. (1994). Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* 263, 1768–1771. doi: 10.1126/science.8134839

Ness, J. K., Romanko, M. J., Rothstein, R. P., Wood, T. L., and Levison, S. W. (2001). Perinatal hypoxia-ischemia induces apoptotic and excitotoxic death of periventricular white matter oligodendrocyte progenitors. *Dev. Neurosci.* 23, 203–208. doi: 10.1159/000046144

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science* 308, 1314–1318. doi: 10.1126/science.1110647

Northington, F. J., Chavez-Valdez, R., and Martin, L. J. (2011). Neuronal cell death in neonatal hypoxia-ischemia. *Ann. Neurol.* 69, 743–758. doi: 10.1002/ana.22419

Northington, F. J., Zelaya, M. E., O'riordan, D. P., Blomgren, K., Flock, D. L., Hagberg, H., et al. (2007). Failure to complete apoptosis following neonatal hypoxia-ischemia manifests as "continuum" phenotype of cell death and occurs with multiple manifestations of mitochondrial dysfunction in rodent forebrain. *Neuroscience* 149, 822–833. doi: 10.1016/j.neuroscience.2007.06.060

Odorcyk, F. K., Nicola, F., Duran-Carabali, L. E., Figueiro, F., Kolling, J., Vizuete, A., et al. (2017). Galantamine administration reduces reactive astrogliosis and upregulates the anti-oxidant enzyme catalase in rats submitted to neonatal hypoxia ischemia. *Int. J. Dev. Neurosci.* 62, 15–24. doi: 10.1016/j.ijdevneu.2017.07.006

Orellana, J. A., Froger, N., Ezan, P., Jiang, J. X., Bennett, M. V., Naus, C. C., et al. (2011). ATP and glutamate released via astroglial connexin 43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels. *J. Neurochem.* 118, 826–840. doi: 10.1111/j.1471-4159.2011.07210.x

Orellana, J. A., Hernandez, D. E., Ezan, P., Velarde, V., Bennett, M. V., Giaume, C., et al. (2010). Hypoxia in high glucose followed by reoxygenation in normal glucose reduces the viability of cortical astrocytes through increased permeability of connexin 43 hemichannels. *Glia* 58, 329–343. doi: 10.1002/glia.20926

O'Shea, T. M., Allred, E. N., Kuban, K. C., Dammann, O., Paneth, N., Fichorova, R., et al. (2012). Elevated concentrations of inflammation-related proteins in postnatal blood predict severe developmental delay at 2 years of age in extremely preterm infants. *J. Pediatr.* 160, 395–401 e394. doi: 10.1016/j.jpeds.2011.08.069

Ozaki, T., Muramatsu, R., Sasai, M., Yamamoto, M., Kubota, Y., Fujinaka, T., et al. (2016). The P2X4 receptor is required for neuroprotection via ischemic preconditioning. *Sci. Rep.* 6:25893. doi: 10.1038/srep25893

Panchin, Y. V. (2005). Evolution of gap junction proteins–the pannexin alternative. *J. Exp. Biol.* 208, 1415–1419. doi: 10.1242/jeb.01547

Patterson, Z. R., and Holahan, M. R. (2012). Understanding the neuroinflammatory response following concussion to develop treatment strategies. *Front. Cell. Neurosci.* 6:58. doi: 10.3389/fncel.2012.00058

Paul, D. L., Ebihara, L., Takemoto, L. J., Swenson, K. I., and Goodenough, D. A. (1991). Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of Xenopus oocytes. *J. Cell Biol.* 115, 1077–1089. doi: 10.1083/jcb.115.4.1077

Pedata, F., Dettori, I., Coppi, E., Melani, A., Fusco, I., Corradetti, R., et al. (2016). Purinergic signalling in brain ischemia. *Neuropharmacology* 104, 105–130. doi: 10.1016/j.neuropharm.2015.11.007

Pelegrin, P., and Surprenant, A. (2006). Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J.* 25, 5071–5082. doi: 10.1038/sj.emboj.7601378

Peng, W., Cotrina, M. L., Han, X., Yu, H., Bekar, L., Blum, L., et al. (2009). Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12489–12493. doi: 10.1073/pnas.0902531106

Pierre, W. C., Smith, P. L., Londono, I., Chemtob, S., Mallard, C., and Lodygensky, G. A. (2017). Neonatal microglia: the cornerstone of brain fate. *Brain. Behav. Immun.* 59, 333–345. doi: 10.1016/j.bbi.2016.08.018

Puka-Sundvall, M., Gajkowska, B., Cholewinski, M., Blomgren, K., Lazarewicz, J. W., and Hagberg, H. (2000). Subcellular distribution of calcium and ultrastructural changes after cerebral hypoxia-ischemia in immature rats. *Brain Res. Dev. Brain Res.* 125, 31–41. doi: 10.1016/S0165-3806(00)00110-3

Qiu, F., and Dahl, G. (2009). A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP. *Am. J. Physiol. Cell Physiol.* 296, C250–C255. doi: 10.1152/ajpcell.00433.2008

Retamal, M. A., Froger, N., Palacios-Prado, N., Ezan, P., Sáez, P. J., Sáez, J. C., et al. (2007). Cx43 hemichannels and gap junction channels in astrocytes are regulated oppositely by proinflammatory cytokines released from activated microglia. *J. Neurosci.* 27, 13781–13792. doi: 10.1523/JNEUROSCI.2042-07.2007

Rice, J. E., Vannucci, R. C., and Brierley, J. B. (1981). The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann. Neurol.* 9, 131–141. doi: 10.1002/ana.410090206

Riquelme, M. A., Cea, L. A., Vega, J. L., Boric, M. P., Monyer, H., Bennett, M. V., et al. (2013). The ATP required for potentiation of skeletal muscle contraction is released via pannexin hemichannels. *Neuropharmacology* 75, 594–603. doi: 10.1016/j.neuropharm.2013.03.022

Romanko, M. J., Rothstein, R. P., and Levison, S. W. (2004). Neural stem cells in the subventricular zone are resilient to hypoxia/ischemia whereas progenitors are vulnerable. *J. Cereb. Blood Flow Metab.* 24, 814–825. doi: 10.1097/01.WCB.0000123906.17746.00

Romero, J., Muñiz, J., Logica Tornatore, T., Holubiec, M., Gonzalez, J., Barreto, G. E., et al. (2014). Dual role of astrocytes in perinatal asphyxia injury and neuroprotection. *Neurosci. Lett.* 565, 42–46. doi: 10.1016/j.neulet.2013.10.046

Rossi, D., and Volterra, A. (2009). Astrocytic dysfunction: insights on the role in neurodegeneration. *Brain Res. Bull.* 80, 224–232. doi: 10.1016/j.brainresbull.2009.07.012

Rothstein, J. D., Martin, L., Levey, A. I., Dykes-Hoberg, M., Jin, L., Wu, D., et al. (1994). Localization of neuronal and glial glutamate transporters. *Neuron* 13, 713–725. doi: 10.1016/0896-6273(94)90038-8

Sáez, J. C., Retamal, M. A., Basilio, D., Bukauskas, F. F., and Bennett, M. V. (2005). Connexin-based gap junction hemichannels: gating mechanisms. *Biochim. Biophys. Acta* 1711, 215–224. doi: 10.1016/j.bbamem.2005.01.014

Santiago, M. F., Veliskova, J., Patel, N. K., Lutz, S. E., Caille, D., Charollais, A., et al. (2011). Targeting pannexin1 improves seizure outcome. *PLoS ONE* 6:e25178. doi: 10.1371/journal.pone.0025178

Schalper, K. A., Sanchez, H. A., Lee, S. C., Altenberg, G. A., Nathanson, M. H., and Saez, J. C. (2010). Connexin 43 hemichannels mediate the Ca^{2+} influx induced by extracellular alkalinization. *Am. J. Physiol. Cell Physiol.* 299, C1504–C1515. doi: 10.1152/ajpcell.00015.2010

Shankaran, S., Laptook, A. R., Ehrenkranz, R. A., Tyson, J. E., Mcdonald, S. A., Donovan, E. F., et al. (2005). Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N. Engl. J. Med.* 353, 1574–1584. doi: 10.1056/NEJMcps050929

Shao, B. Z., Cao, Q., and Liu, C. (2018). Targeting NLRP3 inflammasome in the treatment of CNS diseases. *Front. Mol. Neurosci.* 11:320. doi: 10.3389/fnmol.2018.00320

Sharma, A. A., Jen, R., Kan, B., Sharma, A., Marchant, E., Tang, A., et al. (2015). Impaired NLRP3 inflammasome activity during fetal development regulates IL-1beta production in human monocytes. *Eur. J. Immunol.* 45, 238–249. doi: 10.1002/eji.201444707

Shi, H., Wang, Y., Li, X., Zhan, X., Tang, M., Fina, M., et al. (2016). NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. *Nat. Immunol.* 17, 250–258. doi: 10.1038/ni.3333

Song, L., Pei, L., Yao, S., Wu, Y., and Shang, Y. (2017). NLRP3 inflammasome in neurological diseases, from functions to therapies. *Front. Cell. Neurosci.* 11:63. doi: 10.3389/fncel.2017.00063

Stokes, L., Layhadi, J. A., Bibic, L., Dhuna, K., and Fountain, S. J. (2017). P2X4 receptor function in the nervous system and current breakthroughs in pharmacology. *Front. Pharmacol* 8:291. doi: 10.3389/fphar.2017.00291

Stout, C. E., Costantin, J. L., Naus, C. C., and Charles, A. C. (2002). Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. *J. Biol. Chem.* 277, 10482–10488. doi: 10.1074/jbc.M109902200

Strunk, T., Currie, A., Richmond, P., Simmer, K., and Burgner, D. (2011). Innate immunity in human newborn infants: prematurity means more than immaturity. *J. Matern. Fetal Neonatal Med.* 24, 25–31. doi: 10.3109/14767058.2010.482605

Sullivan, S. M., Björkman, S. T., Miller, S. M., Colditz, P. B., and Pow, D. V. (2010). Morphological changes in white matter astrocytes in response to hypoxia/ischemia in the neonatal pig. *Brain Res.* 1319, 164–174. doi: 10.1016/j.brainres.2010.01.010

Surprenant, A., and North, R. A. (2009). Signaling at purinergic P2X receptors. *Annu. Rev. Physiol.* 71, 333–359. doi: 10.1146/annurev.physiol.70.113006.100630

Suurväli, J., Boudinot, P., Kanellopoulos, J., and Ruutel Boudinot, S. (2017). P2X4: A fast and sensitive purinergic receptor. *Biomed. J.* 40, 245–256. doi: 10.1016/j.bj.2017.06.010

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell* 140, 805–820. doi: 10.1016/j.cell.2010.01.022

Tang, T., Lang, X., Xu, C., Wang, X., Gong, T., Yang, Y., et al. (2017). CLICs-dependent chloride efflux is an essential and proximal upstream event for NLRP3 inflammasome activation. *Nat. Commun.* 8:202. doi: 10.1038/s41467-017-00227-x

Theis, M., Sohl, G., Eiberger, J., and Willecke, K. (2005). Emerging complexities in identity and function of glial connexins. *Trends Neurosci.* 28, 188–195. doi: 10.1016/j.tins.2005.02.006

Thompson, R. J., Jackson, M. F., Olah, M. E., Rungta, R. L., Hines, D. J., Beazely, M. A., et al. (2008). Activation of pannexin-1 hemichannels augments aberrant bursting in the hippocampus. *Science* 322, 1555–1559. doi: 10.1126/science.1165209

Thompson, R. J., Zhou, N., and Macvicar, B. A. (2006). Ischemia opens neuronal gap junction hemichannels. *Science* 312, 924–927. doi: 10.1126/science.1126241

Thornton, J. S., Ordidge, R. J., Penrice, J., Cady, E. B., Amess, P. N., Punwani, S., et al. (1998). Temporal and anatomical variations of brain water

apparent diffusion coefficient in perinatal cerebral hypoxic-ischemic injury: relationships to cerebral energy metabolism. *Magn. Reson. Med.* 39, 920–927. doi: 10.1002/mrm.1910390609

Ting, J. P., Lovering, R. C., Alnemri, E. S., Bertin, J., Boss, J. M., Davis, B. K., et al. (2008). The NLR gene family: a standard nomenclature. *Immunity* 28, 285–287. doi: 10.1016/j.immuni.2008.02.005

Tonkin, R. S., Bowles, C., Perera, C. J., Keating, B. A., Makker, P. G. S., Duffy, S. S., et al. (2018). Attenuation of mechanical pain hypersensitivity by treatment with Peptide5, a connexin-43 mimetic peptide, involves inhibition of NLRP3 inflammasome in nerve-injured mice. *Exp. Neurol.* 300, 1–12. doi: 10.1016/j.expneurol.2017.10.016

Turner, M. D., Nedjai, B., Hurst, T., and Pennington, D. J. (2014). Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochim. Biophys. Acta* 1843, 2563–2582. doi: 10.1016/j.bbamcr.2014.05.014

Unger, V. M., Kumar, N. M., Gilula, N. B., and Yeager, M. (1999). Three-dimensional structure of a recombinant gap junction membrane channel. *Science* 283, 1176–1180. doi: 10.1126/science.283.5405.1176

van den Heuij, L. G., Fraser, M., Miller, S. L., Jenkin, G., Wallace, E. M., Davidson, J. O., et al. (2019). Delayed intranasal infusion of human amnion epithelial cells improves white matter maturation after asphyxia in preterm fetal sheep. *J. Cereb. Blood Flow Metab.* 39, 223–239. doi: 10.1177/0271678X17729954

Vannucci, R. C. (2000). Hypoxic-ischemic encephalopathy. *Am. J. Perinatol.* 17, 113–120. doi: 10.1055/s-2000-9293

Verma, R., Cronin, C. G., Hudobenko, J., Venna, V. R., Mccullough, L. D., and Liang, B. T. (2017). Deletion of the P2X4 receptor is neuroprotective acutely, but induces a depressive phenotype during recovery from ischemic stroke. *Brain. Behav. Immun.* 66, 302–312. doi: 10.1016/j.bbi.2017.07.155

Vogt, A., Hormuzdi, S. G., and Monyer, H. (2005). Pannexin1 and Pannexin2 expression in the developing and mature rat brain. *Brain Res. Mol. Brain Res.* 141, 113–120. doi: 10.1016/j.molbrainres.2005.08.002

Wallisch, J. S., Simon, D. W., Bayir, H., Bell, M. J., Kochanek, P. M., and Clark, R. S. B. (2017). Cerebrospinal fluid NLRP3 is increased after severe traumatic brain injury in infants and children. *Neurocrit. Care* 27, 44–50. doi: 10.1007/s12028-017-0378-7

Wallraff, A., Kohling, R., Heinemann, U., Theis, M., Willecke, K., and Steinhauser, C. (2006). The impact of astrocytic gap junctional coupling on potassium buffering in the hippocampus. *J. Neurosci.* 26, 5438–5447. doi: 10.1523/JNEUROSCI.0037-06.2006

Wang, D., Xu, X., Wu, Y. G., Lyu, L., Zhou, Z. W., and Zhang, J. N. (2018). Dexmedetomidine attenuates traumatic brain injury: action pathway and mechanisms. *Neural Regen. Res.* 13, 819–826. doi: 10.4103/1673-5374.232529

Wang, J., Ma, A., Xi, J., Wang, Y., and Zhao, B. (2014). Connexin 43 and its hemichannels mediate hypoxia-ischemia-induced cell death in neonatal rats. *Child Neurol. Open* 1:2329048X14544955. doi: 10.1177/2329048X14544955

Wang, L., Jacobsen, S. E., Bengtsson, A., and Erlinge, D. (2004). P2 receptor mRNA expression profiles in human lymphocytes, monocytes and $CD34^+$ stem and progenitor cells. *BMC Immunol.* 5:16. doi: 10.1186/1471-2172-5-16

Wang, N., De Bock, M., Decrock, E., Bol, M., Gadicherla, A., Bultynck, G., et al. (2013). Connexin targeting peptides as inhibitors of voltage- and intracellular Ca^{2+}-triggered Cx43 hemichannel opening. *Neuropharmacology* 75, 506–516. doi: 10.1016/j.neuropharm.2013.08.021

Wassink, G., Davidson, J. O., Dhillon, S. K., Fraser, M., Galinsky, R., Bennet, L., et al. (2017). Partial white and grey matter protection with prolonged infusion of recombinant human erythropoietin after asphyxia in preterm fetal sheep. *J. Cereb. Blood Flow Metab.* 37, 1080–1094. doi: 10.1177/0271678X16650455

Wassink, G., Davidson, J. O., Lear, C. A., Juul, S. E., Northington, F., Bennet, L., et al. (2018). A working model for hypothermic neuroprotection. *J. Physiol.* 596, 5641–5654 doi: 10.1113/JP274928

Wassink, G., Gunn, E. R., Drury, P. P., Bennet, L., and Gunn, A. J. (2014). The mechanisms and treatment of asphyxial encephalopathy. *Front. Neurosci.* 8:40. doi: 10.3389/fnins.2014.00040

Wei, R., Wang, J., Xu, Y., Yin, B., He, F., Du, Y., et al. (2015). Probenecid protects against cerebral ischemia/reperfusion injury by inhibiting lysosomal and inflammatory damage in rats. *Neuroscience* 301, 168–177. doi: 10.1016/j.neuroscience.2015.05.070

Weilinger, N. L., Tang, P. L., and Thompson, R. J. (2012). Anoxia-induced NMDA receptor activation opens pannexin channels via Src family kinases. *J. Neurosci.* 32, 12579–12588. doi: 10.1523/JNEUROSCI.1267-12.2012

Willebrords, J., Crespo Yanguas, S., Maes, M., Decrock, E., Wang, N., Leybaert, L., et al. (2016). Connexins and their channels in inflammation. *Crit. Rev. Biochem. Mol. Biol.* 51, 413–439. doi: 10.1080/10409238.2016.1204980

Williams, C. E., Gunn, A., and Gluckman, P. D. (1991). Time course of intracellular edema and epileptiform activity following prenatal cerebral ischemia in sheep. *Stroke* 22, 516–521. doi: 10.1161/01.STR.22.4.516

Williams, C. E., Gunn, A. J., Mallard, C., and Gluckman, P. D. (1992). Outcome after ischemia in the developing sheep brain: an electroencephalographic and histological study. *Ann. Neurol.* 31, 14–21. doi: 10.1002/ana.410310104

Williams, C. E., Mallard, C., Tan, W., and Gluckman, P. D. (1993). Pathophysiology of perinatal asphyxia. *Clin. Perinatol.* 20, 305–325. doi: 10.1016/S0095-5108(18)30395-6

Winerdal, M., Winerdal, M. E., Kinn, J., Urmaliya, V., Winqvist, O., and Aden, U. (2012). Long lasting local and systemic inflammation after cerebral hypoxic ischemia in newborn mice. *PLoS ONE* 7:e36422. doi: 10.1371/journal.pone.0036422

Wixey, J. A., Reinebrant, H. E., Carty, M. L., and Buller, K. M. (2009). Delayed P2X4R expression after hypoxia-ischemia is associated with microglia in the immature rat brain. *J. Neuroimmunol.* 212, 35–43. doi: 10.1016/j.jneuroim.2009.04.016

Wyatt, L. R., Godar, S. C., Khoja, S., Jakowec, M. W., Alkana, R. L., Bortolato, M., et al. (2013). Sociocommunicative and sensorimotor impairments in male P2X4-deficient mice. *Neuropsychopharmacology* 38, 1993–2002. doi: 10.1038/npp.2013.98

Wynn, J. L., and Wong, H. R. (2010). Pathophysiology and treatment of septic shock in neonates. *Clin. Perinatol.* 37, 439–479. doi: 10.1016/j.clp.2010.04.002

Xu, X., Yin, D., Ren, H., Gao, W., Li, F., Sun, D., et al. (2018). Selective NLRP3 inflammasome inhibitor reduces neuroinflammation and improves long-term neurological outcomes in a murine model of traumatic brain injury. *Neurobiol. Dis.* 117, 15–27. doi: 10.1016/j.nbd.2018.05.016

Yan, Z., Khadra, A., Li, S., Tomic, M., Sherman, A., and Stojilkovic, S. S. (2010). Experimental characterization and mathematical modeling of P2X7 receptor channel gating. *J. Neurosci.* 30, 14213–14224. doi: 10.1523/JNEUROSCI.2390-10.2010

Yan, Z., Li, S., Liang, Z., Tomic, M., and Stojilkovic, S. S. (2008). The P2X7 receptor channel pore dilates under physiological ion conditions. *J. Gen. Physiol.* 132, 563–573. doi: 10.1085/jgp.200810059

Ye, X., Shen, T., Hu, J., Zhang, L., Zhang, Y., Bao, L., et al. (2017). Purinergic 2X7 receptor/NLRP3 pathway triggers neuronal apoptosis after ischemic stroke in the mouse. *Exp. Neurol.* 292, 46–55. doi: 10.1016/j.expneurol.2017.03.002

Ye, Z. C., Wyeth, M. S., Baltan-Tekkok, S., and Ransom, B. R. (2003). Functional hemichannels in astrocytes: a novel mechanism of glutamate release. *J. Neurosci.* 23, 3588–3596. doi: 10.1523/JNEUROSCI.23-09-03588.2003

Yi, C., Mei, X., Ezan, P., Mato, S., Matias, I., Giaume, C., et al. (2016). Astroglial connexin43 contributes to neuronal suffering in a mouse model of Alzheimer's disease. *Cell Death Differ.* 23, 1691–1701. doi: 10.1038/cdd.2016.63

Yin, D., Zhou, S., Xu, X., Gao, W., Li, F., Ma, Y., et al. (2018a). Dexmedetomidine attenuated early brain injury in rats with subarachnoid haemorrhage by suppressing the inflammatory response: the TLR4/NF-kappaB pathway and the NLRP3 inflammasome may be involved in the mechanism. *Brain Res.* 1698, 1–10. doi: 10.1016/j.brainres.2018.05.040

Yin, X., Feng, L., Ma, D., Yin, P., Wang, X., Hou, S., et al. (2018b). Roles of astrocytic connexin-43, hemichannels, and gap junctions in oxygen-glucose deprivation/reperfusion injury induced neuroinflammation and the possible regulatory mechanisms of salvianolic acid B and carbenoxolone. *J. Neuroinflammation* 15:97. doi: 10.1186/s12974-018-1127-3

Ystgaard, M. B., Sejersted, Y., Loberg, E. M., Lien, E., Yndestad, A., and Saugstad, O. D. (2015). Early upregulation of NLRP3 in the brain of neonatal mice exposed to hypoxia-ischemia: no early neuroprotective effects of NLRP3 deficiency. *Neonatology* 108, 211–219. doi: 10.1159/000437247

Yu, Q., Guo, Z., Liu, X., Ouyang, Q., He, C., Burnstock, G., et al. (2013). Block of P2X7 receptors could partly reverse the delayed neuronal death in area CA1 of the hippocampus after transient global cerebral ischemia. *Purinergic Signal.* 9, 663–675. doi: 10.1007/s11302-013-9379-y

Zasada, M., Lenart, M., Rutkowska-Zapala, M., Stec, M., Mol, N., Czyz, O., et al. (2018). Analysis of selected aspects of inflammasome function in the monocytes from neonates born extremely and very prematurely. *Immunobiology* 223, 18–24. doi: 10.1016/j.imbio.2017.10.019

Zeinieh, M. P., Talhouk, R. S., El-Sabban, M. E., and Mikati, M. A. (2010). Differential expression of hippocampal connexins after acute hypoxia in the developing brain. *Brain Dev.* 32, 810–817. doi: 10.1016/j.braindev.2009.11.003

Zhao, H. B., Yu, N., and Fleming, C. R. (2005). Gap junctional hemichannel-mediated ATP release and hearing controls in the inner ear. *Proc. Natl. Acad. Sci. U.S.A.* 102, 18724–18729. doi: 10.1073/pnas.0506481102

Conflict of Interest Statement: CG has intellectual property related to connexin hemichannel and pannexin channel modulation for the treatment of inflammatory disease and regulation of the inflammasome pathway and is a co-founder of OcuNexus Therapeutics, which has a focus on the treatment of chronic disease indications.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Zhou, Green, Bennet, Gunn and Davidson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

PROTOCOLS
published: 07 March 2019
doi: 10.3389/fphys.2019.00142

UNICORN Babies: Understanding Circulating and Cerebral Creatine Levels of the Preterm Infant. An Observational Study Protocol

*Mary J. Berry[1,2], Melissa Schlegel[1,2], Greg M. Kowalski[3], Clinton R. Bruce[3], Damien L. Callahan[4], Miranda L. Davies-Tuck[5], Hayley Dickinson[5], Angus Goodson[2], Angie Slocombe[2], Rod J. Snow[3], David W. Walker[6] and Stacey J. Ellery[5]**

[1]Department of Paediatrics and Child Health, University of Otago, Wellington, New Zealand, [2]Capital and Coast District Health Board, Wellington, New Zealand, [3]School of Exercise Sciences, Institute for Physical Activity and Nutrition, Deakin University, Geelong, VIC, Australia, [4]Centre for Cellular and Molecular Biology, School of Life and Environmental Science, Deakin University, Melbourne, VIC, Australia, [5]The Ritchie Centre, Hudson Institute of Medical Research, and Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia, [6]School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC, Australia

OPEN ACCESS

Edited by:
Carina Mallard,
University of Gothenburg,
Sweden

Reviewed by:
Mhoyra Fraser,
The University of Auckland,
New Zealand
Michael Stark,
Women's and Children's Hospital,
Australia

***Correspondence:**
Stacey J. Ellery
stacey.ellery@hudson.org.au

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: 15 October 2018
Accepted: 07 February 2019
Published: 07 March 2019

Citation:
Berry MJ, Schlegel M, Kowalski GM, Bruce CR, Callahan DL, Davies-Tuck ML, Dickinson H, Goodson A, Slocombe A, Snow RJ, Walker DW and Ellery SJ (2019) UNICORN Babies: Understanding Circulating and Cerebral Creatine Levels of the Preterm Infant. An Observational Study Protocol. Front. Physiol. 10:142. doi: 10.3389/fphys.2019.00142

Creatine is an essential metabolite for brain function, with a fundamental role in cellular (ATP) energy homeostasis. It is hypothesized that preterm infants will become creatine deplete in the early postnatal period, due to premature delivery from a maternal source of creatine and a limited supply of creatine in newborn nutrition. This potential alteration to brain metabolism may contribute to, or compound, poor neurological outcomes in this high-risk population. Understanding Creatine for Neurological Health in Babies (UNICORN) is an observational study of circulating and cerebral creatine levels in preterm infants. We will recruit preterm infants at gestational ages 23^{+0}–26^{+6}, 27^{+0}–29^{+6}, 30^{+0}–32^{+6}, 33^{+0}–36^{+6}, and a term reference group at 39^{+0}–40^{+6} weeks of gestation, with 20 infants in each gestational age group. At birth, a maternal capillary blood sample, as well as a venous cord blood sample, will be collected. For preterm infants, serial infant plasma (heel prick), urine, and nutrition samples [total parenteral nutrition (TPN), breast milk, or formula] will be collected between birth and term "due date." Key fetomaternal information, including demographics, smoking status, and maternal diet, will also be collected. At term corrected postnatal age (CPA), each infant will undergo an MRI/^{1}H-MRS scan to evaluate brain structure and measure cerebral creatine content. A general movements assessment (GMA) will also be conducted. At 3 months of CPA, infants will undergo a second GMA as well as further neurodevelopmental evaluation using the Developmental Assessment of Young Children – Second Edition (DAYC-2) assessment tool. The primary outcome measures for this study are cerebral creatine content at CPA and plasma and urine creatine and guanidinoacetate (creatine precursor) concentrations in the early postnatal period. We will also determine associations between (1) creatine levels at term CPA and neurodevelopmental outcomes (MRI, GMA, and DAY-C); (2) dietary creatine intake and circulating and cerebral creatine content; and (3) creatine levels and maternal

characteristics. Novel approaches are needed to try and improve preterm-associated brain injury. Inclusion of creatine in preterm nutrition may better support *ex utero* brain development through improved cerebral cellular energy availability during a period of significant brain growth and development.

Ethics Ref: HDEC 18/CEN/7 New Zealand.

ACTRN: ACTRN12618000871246.

Keywords: premature, nutrition, brain metabolism, cellular energy, neurodevelopment

INTRODUCTION

The global incidence of preterm birth (delivery prior to 37 completed weeks of gestation) is reported to be ~11% (Blencowe et al., 2013). Advances in perinatal care mean that increasing numbers of infants born even at extremes of gestational age (extreme preterm: <28 weeks of gestational age) survive beyond the neonatal period (Davidoff et al., 2006; Mohangoo et al., 2011; Lawn et al., 2013). Additionally, within resource-rich neonatal care settings, the age of human viability continues to fall with increasing survival rates at even 22 and 23 weeks of gestational age. However, this increase in survival has not been coupled with a reduction in long-term morbidity. Neurodevelopmental disorders, including those of executive function, psychiatric, and behavioral sequelae (Blencowe et al., 2013), are more prevalent in those born preterm than at term, with increasing risk, the lesser the gestational age at birth (Crump et al., 2010; Johnson et al., 2010).

The etiology of brain injury in preterm infants is complex and often unexplained. Historically, intraventricular hemorrhage (IVH) with resultant disruption of brain tissue and formation of macrocystic lesions was associated with global developmental delay. With the improvements in perinatal care, high grade IVH and/or periventricular leukomalacia (PVL) has become far less common, and standard cranial ultrasound or magnetic resonance imaging (MRI) examination usually do not demonstrate gross structural lesions (Penn et al., 2016). In Australia and New Zealand, the rate of high grade IVH in infants born prior to 32 weeks' gestation declined from 8% to just over 4% in the period between 2004 and 2013 (Chow et al., 2013). As such, longitudinal studies now indicate that a large percentage of children born preterm have neurodevelopmental problems in the absence of structural brain abnormalities detected with conventional imaging (Constable et al., 2008; Mullen et al., 2011; Aeby et al., 2013). These data suggest that conventional prognostication based on measures of cerebral structure is poorly discriminatory and will not always identify infants at risk of neurodevelopmental morbidity. We, and others, propose that our focus should be widened to explore the role of altered brain metabolism in the pathophysiology of preterm brain injury and neurodevelopmental disability (Koob et al., 2016).

Creatine is a metabolite essential for brain development and energy metabolism (Braissant et al., 2007). It is an amino acid derivative that fuels the creatine kinase circuit; a phosphagen system that mitigates temporal imbalances in energy (ATP) supply and demand (Wallimann et al., 1992). *Via* the creatine kinase circuit, creatine and its phosphorylated derivative (phosphocreatine, PCr) shuttle high-energy phosphate groups from the mitochondria into the cytosol, for the regeneration of ATP from ADP (Ellington, 2001). The creatine kinase isoforms required to run the circuit are found in the cerebellum, choroid plexus, hippocampal granular layer, and pyramidal cells of the brain (Hemmer et al., 1994) and are involved in maintaining ATP homeostasis in the central nervous system (CNS) from early in embryonic development through to adulthood (Braissant et al., 2001; Braissant et al., 2007).

Both creatine and phosphocreatine are spontaneously broken down into creatinine at a rate of ~1.7%/day (Brosnan and Brosnan, 2007). Thus, there is a requirement to replenish creatine and PCr stores on a daily basis. Creatine can be obtained from the diet and is also endogenously synthesized *de novo*. Synthesis involves the production of guanidinoacetate (GAA) from arginine and glycine, in a reaction catalyzed by arginine:glycine aminotransferase (AGAT) that occurs predominately in the kidney and pancreas. GAA is then methylated by guanidinoacetate N-methyltransferase (GAMT), mainly in the liver, to produce creatine. Once synthesized or absorbed, creatine is transported into cells *via* the sodium-dependent SLC6A8 creatine transporter (Wyss and Kaddurah-Daouk, 2000). Creatine is able to cross the blood-brain barrier (Ohtsuki et al., 2002), and the brain itself also has some inherent capacity to synthesize creatine, with both AGAT and GAMT expressed by neurons, astrocytes, and oligodendrocytes (Braissant et al., 2001; Tachikawa et al., 2004; Nakashima et al., 2005). Despite the importance of creatine for brain growth and development, the relative importance of cerebral creatine derived from systemic rather than local synthesis has not yet been established.

In utero fetal magnetic resonance spectroscopy (^{1}H-MRS) studies indicate cerebral creatine accretion occurs from 18 to 40 weeks of gestation (Evangelou et al., 2015). Postnatal studies have also clearly identified that an increase in cerebral creatine concentration occurs during the first 3 months of life (Blüml et al., 2012; Blüml et al., 2014). The importance of a maternal source of creatine for the developing fetus and the continued postnatal accretion of cerebral creatine is made evident by those infants diagnosed with inherited creatine deficiency syndromes. These infants typically raise no concerns antenatally; however, as postnatal creatine levels fall, they become progressively more symptomatic with altered neurological state

(Almeida et al., 2006) and complex neurological symptoms including impaired psychomotor development, seizures, and cognitive impairment (Battini et al., 2006).

In adults, creatine is acquired *via* the diet and *de novo* synthesis, with each contributing ~50% (Brosnan and Brosnan, 2007). However, in infancy, due to low levels of creatine in human breast milk and commercial formulas, a term infant most likely synthesizes between 64 and 93% of their daily creatine requirements (Edison et al., 2013). The age at which a fetus/newborn develops the capacity to synthesize creatine is currently unknown, but it requires sufficient renal, hepatic, and cerebral maturity to express the enzymes necessary for creatine synthesis. Evaluation of AGAT and GAMT expression in the rat embryo found that AGAT was expressed in isolated cells of the CNS from 0.6 gestation; however, widespread expression of AGAT and rudimentary GAMT expression were not apparent before 0.8 gestation (Braissant et al., 2005). Our own studies in the precocial spiny mouse found that the fetal kidney and liver do not express AGAT and GAMT before 0.9 gestation, an age and maturation equivalent to ~35 weeks' human gestation (Ireland et al., 2009). Taken together, these observations raise compelling questions about progressive creatine depletion in preterm infants, and whether this complication of nutritional compromise predisposes or compounds their risk of altered cerebral metabolism and thus long-term neurodevelopmental disability.

To date, a number of small observational studies have identified perturbations in creatine homeostasis in preterm infants (Blüml et al., 2014; Koob et al., 2016). Koob et al. (2016) reported that at term CPA creatine concentrations in the centrum semiovale of infants born preterm (born 29.1 ± 2 weeks) were reduced compared to those born at term (Koob et al., 2016). In addition, Lage et al. (2013) found that by hospital discharge, preterm infants had higher urinary GAA and reduced urinary creatine excretion, suggesting differences in systemic creatine synthesizing capacity. This was particularly apparent in their very preterm group (28–29 weeks) (Lage et al., 2013).

No studies have characterized longitudinal changes in creatine metabolism among preterm infants or the association between cerebral creatine content and neurodevelopmental outcomes. Further to this, no studies have monitored systemic creatine levels (both circulating and excreted) in a single preterm population nor have they assessed nutritional creatine availability for preterm infants fed with total parenteral nutrition (TPN), preterm infant formulas, or breast milk.

We hypothesize that following preterm birth, peripheral and cerebral creatine levels will fall, so that at term CPA, preterm infants will have relative systemic and cerebral creatine deficiency. Furthermore, this reduction in creatine bioavailability will jeopardize normal brain metabolism and development, ultimately predisposing the infant to neurodevelopmental dysfunction.

The overall aim of this study is to assess whether preterm infants become creatine deplete in the early postnatal period, by measuring the preterm infants' circulating and excreted levels of creatine and GAA and comparing these measures to cerebral creatine content, as determined by ^{1}H-MRS, at term corrected age. This study will correlate preterm creatine levels with neurological outcomes using MRI, general movements assessment (GMA), and the Developmental Assessment of Young Children – Second Edition (DAYC-2) Test (Novak et al., 2017). This study will also establish the levels of creatine provided through dietary intake during the days and weeks after preterm birth. Results of this study may support the use of creatine supplementation as standard nutritional care of the preterm infant, in order to improve neurodevelopmental function in this vulnerable population.

Specific Aims

1. Compare cerebral, plasma, and urine creatine and GAA content between preterm and term infants at CPA.
2. Describe preterm infant plasma and urine creatine and GAA concentrations and creatine:GAA ratio in the early postnatal period.
3. Describe associations between cerebral creatine content, plasma, and urine creatine concentrations with maternal and infant characteristics at CPA.
4. Compare creatine, GAA, arginine, glycine, and methionine concentrations in standard preterm nutrition, including TPN, preterm infant formulas, and breast milk.
5. Determine whether dietary source of creatine (TPN, formula, or breast milk) is related to plasma or urine creatine concentrations in the early postnatal period.
6. Describe associations between cerebral creatine content and blood and urine creatine concentrations with neurological outcomes (GMA, MRI, and DAYC-2) at CPA and at 3 months of corrected postnatal age.

METHOD/DESIGN

Study Design

Prospective, single-center, observational study.

Study Setting

Neonatal Intensive Care Unit and Postnatal Wards, Capital Coast District Hospital Board (CCDHB), Wellington, New Zealand.

Primary Outcome Measures

1. Cerebral creatine content at term CPA
2. Plasma (bi-weekly) and urine (weekly) creatine and GAA levels from birth until hospital discharge and at CPA
3. Creatine, GAA, and amino acid levels in infant nutrition (TPN, formula, or breast milk) supplied from birth until discharge and at CPA

Secondary Outcome Measures

1. Global injury score from MRI at term CPA
2. General movement assessment score at term CPA and 3 months of CPA
3. DAYC-2 assessment score at 3 months of CPA

Study Population

Inclusion Criteria

1. Preterm infants receiving care within the Neonatal Intensive Care Unit at CCDHB or healthy term infants at 39–40 weeks' gestation from low-risk pregnancies delivered at CCDHB.

Exclusion Criteria

1. Babies with major congenital, genetic, or chromosomal abnormalities known to affect neurodevelopmental outcomes
2. Hypoxic ischemic encephalopathy
3. Inborn (proven or suspected) errors of metabolism
4. Fetal growth restriction (birth weight < 10th percentile on customized percentiles)

Recruitment

We will recruit infants at gestational ages 23^{+0}–26^{+6}, 27^{+0}–29^{+6}, 30^{+0}–32^{+6}, 33^{+0}–36^{+6}, and 39^{+0}–40^{+6} weeks. We will aim to capture 20 infants at each time point (with equal numbers of males and females). Eligible infants will be identified based on gestational age at delivery and consideration of inclusion and exclusion criteria. Pregnant women will be approached at presentation to Delivery Suite to discuss study participation. If consent is obtained, the mother and infant will be recruited to the study and the consulting obstetric, midwifery, neonatal, and nursing team advised of their involvement. Where pre-delivery consent is not obtained due to rapidity of labor and/or other clinical factors, cord blood will be collected preemptively, and retrospective consent and maternal blood sample sought within 24 h of delivery. If the parents do not wish to participate, the cord blood sample will be disposed of using routine clinical biological waste disposal systems and no further study samples collected.

Sample and Data Collection

Blood, Urine, and Nutrition Sampling

A schematic overview of the sampling regime for each participant is outlined in **Figure 1**. At the time of birth, we will use a finger prick to collect ~120 μl of maternal whole blood using a capillary tube. A 2-ml venous cord blood sample will also be collected from which a ~120 μl sample will be obtained using a capillary tube. Then, while infants are receiving care in the NICU, or on postnatal wards, and at follow-up appointments, the following samples will be collected. Sample frequency will be dictated by gestational age at birth.

Blood sampling: A blood sample will be collected biweekly. We will perform a heel prick to collect ~120 μl of whole blood using a capillary tube. All blood samples (cord, maternal, or infant) will be transferred to a Hemaspot™ SE blood separation device to facilitate collection of the cell-free blood fraction (referred to throughout as plasma for clarity) from a small volume of whole blood. Hemaspots are allowed to dry at room temperature before being stored at −20°C for future analysis.

Urine sampling: Around the same time as the blood collection, and on each alternate week, we will place some gauze into the infant's nappy to facilitate a spot urine collection. Once obtained, this sample will be transferred to an Eppendorf tube and stored at −80°C for future analysis.

Nutrition sampling: A 200 μl sample of the nutrition (TNP, formula, and breast milk) being fed to each infant at the time of the blood and urine sampling will be collected. If in powdered form (formula), this will be reconstituted following standard practices. All samples will be transferred into an Eppendorf tube and stored at −80°C for future analysis.

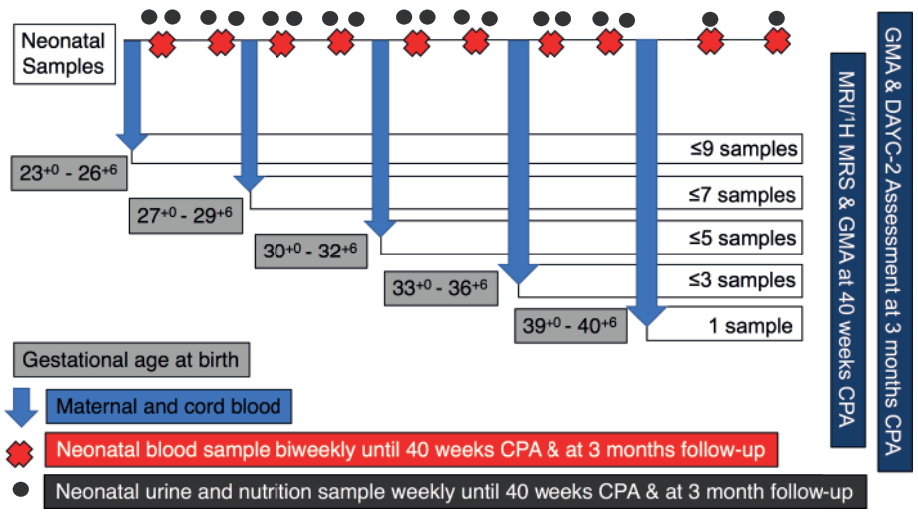


FIGURE 1 | Schematic representation of UNICORN sampling regime. Samples of neonatal blood, urine, and nutrition will be collected weekly or bi-weekly for the duration of the infants stay in hospital. Magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (^{1}H-MRS) will be performed at term corrected postnatal age (CPA). Infants will also undergo a general movements assessment (GMA) at CPA and at 3 months CPA. At the follow-up appointment at 3 months CPA, further neurological assessment will be completed using the Developmental Assessment of Young Children – Second Edition (DAYC-2) assessment tool.

Monitoring and Data Collection

The following covariates/potential confounders will be collected as part of this study:

Maternal characteristics and birth outcomes data:

- Maternal age
- Comorbidities/factors (preeclampsia, gestational diabetes, chorioamnionitis, IVF)
- Country of birth
- Dietary preferences (omnivorous, vegetarian, vegan)
- Smoking status
- Parity
- Gravity
- Mode of delivery
- Placental weight

Infant characteristics:

- Gestational age
- Sex
- Birth weight
- Ethnicity
- Weight and corrected postnatal age at the time of each sample collection
- Presence of postnatal infection (blood culture positive and/or treatment with systemic antibiotics for >24 h) or necrotizing enterocolitis (Modified Bells Classification Grade II-III determined by the treating Pediatric Surgeon at time of diagnosis).
- Feeding schedule prior to each sample collection (<24 h)
- Venous hemoglobin and hematocrit count prior to blood sample collection

MRI/^{1}H-MRS Data Acquisition

At 40 weeks of corrected postnatal age (CPA), each participant will undergo a MRI/^{1}H-MRS scan. MR examinations will be performed on a Siemens 3T Skyra using a 16-channel dedicated pediatric head coil. Infants will be examined without sedation using a "feed and wrap" technique according to standard institutional practice. In brief, infants are brought to the MRI unit, fed, and swaddled comfortably while being monitored with continuous pulse oximetry before being placed in the MRI scanner. Standard, size appropriate, hearing protection will be used for all infants. Sequences will be performed to demonstrate brain anatomy and will include T1 3D 1 mm volumetric, T2 1 mm 3D volumetric scans, T2 TSE FLAIR, and T1SE MTC sequences. These sequences will also contribute to the analysis of any detected brain pathology. T2 SWI axial images will be performed to show calcification and blood products. Sequences for the detection of vascular injury and infarction will include diffusion sequences (b value = 2000), resting BOLD, and arterial spin labeling. ^{1}H-MRS will provide chemical spectra of the brain to further analyze brain pathology and measurement of cerebral creatine content. For MR spectroscopy, the voxel will be placed over the white matter underlying the central sulcus within the centrum semiovale and spectra acquired with a long echo time (TE 135 ms) (Koob et al., 2016).

Neurodevelopmental Observations

At term CPA, prior to the MRI/^{1}H-MRS scan, an experienced neurodevelopmental therapist will record a 5-minute video of the infant to conduct a general movements assessment (GMA); a validated tool to noninvasively identify neurological issues, which may lead to cerebral palsy and other developmental disabilities (Spittle et al., 2018). Infants are placed in a supine position, with minimal clothing to allow freedom of movement. The video will be taken when the infant is fully alert, but not directly interacting with parents, healthcare providers, or receiving stimulus from any external sources.

At 3 months of CPA, we will request participants meet again with a developmental therapist. At this appointment, information about general health (including weight gain trajectories, feeding habits, and number of visits to a GP) will be recorded. A questionnaire about developmental milestones at 3 months of age (developed by the American Academy of Pediatrics) will be completed, as well as a Developmental Assessment of Young Children – Second Edition (DAYC-2) Test, a validated tool to identify children with possible developmental delay in the following domains: cognition, communication, social-emotional development, physical development, and adaptive behavior (Saleh and Smadi, 2017). A 5-minute video of the infant will also be recorded to allow for a second GMA to be completed.

Sample and Data Analysis

Creatine, GAA, and Amino Acid Analysis

Creatine, GAA, arginine, glycine, and methionine will be measured in biological and nutrition samples *via* LC-MS/MS, according to the methods of (Tran et al., 2014), with slight modification (Tran et al., 2014). Briefly, a 6-mm hole punch of the "cell-free" plasma fraction of the Hemaspot™, 7 µl of urine, nutrition, or unlabeled standard, will be transferred to 1.5-ml safety lock Eppendorf tubes. 200 µl of methanol containing 10 µM 2,2-d_3 Creatine (Sigma), 2.5 µM 2,2-d_2 GAA (Sigma), and 2.5 µM of Labeled Amino Acid Standard Set A (Cambridge Isotope Laboratories Inc.) internal standards is added to each tube. Samples are sonicated for 5 min then vortexed for 20 min, before being centrifuged at 20,000× g for 5 min to pellet any precipitate or filter paper at the bottom of the tube. Supernatant (~170 µl) is transferred into 250-µl glass inserts placed in 2-ml glass vials (Agilent), followed by evaporation to complete dryness in a speed vacuum (Labconco, Kansas, MO, USA). After drying, the GAA and creatine carboxyl groups are derivatized (butylated) by the addition of 100 µl of 3 M butanol-HCL followed by incubation at 60°C for 30 min. After cooling to room temperature, excess butanol-HCL is evaporated under speed vacuum, after which the dry residue is resuspended in 200 µl of methanol: water (1:1 v/v) solution prior to LC-MS/MS analysis. The external standards consist of an eight sample serial dilution series of known concentration range (0–25 µM unlabeled GAA and 0–100 µM unlabeled creatine).

The LC-MS/MS system is composed of a vacuum degasser, binary pumps, column oven, and a temperature-controlled autosampler (Shimadzu, Nexera® UPLC). This will be interfaced with a triple quadrupole mass spectrometer (Shimadzu, LCMS-8030) with electrospray ionization. The LC column (Agilent

–2.1 × 100 mm, 1.8 μm C18 Zorbax Eclipse plus) will be maintained at 30°C and elution carried out with a binary gradient mobile phase at a flow rate of 0.4 ml min^{-1}. Mobile phase A will contain water with 0.1% formic acid, and mobile phase B will contain acetonitrile with 0.1% formic acid. The initial mobile phase composition will be 5% B, which is ramped linearly to 50% B over 6 min followed by a 2-min column wash at 100% B, with the column finally undergoing re-equilibration at 5% B for 3 min, thus yielding a total runtime of 11 min. The first 1 min is diverted to waste. The following ESI source conditions will be used: nebulizer gas flow rate 3 L min^{-1}, desolvation line temperature at 250°C, heater block temperature 400°C, and drying gas flow rate of 15 L min^{-1}. The MS will be operated in positive ionization with multiple reaction monitoring (MRM). The collision energy, Q1 and Q3 pre-bias voltages for the MRM transitions have been optimized using authentic creatine and GAA standards (SIGMA) *via* the automatic optimisation software (Labsolutions, Shimadzu), with optimal collision energy for creatine and GAA for the instrument being −15 eV. The precursor and product ion transitions were creatine 188.2–90 *m/z*, d_3-Creatine 191.2–93 *m/z,* GAA 174.2–101.1 *m/z*, and d_2-GAA 176.1–103.1 *m/z*. The sample and standard injection volume will be 5 μl. Peak areas will be determined using Lab solutions post-run browser software (Shimadzu). The creatine, GAA and amino acid concentrations of blood spots, urine, or nutrition samples will be calculated *via* linear regression of the serially diluted external (unlabeled) standard series using the isotope dilution technique.

MRI/^{1}H-MRS

MRI images will be scored according to the Kidokoro assessment tool (Kidokoro et al., 2013). Briefly, cerebral white matter, cortical gray matter, deep gray matter, and cerebellar abnormalities will be independently graded, and then the scores combined to calculate a single global brain injury score. The assessor will also note the presence of hemorrhage or cystic lesions throughout the brain. Hemorrhagic lesions will be graded using the Papile classification system (Papile et al., 1978) and cystic lesions characterized as periventricular leukomalacia, periventricular hemorrhagic infarction, or other (Barkovich, 2000).

Metabolic ratios from ^{1}H-MRS spectra obtained with a TE of 135 ms will be calculated as previously described, with water used as an internal reference to normalize metabolite signal intensities (Koob et al., 2016).

Neurodevelopmental Observations

Motor function will be assessed by a developmental therapist trained in GMA and blinded to the clinical history of the infant. At term CPA, infants will be assessed on withering movements, which should include complex movements of the arms, legs, neck, and trunk. Poor withering movements include rigid or chaotic movements that lack fluency and coordination (Spittle et al., 2013) and are predictive of adverse neurodevelopmental outcomes. At 3 months of CPA, infants will be assessed on fidgety movements, which appear as small continual movements of limbs in all directions. Poor quality fidgety movements include larger amplitude and jerking movements (Spittle et al., 2013) and are again predictive of later outcomes. Infants will be scored independently at each postnatal age.

At 3-month CPA, infants' development will also be reviewed using the DAYC-2 assessment tool (Saleh and Smadi, 2017). An overall score from the five domains tested (cognition, communication, social-emotional development, physical development, and adaptive behavior) will be generated for each infant. Collectively, MRI, GMA, and DAYC-2 scores will be used to assess the potential risk of poor neurological outcomes, based on early diagnosis strategies for cerebral palsy (Novak et al., 2017).

Sample Size

This study will be the first observational study of creatine homeostasis in the preterm infant that encompasses plasma, urine, nutrition, and cerebral creatine measures across time. As the outcomes are mainly descriptive, no formal power calculation has been performed. However, ^{1}H-MRS results reported by Koob et al. (2016) showed comparison of 13 preterm vs term infants allowed for measurement of a statistically significant (−17%) reduction in cerebral creatine content in preterm infants at CPA ($tCr/H_20 \times 10^{-3}$; term 0.12 ± 0.1 vs preterm 0.10 ± 0.1). This same study had a 20–25% failure rate in obtaining accurate MRI/^{1}H-MRS measurements at CPA (Koob et al., 2016). Taking these data into consideration and a potential loss to follow-up at 3-month of 20%, we aim to capture 20 infants in each gestational group.

Statistical Analysis

Characteristics of the population will be tabulated, along with appropriate descriptive statistics of the study sample. Continuous data will be assessed for normality and expressed as means and standard deviations or median and interquartile range as appropriate. Categorical data will be expressed as counts and proportions. Average creatine content from nutrition per week for each baby in the early postnatal period will be calculated.

Differences in creatine and GAA measures (^{1}H-MRS, plasma or urine) between infants born preterm and those born at term will be assessed at CPA using a t-test or Mann-Whitney U test as appropriate. The correlation between gestational age at birth and creatine and GAA measures at CPA will also be determined using a Spearman or Pearson correlation. Preterm infant plasma and urine creatine and GAA concentrations and creatine:GAA ratio in the early postnatal period will be described and plotted. Associations between cerebral creatine content, plasma and urine creatine concentrations with maternal, and infant characteristics at CPA will be determined using Spearman or Pearson correlations for continuous data (maternal age, gestational age, birth weight, parity, gravity, placental weight) or a t-test or Mann-Whitney U test for categorical data (infant sex, mode of delivery, maternal comorbidities, country of birth, ethnicity, and dietary preferences), as appropriate. Differences in creatine, GAA, and amino acid content of nutrition sources (TNP, formula or breast milk) will be determined using a one-way ANOVA or Kruskal-Wallis H test as appropriate. The association between creatine, GAA, and amino acid content of nutrition sources (TNP, formula

or breast milk) and infant plasma and urine creatine at CPA will be assessed using a Spearman or Pearson correlation as appropriate. Finally, the association between cerebral creatine content, plasma creatine levels, and the ratio of plasma:cerebral creatine at CPA with GMA, MRI global injury, and DAYC-2 scores will be determined using linear regression at both CPA and at 3 months' corrected postnatal age. Potential confounders such as gestational age and maternal and infant characteristics will be assessed, along with collinearity, prior to performing multivariable linear regression.

DISCUSSION

Both creatine and the creatine kinase circuit are critical for brain metabolism (Braissant et al., 2011). It is hypothesized that preterm infants will become creatine deplete in the early postnatal period, due to premature delivery from a maternal source of creatine *in utero,* limited capacity for endogenous synthesis postnatally, and a limited supply of creatine in newborn nutrition. This creatine depletion may alter cerebral creatine metabolism, contributing to or compounding the risk of neurological disability in this population.

We anticipate that preterm babies will have altered creatine homeostasis and reduced creatine accretion in the early postnatal period compared to an infant of the same postconceptional age. Whether or not relative creatine deficiency is associated with neurological deficit will be a secondary outcome of this study. As the results are mainly descriptive and a formal power calculation could not be completed, there is the possibility that sample size may become a limitation for some of the proposed analyses in this observational study. However, the data obtained will enable calculation of effect size for future studies. If our overall hypothesis is supported, this observational study will inform a future randomized control trial of infant dietary creatine supplementation following premature birth, with the aim of improving brain metabolism during a key stage of development and thereby reducing the burden of neurodevelopmental disability in this high-risk population.

AUTHOR CONTRIBUTIONS

MB, SE, HD, and DW conceptualized the study. MS is a research nurse coordinating recruitment, sample collection, and data management. AG was involved in ethics development and study design. GK, CB, DC, and RS developed the LC-MS/MS protocol for creatine and metabolite assessment. AS developed the MR data acquisition protocol. MD-T designed the statistical approaches to the study. MB and SE wrote the manuscript. All authors contributed to the drafting of the manuscript.

FUNDING

The authors would like to acknowledge funding support from the Cerebral Palsy Alliance (PG2715) and Rebecca L Cooper Medical Research Foundation (10624) for the completion of this study.

REFERENCES

Aeby, A., De Tiège, X., Creuzil, M., David, P., Balériaux, D., Van Overmeire, B., et al. (2013). Language development at 2 years is correlated to brain microstructure in the left superior temporal gyrus at term equivalent age: a diffusion tensor imaging study. *Neuroimage* 78, 145–151. doi: 10.1016/j.neuroimage.2013.03.076

Almeida, L. G. S., Rosenberg, E. H., Verhoeven, N. M., Jakobs, C., and Salomons, G. S. (2006). Are cerebral creatine deficiency syndromes on the radar screen? *Future Neurol.* 1, 637–649. doi: 10.2217/14796708.1.5.637

Barkovich, A. J. (2000). Brain and spine injury in infancy and childhood. *J. Pediatr. Neurol.* 30, 157–250.

Battini, R., Alessandri, M., Leuzzi, V., Moro, F., Tosetti, M., Bianchi, M., et al. (2006). Arginine:glycine amidinotransferase (AGAT) deficiency in a newborn: early treatment can prevent phenotypic expression of the disease. *J. Pediatr.* 148, 828–830. doi: 10.1016/j.jpeds.2006.01.043

Blencowe, H., Cousens, S., Chou, D., Oestergaard, M., Say, L., Moller, A. -B., et al. (2013). Born too soon: the global epidemiology of 15 million preterm births. *Reprod. Health* 10:S2. doi: 10.1186/1742-4755-10-S1-S2

Blüml, S., Wisnowski, J. L., Nelson, M. D. Jr., Paquette, L., Gilles, F. H., Kinney, H. C., et al. (2012). Metabolic maturation of the human brain from birth through adolescence: insights from in vivo magnetic resonance spectroscopy. *Cereb. Cortex* 23, 2944–2955. doi: 10.1093/cercor/bhs283

Blüml, S., Wisnowski, J. L. Nelson, M. D. Jr., Paquette, L., and Panigrahy, A. (2014). Metabolic maturation of white matter is altered in preterm infants. *PLoS One* 9:E85829. doi: 10.1371/journal.pone.0085829

Braissant, O., Bachmann, C., and Henry, H. (2007). "Expression and function of AGAT, GAMT and CT1 in the mammalian brain" in *Creatine and creatine kinase in health and disease*. Subcellular Biochemistry. eds. G. S. Salomons, and M. Wyss vol 46, (Dordrecht: Springer). doi: 10.1007/978-1-4020-6486-9_4

Braissant, O., Henry, H., Béard, E., and Uldry, J. (2011). Creatine deficiency syndromes and the importance of creatine synthesis in the brain. *Amino Acids* 40, 1315–1324. doi: 10.1007/s00726-011-0852-z

Braissant, O., Henry, H., Loup, M., Eilers, B., and Bachmann, C. (2001). Endogenous synthesis and transport of creatine in the rat brain: an in situ hybridization study. *Mol. Brain Res.* 86, 193–201. doi: 10.1016/S0169-328X(00)00269-2

Braissant, O., Henry, H., Villard, A. -M., Speer, O., Wallimann, T., and Bachmann, C. (2005). Creatine synthesis and transport during rat embryogenesis: spatiotemporal expression of AGAT, GAMT and CT1. *BMC Dev. Biol.* 5:9. doi: 10.1186/1471-213X-5-9

Brosnan, J., and Brosnan, M. (2007). Creatine: endogenous metabolite, dietary, and therapeutic supplement. *Annu. Rev. Nutr.* 27, 241–261. doi: 10.1146/annurev.nutr.27.061406.093621

Chow, S., Le Marsney, R., Hossain, S., Haslam, R., and Lui, K. (2013). *Report Of The Australian And New Zealand Neonatal Network 2012*. Sydney: Anznn.

Constable, R. T., Ment, L. R., Vohr, B. R., Kesler, S. R., Fulbright, R. K., Lacadie, C., et al. (2008). Prematurely born children demonstrate white matter microstructural differences at 12 years of age, relative to term control subjects: an investigation of group and gender effects. *Pediatrics* 121, 306–316. doi: 10.1542/peds.2007-0414

Crump, C., Winkleby, M. A., Sundquist, K., and Sundquist, J. (2010). Preterm birth and psychiatric medication prescription in young adulthood: a Swedish national cohort study. *Int. J. Epidemiol.* 39, 1522–1530. doi: 10.1093/ije/dyq103

Davidoff, M. J., Dias, T., Damus, K., Russell, R., Bettegowda, V. R., Dolan, S., et al. (2006). Changes in the gestational age distribution among us singleton births: impact on rates of late preterm birth, 1992 to 2002. *Semin. Perinatol.* 30, 8–15. doi: 10.1053/j.semperi.2006.01.009

Edison, E. E., Brosnan, M. E., Aziz, K., and Brosnan, J. T. (2013). Creatine and guanidinoacetate content of human milk and infant formulas: implications

for creatine deficiency syndromes and amino acid metabolism. *Br. J. Nutr.* 110, 1075–1078. doi: 10.1017/S000711451300010X

Ellington, W. R. (2001). Evolution and physiological roles of phosphagen systems. *Annu. Rev. Physiol.* 63, 289–325. doi: 10.1146/annurev.physiol.63.1.289

Evangelou, I. E., Du Plessis, A. J., Vezina, G., Noeske, R., and Limperopoulos, C. (2015). Elucidating metabolic maturation in the healthy fetal brain using 1h-mr spectroscopy. *Am. J. Neuroradiol.* doi: 10.3174/ajnr.A4512

Hemmer, W., Zanolla, E., Furter-Graves, E. M., Eppenberger, H. M., and Wallimann, T. (1994). Creatine kinase isoenzymes in chicken cerebellum: specific localization of brain-type creatine kinase in Bergmann glial cells and muscle-type creatine kinase in Purkinje neurons. *Eur. J. Neurosci.* 6, 538–549. doi: 10.1111/j.1460-9568.1994.tb00298.x

Ireland, Z., Russell, A., Wallimann, T., Walker, D. and Snow, R. (2009). Developmental changes in the expression of creatine synthesizing enzymes and creatine transporter in a precocial rodent, the spiny mouse. *BMC Dev. Biol.* 9, 39–50. doi: 10.1186/1471-213X-9-39

Johnson, S., Hollis, C., Kochhar, P., Hennessy, E., Wolke, D., and Marlow, N. (2010). Psychiatric disorders in extremely preterm children: longitudinal finding at age 11 years in the epicure study. *J. Am. Acad. Child Adolesc. Psychiatry* 49, 453–463.e1. doi: 10.1016/j.jaac.2010.02.002

Kidokoro, H., Neil, J., and Inder, T. (2013). New MR imaging assessment tool to define brain abnormalities in very preterm infants at term. *Am. J. Neuroradiol.* doi: 10.1681/ASN.2012080783

Koob, M., Viola, A., Le Fur, Y., Viout, P., Ratiney, H., Confort-Gouny, S., et al. (2016). Creatine, glutamine plus glutamate, and macromolecules are decreased in the central white matter of premature neonates around term. *PLoS One* 11:E0160990. doi: 10.1371/journal.pone.0160990

Lage, S., Andrade, F., Prieto, J. A., Asla, I., Rodríguez, A., Ruiz, N., et al. (2013). Arginine-guanidinoacetate-creatine pathway in preterm newborns: creatine biosynthesis in newborns. *J. Pediatr. Endocrinol. Metab.* 26, 53–60. doi: 10.1515/jpem-2012-0293

Lawn, J. E., Davidge, R., Paul, V. K., Von Xylander, S., De Graft Johnson, J., Costello, A., et al. (2013). Born too soon: care for the preterm baby. *Reprod. Health* 10:S5. doi: 10.1186/1742-4755-10-S1-S5

Mohangoo, A. D., Buitendijk, S. E., Szamotulska, K., Chalmers, J., Irgens, L. M., Bolumar, F., et al. (2011). Gestational age patterns of fetal and neonatal mortality in Europe: results from the Euro-Peristat project. *PLoS One* 6:E24727. doi: 10.1371/journal.pone.0024727

Mullen, K. M., Vohr, B. R., Katz, K. H., Schneider, K. C., Lacadie, C., Hampson, M., et al. (2011). Preterm birth results in alterations in neural connectivity at age 16 years. *NeuroImage* 54, 2563–2570. doi: 10.1016/j.neuroimage.2010.11.019

Nakashima, T., Tomi, M., Tachikawa, M., Watanabe, M., Terasaki, T., and Hosoya, K. I. (2005). Evidence for creatine biosynthesis in Müller glia. *Glia* 52, 47–52. doi: 10.1002/glia.20222

Novak, I., Morgan, C., Adde, L., Blackman, J., Boyd, R. N., Brunstrom-Hernandez, J., et al. (2017). Early, accurate diagnosis and early intervention in cerebral palsy: advances in diagnosis and treatment. *JAMA Pediatr.* 171, 897–907. doi: 10.1001/jamapediatrics.2017.1689

Ohtsuki, S., Tachikawa, M., Takanaga, H., Shimizu, H., Watanabe, M., Hosoya, K. -I., et al. (2002). The blood-brain barrier creatine transporter is a major pathway for supplying creatine to the brain. *J. Cereb. Blood Flow Metab.* 22, 1327–1335. doi: 10.1097/01.WCB.0000033966.83623.7D

Papile, L. -A., Burstein, J., Burstein, R., and Koffler, H. (1978). Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 Gm. *J. Pediatr.* 92, 529–534. doi: 10.1016/S0022-3476(78)80282-0

Penn, A. A., Gressens, P., Fleiss, B., Back, S. A., and Gallo, V. (2016). Controversies in preterm brain injury. *Neurobiol. Dis.* 92, 90–101. doi: 10.1016/j.nbd.2015.10.012

Saleh, R. M. A., and Smadi, J. M. (2017). The efficacy of arabic version of the developmental assessment of young children second edition (Dayc-2) scale in detecting developmental delay among Jordanian children aged birth to 71 months. *Int. Educ. Stud.* 10, 113–132. doi: 10.5539/ies.v10n4p113

Spittle, A. J., Morgan, C., Olsen, J. E., Novak, I., and Cheong, J. L. (2018). Early diagnosis and treatment of cerebral palsy in children with a history of preterm birth. *Clin Perinatol.* doi: 10.1111/jpc.14149

Spittle, A. J., Spencer-Smith, M. M., Cheong, J. L., Eeles, A. L., Lee, K. J., Anderson, P. J., et al. (2013). General movements in very preterm children and neurodevelopment at 2 and 4 years. *Pediatr. Peds.* 45. doi: 10.1016/j.clp.2018.05.011

Tachikawa, M., Fukaya, M., Terasaki, T., Ohtsuki, S., and Watanabe, M. (2004). Distinct cellular expressions of creatine synthetic enzyme gamt and creatine kinases UCK-MI And CK-B suggest a novel neuron–glial relationship for brain energy homeostasis. *Eur. J. Neurosci.* 20, 144–160. doi: 10.1111/j.1460-9568.2004.03478.x

Tran, C., Yazdanpanah, M., Kyriakopoulou, L., Levandovskiy, V., Zahid, H., Naufer, A., et al. (2014). Stable isotope dilution microquantification of creatine metabolites in plasma, whole blood and dried blood spots for pharmacological studies in mouse models of creatine deficiency. *Clin. Chim. Acta* 436, 160–168. doi: 10.1016/j.cca.2014.05.007

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., and Eppenberger, H. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem. J.* 281, 21–40. doi: 10.1042/bj2810021

Wyss, M., and Kaddurah-Daouk, R. (2000). Creatine and creatinine metabolism. *Physiol. Rev.* 80, 1107–1213. doi: 10.1152/physrev.2000.80.3.1107

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Berry, Schlegel, Kowalski, Bruce, Callahan, Davies-Tuck, Dickinson, Goodson, Slocombe, Snow, Walker and Ellery. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 19 March 2019
doi: 10.3389/fphys.2019.00282

Mesenchymal Stromal Cell Derived Extracellular Vesicles Reduce Hypoxia-Ischaemia Induced Perinatal Brain Injury

Claudia Sisa[1], Sharad Kholia[2], Jordan Naylor[1], Maria Beatriz Herrera Sanchez[3], Stefania Bruno[2], Maria Chiara Deregibus[3], Giovanni Camussi[2], Jameel M. Inal[4], Sigrun Lange[5] and Mariya Hristova[1]**

[1] Perinatal Brain Protection and Repair Group, EGA Institute for Women's Health, University College London, London, United Kingdom, [2] Department of Medical Sciences, University of Turin, Turin, Italy, [3] 2i3T, Incubator and Technology Transfer, Molecular Biotechnology Center, University of Turin, Turin, Italy, [4] Extracellular Vesicle Research Unit and Bioscience Research Group, School of Life and Medical Sciences, University of Hertfordshire, Hatfield, United Kingdom, [5] Tissue Architecture and Regeneration Research Group, School of Life Sciences, University of Westminster, London, United Kingdom

OPEN ACCESS

Edited by:
Carina Mallard, University of Gothenburg, Sweden

Reviewed by:
Angela Leigh Cumberland, RMIT University, Australia
Sandra Buratta, University of Perugia, Italy

***Correspondence:**
Sigrun Lange
S.lange@westminster.ac.uk
Mariya Hristova
m.hristova@ucl.ac.uk

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: 13 December 2018
Accepted: 04 March 2019
Published: 19 March 2019

Citation:
Sisa C, Kholia S, Naylor J, Herrera Sanchez MB, Bruno S, Deregibus MC, Camussi G, Inal JM, Lange S and Hristova M (2019) Mesenchymal Stromal Cell Derived Extracellular Vesicles Reduce Hypoxia-Ischaemia Induced Perinatal Brain Injury. Front. Physiol. 10:282. doi: 10.3389/fphys.2019.00282

Background: Neonatal hypoxic-ischemic (HI) insult is a leading cause of disability and death in newborns, with therapeutic hypothermia being the only currently available clinical intervention. Thus there is a great need for adjunct and novel treatments for enhanced or alternative post-HI neuroprotection. Extracellular vesicles (EVs) derived from mesenchymal stromal/stem cells (MSCs) have recently been shown to exhibit regenerative effects in various injury models. Here we present findings showing neuroprotective effects of MSC-derived EVs in the Rice–Vannucci model of severe HI-induced neonatal brain insult.

Methods: Mesenchymal stromal/stem cell-derived EVs were applied intranasally immediately post HI-insult and behavioral outcomes were observed 48 h following MSC-EV treatment, as assessed by negative geotaxis. Brains were thereafter excised and assessed for changes in glial responses, cell death, and neuronal loss as markers of damage at 48 h post HI-insult.

Results: Brains of the MSC-EV treated group showed a significant decrease in microglial activation, cell death, and percentage tissue volume loss in multiple brain regions, compared to the control-treated groups. Furthermore, negative geotaxis test showed improved behavioral outcomes at 48 h following MSC-EV treatment.

Conclusion: Our findings highlight the clinical potential of using MSC-derived EVs following neonatal hypoxia-ischaemia.

Keywords: hypoxia, ischaemia, extracellular vesicles, mesenchymal stromal/stem cells, microglia, neuroprotection

INTRODUCTION

Neonatal hypoxic-ischaemic (HI) brain injury is a major contributing factor to cerebral palsy and other neurological disabilities and is estimated to occur in 3 in 1000 live births in the Western world and even at higher frequency in less developed countries (Perlman, 2006; Kurinczuk et al., 2010). Oxygen deprivation has been identified as a major cause of brain injury both in term and preterm babies (Wu and Colford, 2000; Hagberg et al., 2002; Mallard et al., 2003). While 15–20% of affected neonates will die postnatally, it has been found that in surviving babies 5–10% will develop persistent motor deficiencies. Furthermore, 20–50% will suffer from sensory or cognitive abnormalities; for example approximately 15% of cerebral palsy cases are due to severe neonatal HI (Hack et al., 1992; Vohr et al., 2000; Volpe, 2012; Lee et al., 2013).

Following HI insult, changes in cellular transcription, mitochondrial function, *de novo* protein synthesis and post-translational modifications all play pivotal roles (Culman et al., 2007; Pirianov et al., 2007; Yi et al., 2007). In experimental murine models of HI, implications have for example been shown for epigenetic mechanisms (Kumral et al., 2012; Lange et al., 2014), pH changes (Kendall et al., 2011b; Uria-Avellanal and Robertson, 2014), cytokines (Kendall et al., 2011a), transcription factors (Hristova et al., 2016), and protein kinases (Thei et al., 2018). Reperfusion injury and associated reactive oxygen species, together with persistent inflammation, are also a significant contributor to brain damage in neonates following HI insult (Rocha-Ferreira and Hristova, 2016). Following positive outcomes in clinical studies of hypothermia (Wyatt et al., 2007), cooling brain or whole body to 33°C (moderate hypothermia) is currently the only strategy with a demonstrated clinical benefit. In full-term infants with moderate to severe HI, cooling significantly improves survival and disability by 11% (Shankaran et al., 2012; Jacobs et al., 2013; Azzopardi et al., 2014). Importantly, as cooling is not always effective, with 40% of treated infants still suffering neurodevelopmental disabilities, recent studies have underscored the importance of combining hypothermia with safe adjunct therapies for more substantial neuroprotection. In addition, as hypothermia equipment is not readily available worldwide, novel safe and easy to apply treatments are of pivotal importance.

Mesenchymal stromal/stem cells (MSCs) have gained popularity over the years for their potential to implement regenerative medical treatment to damaged tissues of the body (Uccelli et al., 2008; Bruno et al., 2009; Gatti et al., 2011; Bruno et al., 2012; Lee et al., 2012; Tan et al., 2014; Collino et al., 2015; Heldring et al., 2015). Stem cells participate in the maintenance of homeostasis and restoration of tissues after injury through secretion of soluble factors and extracellular vesicles (EVs). EVs (exosomes and microvesicles) are 30–1000 nm lipid bilayer-enclosed structures which are released from parental cells and participate in cell-to-cell signaling processes. EVs have been shown to transport various biologically active molecules such as proteins, mRNAs, miRNAs, lncrRNAs, DNA, and lipids to target cells (Inal et al., 2012; Yeo et al., 2013; György et al., 2015; Bruno et al., 2017; Tricarico et al., 2017; van Niel et al., 2018). These molecules can act on various cell types by signaling proliferative and/or regenerative pathways including angiogenesis, cell proliferation, and immune tolerance (Deregibus et al., 2007; Camussi et al., 2010; Ranghino et al., 2012; de Jong et al., 2014; Robbins and Morelli, 2014; Lamichhane et al., 2015; Kholia et al., 2016; Merino-González et al., 2016; Zhang et al., 2016). Anti-inflammatory factors are one key group of molecules released by MSCs that are important in mediating repair and include interleukin 1 receptor agonist (IL-1ra) (Prockop and Oh, 2012), interleukin 10 (IL-10) (Németh et al., 2009), TNF-α-stimulated gene-6 (TSG6) (Drago et al., 2013; English, 2013; Madrigal et al., 2014), prostaglandin (PG) E2 (Prockop and Oh, 2012; Drago et al., 2013; English, 2013; Németh et al., 2009), and indoleamine 2,3-dioxygenase (IDO) (Meisel et al., 2004; Croitoru-Lamoury et al., 2011; Kang et al., 2012; Lin et al., 2012; Rong et al., 2012).

Recent studies have suggested that EVs released from MSCs may be more important than the actual stem cells themselves in mediating tissue-protective effects due to the therapeutic factors secreted by EVs, including anti-inflammatory mediators, cytokines and growth factors, as well as microRNAs (Collino et al., 2015; Vallabhaneni et al., 2015; Bruno et al., 2017).

Studies in regenerative models, using total EVs or separate applications of either 100K EVs (population enriched with exosomes) or 10K EVs (population enriched with microvesicles), have led to indicate that 100K EVs provide the bulk of pro-regenerative effects, although when total EVs were used it was found that 10K EVs do not interfere negatively with 100K EVs function (Bruno et al., 2017). Other studies have found that the application of total EVs was more effective than using isolated vesicle populations of 100K EVs or 10K EVs, respectively (Wen et al., 2016). Variations in these findings have not been fully explained yet but may possibly be due to differing target tissues.

HI studies using MSCs as putative treatment, have found that these stem cells have neuroprotective potential (van Velthoven et al., 2010; Kim et al., 2012; Donega et al., 2014; Park et al., 2015; Ahn et al., 2016; Corcelli et al., 2018) and the therapeutic time window was shown to be further extended when combining MSC treatment with hypothermia (Ahn et al., 2018). Furthermore, a potential for using stem cell derived EVs was recently shown in an ovine HI study, using intravenous administration of MSC-derived EVs following transient umbilical cord occlusion *in utero*, which lead to improved brain function following MSC-EV treatment (Ophelders et al., 2016).

As intranasal administration has shown to be more efficient compared to intravenous administration in experimental HI models, due to direct transport to the CNS and bypassing peripheral elimination (Merkus and van den Berg, 2007; Hanson and Frey, 2008), we thus aimed in the present study at assessing whether intranasal administration of MSC-EVs following HI-insult would have neuroprotective effects. We used the Rice-Vannucci neonatal mouse model, which involves permanent unilateral common carotid artery occlusion followed by severe (1 h) hypoxia (Vannucci and Vannucci, 1997).

Here we provide evidence that compared to control-treated littermates, the brains of animals treated intranasally with MSC-derived EVs, immediately following HI insult, show

significant neuroprotection and reduced neuroinflammation, as well as improved behavioral outcome, when assessed at 48 h post-treatment.

MATERIALS AND METHODS

Animals and Procedures

All animal experiments and care protocols were approved by the UK Home Office (PPL 70/8784) and UCL Animal Welfare and Ethical Review Board, carried out according to the United Kingdom Animals (Scientific Procedures) Act 1986. The ARRIVE guidelines were followed. Operations were performed at post-natal day 9 (P9) on C57/BI6 mice (Charles River, United Kingdom), bred in house, using a modification of the Rice–Vannucci model of neonatal HI as previously described (Hristova et al., 2010; Lange et al., 2014; Rocha-Ferreira et al., 2018). The parental animals were bred in an environment providing 12 h light/dark cycle and food and water *ad libitum*. The HI procedure was carried out as follows: P9 mice (males and females) were anesthetized using isoflurane (5% induction, 1.5% maintenance). Permanent occlusion of the left common carotid artery was established with 8/0 polypropylene sutures followed by wound closure with tissue glue. The pups were recovered at 36°C and then were returned to the dam for 2 h, whereafter they were placed in a 36°C hypoxic chamber for 60 min, in humidified 10% oxygen/90% nitrogen at 3 L/min. Immediately following hypoxic exposure, the animals were randomly allocated to HI, EV- or PBS-treatment group. The experimental (EV) group (n = 15) received one intranasal dose of 6 μL of EVs (100KTOT-EV; 1.25 × 10^9 particles/dose) in PBS, obtained from human bone marrow derived MSCs (Poietics hMSC, Cat no: PT-2501, Lot no: 0000446319, Lonza, Switzerland) and prepared according to Bruno et al. (2017). In brief, total EVs were isolated from characterized MSC cultures (Bruno et al., 2009), which were positive for the typical MSC markers (CD105, CD29, CD73, CD44, and CD90). The total EVs were collected from the supernatants of MSCs, cultured in fetal calf serum (FCS)-depleted RPMI medium, overnight. The EVs were a pool of 5 EV isolations with each isolation comprised of 10 flasks with a passage number ranging from P3 to P5 progressively. Cell debris and apoptotic bodies were removed by centrifugation at 3000 g for 20 min and thereafter total EVs were isolated by ultracentrifugation for 2 h at 100,000 g at 4°C and stored at −80°C until used. Details on EV characterization by nanoparticle tracking analysis (NTA, Nanosight, Malvern, United Kingdom), electron microscopy, FACS analysis and Western blotting for MSC-EV specific markers are shown in **Supplementary Figure S1**. EV payload characterization of proteomic and genomic content of these EVs was previously published in Bruno et al. (2017). The control treated groups received either 6 μL intranasal phosphate-buffered saline (PBS) (n = 14; vehicle only group) or no intervention (n = 16; HI only group). The pups where returned to the dam and left for 48 h before behavioral assessment and subsequent histological analysis of extracted brains. All animals (both males and females) from all litters were used in the experiments. Following the HI protocol the mums and pups were observed and scored for welfare of neonatal rodents (Lloyd et al., 2000). All pups were taken care of by the mums and achieved overall scores 0–1.

Post-natal day 9 mice were used in this particular experiment as they most closely resemble term neonates (Mallard and Vexler, 2015) and also possess injury responses phenotypically similar to white and gray matter damage, such as tissue loss, microglia-mediated immunity, cell-death-mediated apoptosis, astrogliosis, and neurobehavioral alterations (Vannucci and Vannucci, 1997). The hypoxic chamber conditions were 10% oxygen/90% nitrogen according to previously described protocols (Rocha-Ferreira et al., 2018). Differences between male and female mice were not taken into account and male and female pups were combined in the analysis, as testosterone levels in mice are similar in both genders at this age (P9–P11) (Clarkson and Herbison, 2016).

Tissue Sample Preparation

For histological assessment, animals were sacrificed 48 h post-HI insult with an intraperitoneal injection of pentobarbitone and perfusion with 30 mL of 4% paraformaldehyde in PBS. Brains were extracted and fixed in 4% paraformaldehyde in PBS at 4°C for 1 h, followed by cryoprotection in 30% sucrose dissolved in phosphate buffer (PB) for 24 h. Thereafter brains were frozen on dry ice, sectioned by cryostat into sequential 40 μm sections and stored at −80°C until histological analysis.

Histological Analysis

Coronal brain sections were compared between animals receiving MSC-derived EVs and control-treated animals (PBS-vehicle or HI only), for microglial activation, cell death, and infarct size. Tissue sections were scored blindly by two independent observers.

Microglial Activation Assessment

Tissue staining was performed according to established protocols as previously described (Hristova et al., 2010; Lange et al., 2014; Rocha-Ferreira et al., 2018). In brief, cryosections were thawed and rehydrated in distilled water, spread onto glass slides coated with 0.5% gelatine under a dissecting microscope, dried for 10 min, fixed in 4% formaldehyde in 100 mM PB for 5 min, treated with acetone (50, 100, 50%: 2 min each), 0.1% bovine serum albumin (PB/BSA) and washed twice in PB. The sections were pre-incubated with 5% goat serum (Sigma, St. Louis, MO, United States) in PB for 30 min and incubated overnight at 4°C with an antibody against αMβ2 integrin (Serotec, Oxford, United Kingdom) 1/5000. The sections were then washed in PB/BSA, PB, PB, PB/BSA (2 min each), incubated with biotin-labeled anti-rat IgG (Vector Laboratories, Inc., Burlingame, CA, United States) and visualized with Avidin-Biotinylated peroxidase Complex (ABC, Vector Laboratories, Inc.) and diaminobenzidine/hydrogen peroxide (DAB) stain. Sections were processed through alcohol and xylene and mounted with DEPEX (Sigma). Per animal, five cryosections (400 μm apart) of each brain were stained. Semi-quantitative scores for microglial activation were obtained as follows: 0 = no activation; 1 = focal activation; 2 = mild phagocytic activation affecting <50% of the region, thus showing diffuse activation with

occasional phagocytic macrophages; 3 = phagocytic activation affecting >50% of the region, thus showing widespread activation with predominant phagocytic macrophages; 4 = total phagocytic activation (Rocha-Ferreira et al., 2015, 2018; Hristova et al., 2016). Microglial activation was scored for the following brain regions: cortex, pyriform cortex, hippocampus, striatum, thalamus, and external capsule. These regions were selected based on known selective vulnerability as a result of increased metabolic rate (Martin et al., 1997; Barkovich et al., 1998; Johnston et al., 2001, 2002; Volpe, 2001; McQuillen et al., 2003; Castillo, 2007; Leisman et al., 2014; Schmidt-Kastner, 2015).

TUNEL Staining

For assessment of changes in cell death, brain tissue sections were stained at 400 μm intervals for DNA fragmentation using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) according to the manufacturer's instructions (Roche, United Kingdom). Cell death was quantified by averaging the numerical count of TUNEL-positive nuclei in three representative optical fields in each brain region, comparing EV-treated versus control-treated brains.

Infarct Volume Measurement

Infarct volume was measured by Nissl stain in five coronal sections at 400 μm intervals from each forebrain, stained with cresyl violet (VWR, United Kingdom). The Optimas 6.5 imaging analysis software (Bothell, WA, United States) was used to calculate the surviving brain tissue in each brain region as percentage between experimental and control side to estimate reduction in infarct size following EV treatment compared to control groups, according to previously described methods (Kendall et al., 2006).

Behavioral Assessment by Negative Geotaxis

The negative geotaxis test, assessing the labyrinthine reflex which reflects the strength and co-ordination of neonatal mice, was used for behavioral analysis in EV treated and control-treated or non-treated groups at 48 h post insult, before sacrificing the animals for brain extraction and subsequent histological assessment. The order of testing of the animals within the litters was randomly assigned. The mice were placed in the center of a 45° incline board with their heads facing downwards. The time for the animal to make a 180° turn to face upward and begin to move up the hill was recorded. Time was capped at 30 s. If the animal failed to complete the task by this time, 30 s was the recorded outcome. Each animal attempted this task three times and these values were averaged per animal (Rocha-Ferreira et al., 2018). The animal numbers tested per group were as follows: MSC-EV treated: n = 10; PBS-control treated: n = 10; and HI-untreated control: n = 13.

Statistical Analysis

Statistical analysis was carried out with GraphPad Prism 7 (La Jolla, CA, United States), using brain region as the repeated measure for the following statistical analyses: The same six forebrain regions (cortex, pyriform cortex, hippocampus, striatum, thalamus, external capsule) were used for each outcome and each assay. Only the TUNEL data-set passed the D'Agostino and Pearson normality test. Normalized data (only one data set: TUNEL-thalamus) were analyzed using the one-way ANOVA test and the Tukey's multiple comparisons test.

It is likely that with repeated measures such as these the observations from a single subject are correlated, therefore the first stage of the analysis included the observations from all the regions tested in a single mixed model with a random subject effect, to produce an estimate of the treatment effect and associated inference that accounts for the correlations in the data arising from the repeated measures. Further *post hoc* Tukey's multiple comparisons test was carried out to assess evidence for subregional differences, $p < 0.05$. Other regional data sets, being non-normalized data, were analyzed using the Kruskal–Wallis test and the Dunn's multiple comparisons test. For each outcome, the overall effect from the mixed linear model is reported, followed by the results from the individual regional *post hoc* tests. All data is presented as means and standard error of the mean (SEM) for each group. Group sizes (n = 15 EV, n = 14 PBS, n = 16 HI), were based on power calculations for test power >0.80 and significance <0.05. Statistical significance was determined for p-value <0.05.

Statistical significance in the negative geotaxis behavioral testing was assessed through Kruskal–Wallis with Dunn's multiple comparison test, as the data was non-normally distributed. Normal distribution was assessed using Shapiro–Wilk normality test. The data is presented as individual values and median ± interquartile bars.

RESULTS

Intranasal Application of MSC-Derived EVs Decreases Microglial Activation in HI

Microglial activation (AlphaM), as assessed through αMβ2 integrin immunoreactivity, was significantly reduced in the EV-treated, compared to control PBS-treated groups overall (Kruskal–Wallis test p = 0.0168, Dunn's multiple comparison test p = 0.0175) with subregional differences in cortex (p = 0.0319), hippocampus (p = 0.0392), and striatum (p = 0.0376) (**Figure 1**). Only a trend toward reduction, but not reaching significance, was observed in the other brain regions (**Figure 1**). Combining all six regions, using the Kruskal–Wallis test, the mean AlphaM scores varied significantly between treatment groups (p = 0.0168) (**Figure 1**).

Intranasal Application of MSC-Derived EVs Decreases HI-Mediated Cell Death

Intranasal application of MSC-derived EVs significantly reduced TUNEL+ cell death in cortex and external capsule (**Figure 2**). Using the Kruskal–Wallis test, the mean ipsilateral TUNEL+ cell counts per representative area in the cortex varied significantly between treatment groups (EV: 12.86; PBS: 26.09; HI: 32.82;

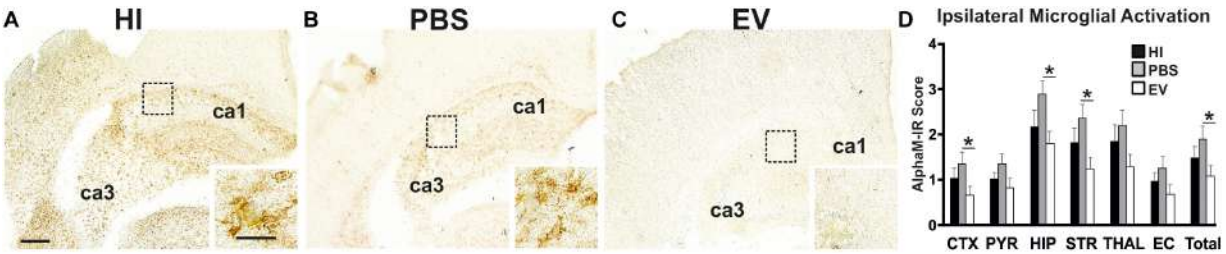


FIGURE 1 | Intranasal application of MSC-derived EVs, immediately following HI, significantly reduces microglial activation after 48 h. **(A–C)** Ipsilateral overview of early microglial activation in EV- and control treated animals. Note the strong microglial activation in the HI **(A)** and PBS-treated **(B)** groups with αM+ cells showing phagocytic morphology at high magnification (**A,B** – inserts, hippocampus), compared to the EV-treated brains exhibiting a ramified phenotype (**C** – insert). The ca1 and ca3 areas of hippocampus are indicated for reference. **(D)** Intranasal EV treatment significantly reduced ipsilateral microglial activation score (**D**, Mean + SEM) overall compared to control-treated groups (PBS or HI) (Kruskal–Wallis test, $p = 0.0168$; Dunn's test EV vs. PBS $^*p = 0.0175$) with significant individual decrease in cortex (Kruskal–Wallis test, $p = 0.0371$; Dunn's test EV vs. PBS $^*p = 0.0319$), hippocampus (Kruskal–Wallis test, p = 0.0451; Dunn's test EV vs. PBS $^*p = 0.0392$), striatum (Kruskal–Wallis test, $p = 0.0442$; Dunn's test EV vs. PBS $^*p = 0.0376$). CTX, cerebral iscocortex; PYR, pyriform cortex; HIP, hippocampus; STR, striatum, Thal, thalamus; EC, external capsule. Scale bars: **A–C** = 150 μm; inserts = 15 μm.

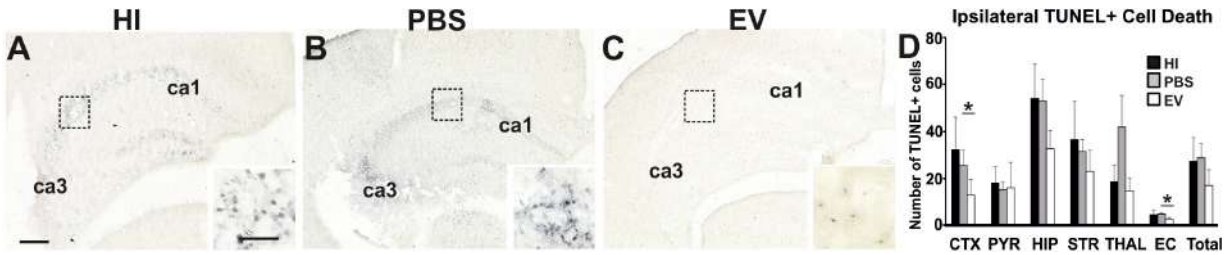


FIGURE 2 | Intranasal application of MSC-derived EVs, immediately following HI, significantly reduces TUNEL+ cell death after 48 h. **(A–C)** Histochemical overview of TUNEL+ cell death in the ipsilateral forebrain of HI **(A)**, PBS- **(B)**, and EV-treated **(C)** animals. Note the typical pyknotic nuclear morphology of the TUNEL+ cells observed in the HI and PBS groups (**A,B** – inserts, hippocampus) and the lack of such cells in the EV-treated group (**C** – insert). The ca1 and ca3 areas of hippocampus are indicated for reference. **(D)** Quantification of the number of TUNEL+ cells per 20× eye-field (Mean + SEM). Compared to HI and PBS-treated control animals, Intranasal EV treatment significantly reduced ipsilateral TUNEL+ cell death in cortex (Kruskal–Wallis test, $p = 0.0305$; Dunn's test EV vs. PBS $^*p = 0.0320$) and external capsule (Kruskal–Wallis test, $p = 0.0131$; Dunn's test EV vs. PBS $^*p = 0.0117$). CTX, cerebral iscocortex; PYR, pyriform cortex; HIP, hippocampus; STR, striatum; Thal, thalamus; EC, external capsule. Scale bars: **A–C** = 150 μm; inserts = 15 μm.

$p = 0.0305$). Using the Dunn's multiple comparisons test, significant decrease was found in EV-treated compared to the PBS treated control group ($p = 0.0320$).

The mean ipsilateral TUNEL+ cell counts per representative area in the external capsule varied significantly between treatment groups (Kruskal–Wallis test, EV: 2.125; PBS: 4.556; HI: 4.09; $p = 0.0131$). Dunn's multiple comparisons test showed significant decrease in the TUNEL+ cell counts in the EV-treated compared to control PBS-treated group ($p = 0.0117$) (**Figure 2**).

Intranasal Application of MSC-Derived EVs Decreases HI-Mediated Volume Loss

Intranasal EV-treatment resulted in significant reduction of tissue loss as assessed through NISSL staining, compared to control treated littermates overall (Kruskal–Wallis test, $p = 0.0453$), as well as in pyriform cortex, thalamus, and external capsule (**Figure 3**).

The mean ipsilateral volume loss in the pyriform cortex varied significantly between treatment groups (Kruskal–Wallis test, EV: 16.13%; PBS: 25.5%; HI: 19.63%; $p = 0.0273$). Dunn's multiple comparisons test showed significant decrease in the EV-treated compared to the PBS-treated groups ($p = 0.0219$). The mean ipsilateral volume loss in the thalamus varied significantly between treatment groups (Kruskal–Wallis test, EV: 17.53%; PBS: 23.43%; HI: 30.44%; $p = 0.0445$). Dunn's multiple comparisons test showed significant reduction in the EV-treated compared to HI groups ($p = 0.0348$). The mean ipsilateral volume loss in the external capsule varied significantly between treatment groups (Kruskal–Wallis test, EV: 10.8%; PBS: 25.21%; HI: 21.63%; $p = 0.0114$). Dunn's multiple comparisons test showed significant decrease in the EV-treated compared to the PBS-treated control groups ($p = 0.0126$) (**Figure 3**).

Intranasal Application of MSC-Derived EVs Improves Behavioral Outcomes

Intranasal administration of MSC-derived EVs significantly improved behavioral outcomes using the negative geotaxis test at 48 h (postnatal day 11, P11) postneonatal HI insult compared to control-treated animals (**Figure 4**). The Kruskal–Wallis test showed significant variation in the mean-time in seconds between treatment groups ($p = 0.0151$). Dunn's

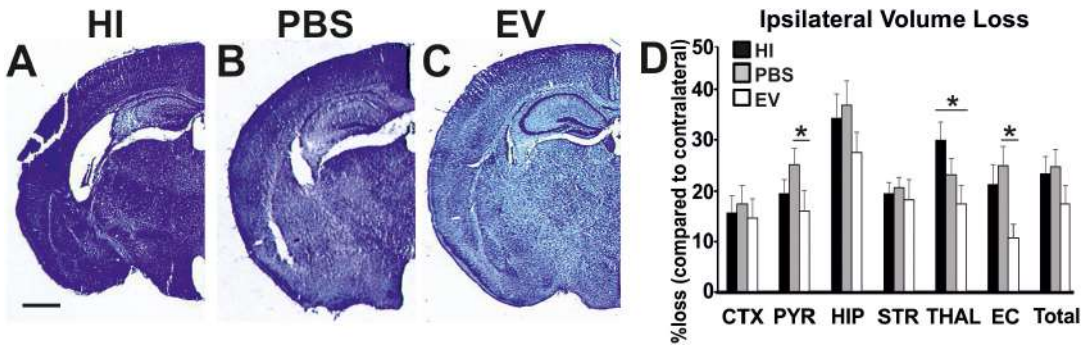


FIGURE 3 | Intranasal application of MSC-derived EVs, immediately following HI, significantly reduces tissue loss after 48 h. Ipsilateral forebrain Nissl staining (Cresyl-Violet, at rostral parietal level) of HI **(A)**, PBS- **(B)**, and EV-treated **(C)** animals and quantification of ipsilateral brain tissue volume loss **(D)** at 48 h following HI-insult. Intranasal EV treatment reduced volume loss (Mean + SEM) compared to HI and PBS-treated littermates (Kruskal–Wallis test, $p = 0.0453$) with significant, individual decrease in pyriform cortex (Kruskal–Wallis test, $p = 0.0273$; Dunn's test EV vs. PBS $^*p = 0.0219$), thalamus (Kruskal–Wallis test, $p = 0.0445$; Dunn's test EV vs. HI $^*p = 0.0348$), and external capsule (Kruskal–Wallis test, $p = 0.0114$; Dunn's test EV vs. PBS $^*p = 0.0126$). CTX, cerebral iscocortex; PYR, pyriform cortex; HIP, hippocampus; STR, striatum; Thal, thalamus; EC, external capsule. Scale bars: **A–C** = 470 μm.

multiple comparisons test showed significant decrease of the time necessary for change of orientation in the EV-treated compared to the HI-alone group ($p = 0.0114$) (**Figure 4**).

DISCUSSION

Our study shows evidence for neuroprotective effects of MSC-derived EVs via intranasal administration following neonatal HI brain injury, using the Rice–Vannucci mouse model in P9 mice. Intranasal treatment with MSC-derived EVs, immediately after severe (1 h) hypoxia following unilateral carotid artery occlusion, significantly reduced microglial activation, cell death, and tissue loss in the various brain regions tested, compared to brains in control-treated or untreated groups. In addition, compared to control littermates, intranasal EV treatment significantly improved short term behavioral outcomes as assessed through negative geotaxis.

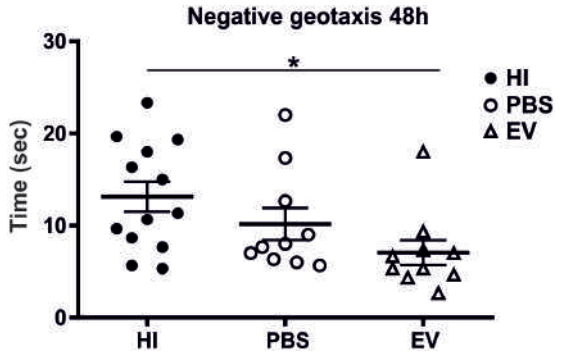


FIGURE 4 | Intranasal application of MSC-derived EVs, immediately following HI reduces the time required to change orientation in negative geotaxis following neonatal HI at 48 h (postnatal day 11). Intranasal treatment with MSC-derived EVs immediately following HI significantly reduces the time necessary for change of orientation compared to HI and PBS-treated littermate controls (Kruskal–Wallis test, $p = 0.0151$, Dunn's test EV vs. HI $^*p = 0.0114$).

Microglial activation was significantly reduced in cortex, hippocampus and striatum, while reduction in cell death was found in cortex and external capsule in the EV-treated group. Tissue volume loss was significantly reduced in pyriform cortex, thalamus and external capsule in the EV-treated group. Assessing all brain regions, the total brain regional means were also significantly decreased for microglial activation and tissue volume loss in the EV-treated groups.

These results are in line with findings from previous studies, showing differential vulnerability of certain brain regions to injury (Rocha-Ferreira and Hristova, 2016). HI injury at term, as modeled in this study, tends to damage the entire brain, most notably gray matter in cortex, hippocampus, and/or thalamus (Alexander et al., 2014).

Damage to the cortex, as well as to the thalamus and striatum (Lubics et al., 2005) have been associated with sensorimotor deficits in animal models of HI (Mercuri and Barnett, 2003; Mercuri et al., 2004; Martinez-Biarge et al., 2011). HI damage in the hippocampus and associated projections to the cortex have been shown to result in disrupted memory function and spatial processing (Aylward, 2005). Significant reductions in hippocampus volume have been shown to reduce long-term reference memory, short-term working memory (Ikeda et al., 2001) and impact the necessary role of the hippocampus in spatial navigation and recollection (Packard and McGaugh, 1996; White and McDonald, 2002). The dorsal striatum, namely the nucleus accumbens, may impact non-spatial navigation, and learning (Packard and Knowlton, 2002; White and McDonald, 2002), thus damage to this structure may account for the non-spatial memory deficits seen for example in HI injured rats (Alexander et al., 2014). Fronto-striato-thalamic circuitry damage from HI injury may lead to deficits in attention, executive function, and activity modulation (Aylward, 2005). As studies have shown anxiety-like behavior in mice following HI injury (Sanches et al., 2013), it has been hypothesized that HI injury may be associated

with susceptibility to other pathologies such as attention-deficit hyperactivity disorder (ADHD), autism and schizophrenia, as the hippocampus and striatum are associated with related cognitive functions (DeLong, 1992; Lou, 1996; Dilenge et al., 2001; Van Petten, 2004; de Haan et al., 2006).

The results of the present study, emphasize the clinical potential of MSC-derived EVs for neuroprotection following neonatal HI injury and are in line with studies using MSC treatment in neonatal HI murine models (van Velthoven et al., 2010, 2012; Kim et al., 2012; Donega et al., 2014) as well as MSC-derived EVs treatment in an ovine neonatal HI model (Ophelders et al., 2016). The use of EVs as therapeutic vesicles is thus of great clinical interest. Previous HI studies using whole MSCs have found neuroprotective potential for these stem cells (van Velthoven et al., 2010; Donega et al., 2014; Park et al., 2015; Ahn et al., 2016; Corcelli et al., 2018) and the therapeutic time window was shown to be extended when combining MSC treatment with hypothermia (Ahn et al., 2018).

As there may be practical drawbacks of using whole MSCs in clinic, the use of MSC-derived EVs has been gaining increased interest. Neuroprotective effects were for example shown in a recent HI study following intravenous administration of MSC-derived EVs in an ovine model of transient umbilical cord occlusion *in utero*, with improved brain function and reduction of the number and duration of seizures following treatment (Ophelders et al., 2016). It has been shown that intravenous MSC delivery to the brain is hampered by the blood brain barrier (BBB) and results in a tendency of the MSCs to accumulate in other organs, such as the lungs (Lappalainen et al., 2008; Fischer et al., 2009). Intra-arterial administration can deliver high numbers of MSCs to the brain (Lappalainen et al., 2008; Li et al., 2010; Pendharkar et al., 2010), however, this method also has high incidences of mortality and impaired cerebral blood flow in rat HI models (Walczak et al., 2008; Pendharkar et al., 2010). Attempts to increase cell delivery by disrupting the BBB can leave the animal susceptible to infection or toxins (Burgess et al., 2011). Intranasal administration has shown to be superior to intravenous administration in experimental HI models due to direct transport to the CNS through intranasal drug delivery (Merkus and van den Berg, 2007) via the olfactory and trigeminal neural pathways, which innervate the nasal cavity and create a direct pathway to the CNS (Hanson and Frey, 2008). Thus in this study we delivered MSC-derived EVs intranasally, as this method is more efficient and far less invasive than intracranial, intravenous or intraarterial deliveries and avoids inactivation by the gastrointestinal and hepatic first-pass metabolism (Hanson et al., 2013; Djupesland et al., 2014; Kozlovskaya et al., 2014). The MSC-derived EVs used in this study are well characterized (Collino et al., 2015; Bruno et al., 2017; GEO GSE59958; **Supplementary Figure S1**) and have previously shown to regenerative potential in murine acute renal injury models (Bruno et al., 2012; Bruno et al., 2017).

In the current study, we assessed the neuroprotective effects of MSC-derived EVs following intranasal administration after a HI-insult. We observed significant neuroprotective effects and improved short-term behavioral outcomes. This bodes well for future applications of MSC-derived EVs, or EVs derived from other stem cells, following neonatal HI and may also be translatable to other types of neurotrauma. Tracing of MSC-derived EVs, using PKH26 (Long et al., 2017), would further provide information on the exact fate of intranasally delivered EVs to specific brain-regions and such tracing of EV fate is indeed planned by our group, alongside assessment on longer-term outcomes following MSC-EV treatment. The application of stem-cell derived EVs in combination with hypothermia, for an extended therapeutic time window, should also be explored. The preparation of the EVs is relatively inexpensive and simple, but for successful translation into the clinic strict quality control of MSC cultures and well characterized MSC-derived EVs will be required. Overall our data suggests that intranasal application of MSC-derived EVs following neonatal HI insult has neuroprotective effects and thus possesses potential as clinical treatment for neonatal HI brain damage.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

CS and MH carried out the animal experiments. SK, MHS, SB, MD, GC, and JI prepared and characterized the MSC-EVs. CS, JN, and MH generated the histological and behavioral data. All authors contributed to data analysis and read and approved the final manuscript. SL and MH co-designed the study and wrote the manuscript.

FUNDING

This work was supported in parts by the BBSRC LIDo, UCLB Apolo PoC, the UCL graduate school and a University of Westminster Start-Up Grant. The funding bodies had no role in the design of the study, analysis, interpretation of data, or writing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00282/full#supplementary-material

FIGURE S1 | Characterization of the MSC-derived EVs used in the current study for intranasal application following HI. **(A)** Nanosight analysis showing EVs in the size range of 30–1000 nm. **(B)** Electron microscopy showing purified MSC-derived EVs, the scale bar represents 0.5 μm. **(C)** FACS analysis verifying the presence of the following MSC-EV specific markers: CD105, CD44, CD73, CD29, CD63, and CD81. **(D)** Western blotting showing MSC-EV specific markers CD63 and CD9.

REFERENCES

Ahn, S. Y., Chang, Y. S., and Park, W. S. (2016). Stem cells for neonatal brain disorders. *Neonatology* 109, 377–383. doi: 10.1159/000444905

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., and Park, W. S. (2018). Hypothermia broadens the therapeutic time window of mesenchymal stem cell transplantation for severe neonatal hypoxic ischemic encephalopathy. *Sci. Rep.* 8:7665. doi: 10.1038/s41598-018-25902-x

Alexander, M., Garbus, H., Smith, A. L., Rosenkrantz, T. S., and Fitch, R. H. (2014). Behavioral and histological outcomes following neonatal HI injury in a preterm (P3) and term (P7) rodent model. *Behav. Brain Res.* 259, 85–96. doi: 10.1016/j.bbr.2013.10.038

Aylward, G. P. (2005). Neurodevelopmental outcomes of infants born prematurely. *J. Dev. Behav. Pediatr.* 26, 427–440. doi: 10.1097/00004703-200512000-00008

Azzopardi, D., Strohm, B., Marlow, N., Brocklehurst, P., Deierl, A., Eddama, O., et al. (2014). Effects of hypothermia for perinatal asphyxia on childhood outcomes. *N. Engl. J. Med.* 371, 140–149. doi: 10.1056/NEJMoa1315788

Barkovich, A. J., Hajnal, B. L., Vigneron, D., Sola, A., Partridge, J. C., Allen, F., et al. (1998). Prediction of neuromotor outcome in perinatal asphyxia: evaluation of MR scoring systems. *AJNR Am. J. Neuroradiol.* 19, 143–149.

Bruno, S., Grange, C., Collino, F., Deregibus, M. C., Cantaluppi, V., Biancone, L., et al. (2012). Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury. *PLoS One* 7:e33115. doi: 10.1371/journal.pone.0033115

Bruno, S., Grange, C., Deregibus, M. C., Calogero, R. A., Saviozzi, S., Collino, F., et al. (2009). Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J. Am. Soc. Nephrol.* 20:1053. doi: 10.1681/ASN.2008070798

Bruno, S., Tapparo, M., Collino, F., Chiabotto, G., Deregibus, M. C., Soares Lindoso, R., et al. (2017). Renal regenerative potential of different extracellular vesicle populations derived from bone marrow mesenchymal stromal cells. *Tissue Eng. Part A* 23, 1262–1273. doi: 10.1089/ten.TEA.2017.0069

Burgess, A., Ayala-Grosso, C. A., Ganguly, M., Jordão, J. F., Aubert, I., and Hynynen, K. (2011). Targeted delivery of neural stem cells to the brain using MRI-guided focused ultrasound to disrupt the blood-brain barrier. *PLoS One* 6:e27877. doi: 10.1371/journal.pone.0027877

Camussi, G., Deregibus, M. C., and Tetta, C. (2010). Paracrine/endocrine mechanism of stem cells on kidney repair: role of microvesicle-mediated transfer of genetic information. *Curr. Opin. Nephrol. Hypertens.* 19, 7–12. doi: 10.1097/MNH.0b013e328332fb6f

Castillo, M. (2007). Selective vulnerability and the cerebellum in neonates. *AJNR Am. J. Neuroradiol.* 28, 20–21.

Clarkson, J., and Herbison, A. E. (2016). Hypothalamic control of the male neonatal testosterone surge. *Philos. Trans. R. Soc. London B Biol. Sci.* 371:20150115. doi: 10.1098/rstb.2015.0115

Collino, F., Bruno, S., Incarnato, D., Dettori, D., Neri, F., Provero, P., et al. (2015). AKI recovery induced by mesenchymal stromal cell-derived extracellular vesicles carrying MicroRNAs. *J. Am. Soc. Nephrol.* 26, 2349–2360. doi: 10.1681/ASN.2014070710

Corcelli, M., Hawkins, K., Vlahova, F., Hunjan, A., Dowding, K., De Coppi, P., et al. (2018). Neuroprotection of the hypoxic-ischemic mouse brain by human CD117+CD90+CD105+ amniotic fluid stem cells. *Sci. Rep.* 8:2425. doi: 10.1038/s41598-018-20710-9

Croitoru-Lamoury, J., Lamoury, F. M. J., Caristo, M., Suzuki, K., Walker, D., Takikawa, O., et al. (2011). Interferon-γ regulates the proliferation and differentiation of mesenchymal stem cells via activation of indoleamine 2,3 dioxygenase (IDO). *PLoS One* 6:e14698. doi: 10.1371/journal.pone.0014698

Culman, J., Zhao, Y., Gohlke, P., and Herdegen, T. (2007). PPAR-gamma: therapeutic target for ischemic stroke. *Trends Pharmacol. Sci.* 28, 244–249. doi: 10.1016/j.tips.2007.03.004

de Haan, M., Wyatt, J. S., Roth, S., Vargha-Khadem, F., Gadian, D., and Mishkin, M. (2006). Brain and cognitive-behavioural development after asphyxia at term birth. *Dev. Sci.* 9, 350–358. doi: 10.1111/j.1467-7687.2006.00499.x

de Jong, R., Houtgraaf, J. H., Samiei, S., Boersma, E., and Duckers, H. J. (2014). Intracoronary stem cell infusion after acute myocardial infarction: a meta-analysis and update on clinical trials. *Circ. Cardiovasc. Interv.* 7, 156–167. doi: 10.1161/CIRCINTERVENTIONS.113.001009

DeLong, G. R. (1992). Autism, amnesia, hippocampus, and learning. *Neurosci. Biobehav. Rev.* 16, 63–70. doi: 10.1016/S0149-7634(05)80052-1

Deregibus, M. C., Cantaluppi, V., Calogero, R., Lo Iacono, M., Tetta, C., Biancone, L., et al. (2007). Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* 110, 2440–2448. doi: 10.1182/blood-2007-03-078709

Dilenge, M. E., Majnemer, A., and Shevell, M. I. (2001). Long-term developmental outcome of asphyxiated term neonates. *J. Child Neurol.* 16, 781–792. doi: 10.1177/088307380101601102l

Djupesland, P. G., Messina, J. C., and Mahmoud, R. A. (2014). The nasal approach to delivering treatment for brain diseases: an anatomic, physiologic, and delivery technology overview. *Ther. Deliv.* 5, 709–733. doi: 10.4155/tde.14.41

Donega, V., Nijboer, C. H., van Tilborg, G., Dijkhuizen, R. M., Kavelaars, A., and Heijnen, C. J. (2014). Intranasally administered mesenchymal stem cells promote a regenerative niche for repair of neonatal ischemic brain injury. *Exp. Neurol.* 261, 53–64. doi: 10.1016/j.expneurol.2014.06.009

Drago, D., Cossetti, C., Iraci, N., Gaude, E., Musco, G., Bachi, A., et al. (2013). The stem cell secretome and its role in brain repair. *Biochimie* 95, 2271–2285. doi: 10.1016/j.biochi.2013.06.020

English, K. (2013). Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol. Cell Biol.* 91, 19–26. doi: 10.1038/icb.2012.56

Fischer, U. M., Harting, M. T., Jimenez, F., Monzon-Posadas, W. O., Xue, H., Savitz, S. I., et al. (2009). Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev.* 18, 683–692. doi: 10.1089/scd.2008.0253

Gatti, S., Bruno, S., Deregibus, M. C., Sordi, A., Cantaluppi, V., Tetta, C., et al. (2011). Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrol. Dial Transplant.* 26:1474. doi: 10.1093/ndt/gfr015

György, B., Hung, M. E., Breakefield, X. O., and Leonard, J. N. (2015). Therapeutic applications of extracellular vesicles: clinical promise and open questions. *Annu. Rev. Pharmacol. Toxicol.* 55, 439–464. doi: 10.1146/annurev-pharmtox-010814-124630

Hack, M., Breslau, N., Aram, D., Weissman, B., Klein, N., and Borawski-Clark, E. (1992). The effect of very low birth weight and social risk on neurocognitive abilities at school age. *J. Dev. Behav. Pediatr.* 13, 412–420. doi: 10.1097/00004703-199212000-00005

Hagberg, H., Peebles, D., and Mallard, C. (2002). Models of white matter injury: comparison of infectious, hypoxic-ischaemic, and excitotoxic insults. *Ment. Retard. Dev. Disabil. Res. Rev.* 8, 30–38. doi: 10.1002/mrdd.10007

Hanson, L. R., Fine, J. M., Svitak, A. L., and Faltesek, K. A. (2013). Intranasal administration of CNS therapeutics to awake mice. *J. Vis. Exp.* 74:4440. doi: 10.3791/4440

Hanson, L. R., and Frey, W. H. (2008). Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease. *BMC Neurosci.* 9(Suppl. 3):S5. doi: 10.1186/1471-2202-9-S3-S5

Heldring, N., Mager, I., Wood, M. J. A., and Le Blanc, K. (2015). Therapeutic potential of multipotent mesenchymal stromal cells and their extracellular vesicles. *Hum. Gene Ther.* 26:506. doi: 10.1089/hum.2015.072

Hristova, M., Cuthill, D., Zbarsky, V., Acosta-Saltos, A., Wallace, A., Blight, K., et al. (2010). Activation and deactivation of periventricular white matter phagocytes during postnatal mouse development. *Glia* 58, 11–28. doi: 10.1002/glia.20896

Hristova, M., Rocha-Ferreira, E., Fontana, X., Thei, L., Buckle, R., Christou, M., et al. (2016). Inhibition of Signal Transducer and Activator of Transcription 3 (STAT3) reduces neonatal hypoxic-ischaemic brain damage. *J. Neurochem.* 136, 981–994. doi: 10.1111/jnc.13490

Ikeda, T., Mishima, K., Yoshikawa, T., Iwasaki, K., Fujiwara, M., Xia, Y. X., et al. (2001). Selective and long-term learning impairment following neonatal hypoxic-ischemic brain insult in rats. *Behav. Brain Res.* 118, 17–25. doi: 10.1016/S0166-4328(00)00287-4

Inal, J. M., Ansa-Addo, E. A., Stratton, D., Kholia, S., Antwi-Baffour, S. S., Jorfi, S., et al. (2012). Microvesicles in health and disease. *Arch. Immunol. Ther. Exp.* 60, 107–121. doi: 10.1007/s00005-012-0165-2

Jacobs, S. E., Berg, M., Hunt, R., Tarnow-Mordi, W. O., Inder, T. E., and Davis, P. G. (2013). Cooling for newborns with hypoxic ischaemic encephalopathy.

Cochrane Database Syst. Rev. 1: CD003311. doi: 10.1002/14651858.CD003311.pub3

Johnston, M. V., Nakajima, W., and Hagberg, H. (2002). Mechanisms of hypoxic neurodegeneration in the developing brain. *Neuroscientist* 8, 212–220. doi: 10.1177/1073858402008003007

Johnston, M. V., Trescher, W. H., Ishida, A., and Nakajima, W. (2001). Neurobiology of hypoxicischemic injury in the developing brain. *Pediatr. Res.* 49, 735–741. doi: 10.1203/00006450-200106000-00003

Kang, J. W., Koo, H. C., Hwang, S. Y., Kang, S. K., Ra, J. C., Lee, M. H., et al. (2012). Immunomodulatory effects of human amniotic membrane-derived mesenchymal stem cells. *J. Vet. Sci.* 13, 23–31. doi: 10.4142/jvs.2012.13.1.23

Kendall, G. S., Hristova, M., Horn, S., Dafou, D., Acosta-Saltos, A., Almolda, B., et al. (2011a). TNF gene cluster deletion abolishes lipopolysaccharide-mediated sensitization of the neonatal brain to hypoxic ischemic insult. *Lab. Invest.* 91, 328–341. doi: 10.1038/labinvest.2010.192

Kendall, G. S., Hristova, M., Zbarsky, V., Clements, A., Peebles, D. M., Robertson, N. J., et al. (2011b). Distribution of pH changes in mouse neonatal hypoxic-ischaemic insult. *Dev. Neurosci.* 33, 505–518. doi: 10.1159/000333850

Kendall, G. S., Robertson, N. J., Iwata, O., Peebles, D., and Raivich, G. (2006). N-methylisobutyl-amiloride ameliorates brain injury when commenced before hypoxia ischemia in neonatal mice. *Pediatr. Res.* 59, 227–231. doi: 10.1203/01.pdr.0000196805.68082.22

Kholia, S., Ranghino, A., Garnieri, P., Lopatina, T., Deregibus, M. C., Rispoli, P., et al. (2016). Extracellular vesicles as new players in angiogenesis. *Vascul. Pharmacol.* 86, 64–70. doi: 10.1016/j.vph.2016.03.005

Kim, E. S., Ahn, S. Y., Im, G. H., Sung, D. K., Park, Y. R., Choi, S. H., et al. (2012). Human umbilical cord blood-derived mesenchymal stem cell transplantation attenuates severe brain injury by permanent middle cerebral artery occlusion in newborn rats. *Pediatr. Res.* 72, 277–284. doi: 10.1038/pr.2012.71

Kozlovskaya, L., Abou-Kaoud, M., and Stepensky, D. (2014). Quantitative analysis of drug delivery to the brain via nasal route. *J. Control Release* 189, 133–140. doi: 10.1016/j.jconrel.2014.06.053

Kumral, A., Tuzun, F., Tugyan, K., Ozbal, S., Yılmaz, O., Yesilirmak, C. D., et al. (2012). Role of epigenetic regulatory mechanisms in neonatal hypoxic-ischemic brain injury. *Early Hum. Dev.* 89, 165–173. doi: 10.1016/j.earlhumdev.2012.09.016

Kurinczuk, J. J., White-Koning, M., and Badawi, N. (2010). Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early Hum. Dev.* 86, 329–338. doi: 10.1016/j.earlhumdev.2010.05.010

Lamichhane, T. N., Sokic, S., Schardt, J. S., Raiker, R. S., Lin, J. W., and Jay, S. M. (2015). Emerging roles for extracellular vesicles in tissue engineering and regenerative medicine. *Tissue Eng. Part B Rev.* 21, 45–54. doi: 10.1089/ten.TEB.2014.0300

Lange, S., Rocha-Ferreira, E., Thei, L., Mawjee, P., Bennett, K., Thompson, P. R., et al. (2014). Peptidylarginine deiminases: novel drug targets for prevention of neuronal damage following hypoxic ischemic insult (HI) in neonates. *J. Neurochem.* 130, 555–562. doi: 10.1111/jnc.12744

Lappalainen, R. S., Narkilahti, S., Huhtala, T., Liimatainen, T., Suuronen, T., Närvänen, A., et al. (2008). The SPECT imaging shows the accumulation of neural progenitor cells into internal organs after systemic administration in middle cerebral artery occlusion rats. *Neurosci. Lett.* 440, 246–250. doi: 10.1016/j.neulet.2008.05.090

Lee, A. C. C., Kozuki, N., Blencowe, H., Vos, T., Bahalim, A., Darmstadt, G. L., et al. (2013). Intrapartum-related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990. *Pediatr. Res.* 74(Suppl. 1), 50–72. doi: 10.1038/pr.2013.206

Lee, C., Mitsialis, S. A., Aslam, M., Vitali, S. H., Vergadi, E., Konstantinou, G., et al. (2012). Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 126:2601. doi: 10.1161/CIRCULATIONAHA.112.114173

Leisman, G., Braun-Benjamin, O., and Melillo, R. (2014). Cognitive-motor interactions of the basal ganglia in development. *Front. Syst. Neurosci.* 8:16. doi: 10.3389/fnsys.2014.00016

Li, L., Jiang, Q., Ding, G., Zhang, L., Zhang, Z. G., Li, Q., et al. (2010). Effects of administration route on migration and distribution of neural progenitor cells transplanted into rats with focal cerebral ischemia, an MRI study. *J. Cereb. Blood Flow Metab.* 30, 653–662. doi: 10.1038/jcbfm.2009.238

Lin, W., Oh, S. K. W., Choo, A. B. H., and George, A. J. T. (2012). Activated T cells modulate immunosuppression by embryonic-and bone marrow-derived mesenchymal stromal cells through a feedback mechanism. *Cytotherapy* 14, 274–284. doi: 10.3109/14653249.2011.635853

Lloyd, M., Wolfensohn, S., and Thornton, P. (2000). "Quantitative assessment of welfare in experimental animals: the development and use of scoring systems," in *Proceedings of the 3rd World Congress on Alternatives and Animal Use in the Life Science: Progress in the Reduction, Refinement and Replacement of Animal Experimentation*, eds M. Balls, A.-M. van Zeller, and M. E. Halder (Amsterdam: Elsevier Science), 1107–1117.

Long, Q., Upadhya, D., Hattiangady, B., Kim, D. K., An, S. Y., Shuai, B., et al. (2017). Intranasal MSC-derived A1-exosomes ease inflammation, and prevent abnormal neurogenesis and memory dysfunction after status epilepticus. *Proc. Natl. Acad. Sci. U.S.A.* 114, E3536–E3545. doi: 10.1073/pnas.1703920114

Lou, H. C. (1996). Etiology and pathogenesis of attention-deficit hyperactivity disorder (ADHD): significance of prematurity and perinatal hypoxic-haemodynamic encephalopathy. *Acta Paediatr.* 85, 1266–1271. doi: 10.1111/j.1651-2227.1996.tb13909.x

Lubics, A., Reglodi, D., Tamás, A., Kiss, P., Szalai, M., Szalontay, L., et al. (2005). Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic-ischemic injury. *Behav. Brain Res.* 157, 157–165. doi: 10.1016/j.bbr.2004.06.019

Madrigal, M., Rao, K. S., and Riordan, N. H. (2014). A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J. Transl. Med.* 12:260. doi: 10.1186/s12967-014-0260-8

Mallard, C., and Vexler, Z. S. (2015). Modeling ischemia in the immature brain: how translational are animal models? *Stroke* 46, 3006–3011. doi: 10.1161/STROKEAHA.115.007776

Mallard, C., Welin, A. K., Peebles, D., Hagberg, H., and Kjellmer, I. (2003). White matter injury following systemic endotoxemia or asphyxia in the fetal sheep. *Neurochem. Res.* 28, 215–223. doi: 10.1023/A:1022368915400

Martin, L. J., Brambrink, A., Koehler, R. C., and Traystman, R. J. (1997). Primary sensory and forebrain motor systems in the newborn brain are preferentially damaged by hypoxia-ischemia. *J. Comp. Neurol.* 377, 262–285. doi: 10.1002/(SICI)1096-9861(19970113)377:2<262::AID-CNE8>3.0.CO;2-1

Martinez-Biarge, M., Diez-Sebastian, J., Kapellou, O., Gindner, D., Allsop, J. M., Rutherford, M. A., et al. (2011). Predicting motor outcome and death in term hypoxic-ischemic encephalopathy. *Neurology* 76, 2055–2061. doi: 10.1212/WNL.0b013e31821f442d

McQuillen, P. S., Sheldon, R. A., Shatz, C. J., and Ferriero, D. M. (2003). Selective vulnerability of subplate neurons after early neonatal hypoxia-ischemia. *J. Neurosci.* 23, 3308–3315. doi: 10.1523/JNEUROSCI.23-08-03308.2003

Meisel, R., Zibert, A., Laryea, M., Göbel, U., Däubener, W., and Dilloo, D. (2004). Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenasemediated tryptophan degradation. *Blood* 103, 4619–4621. doi: 10.1182/blood-2003-11-3909

Mercuri, E., Barnett, A., Rutherford, M., Guzzetta, A., Haataja, L., Cioni, G., et al. (2004). Neonatal cerebral infarction and neuromotor outcome at school age. *Pediatrics* 113, 95–100. doi: 10.1542/peds.113.1.95

Mercuri, E., and Barnett, A. L. (2003). Neonatal brain MRI and motor outcome at school age in children with neonatal encephalopathy: a review of personal experience. *Neural Plast.* 10, 51–57. doi: 10.1155/NP.2003.51

Merino-González, C., Zuñiga, F. A., Escudero, C., Ormazabal, V., Reyes, C., Nova-Lamperti, E., et al. (2016). Mesenchymal stem cell-derived extracellular vesicles promote angiogenesis: potencial clinical application. *Front. Physiol.* 7:24. doi: 10.3389/fphys.2016.00024

Merkus, F. W. H. M., and van den Berg, M. P. (2007). Can nasal drug delivery bypass the bloodbrain barrier: questioning the direct transport theory. *Drugs R. D.* 8, 133–144. doi: 10.2165/00126839-200708030-00001

Németh, K., Leelahavanichkul, A., Yuen, P. S. T., Mayer, B., Parmelee, A., Doi, K., et al. (2009). Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat. Med.* 15, 42–49. doi: 10.1038/nm.1905

Ophelders, D. R. M. G., Wolfs, T. G. A. M., Jellema, R. K., Zwanenburg, A., Andriessen, P., Delhaas, T., et al. (2016). Mesenchymal stromal cell-derived

extracellular vesicles protect the fetal brain after hypoxia-ischemia. *Stem Cells Transl. Med.* 5, 754–763. doi: 10.5966/sctm.2015-0197

Packard, M. G., and Knowlton, B. J. (2002). Learning and memory functions of the Basal Ganglia. *Annu. Rev. Neurosci.* 25, 563–593. doi: 10.1146/annurev.neuro.25.112701.142937

Packard, M. G., and McGaugh, J. L. (1996). Inactivation of hippocampus or caudate nucleus with lidocaine differentially affects expression of place and response learning. *Neurobiol. Learn. Mem.* 65, 65–72. doi: 10.1006/nlme.1996.0007

Park, W. S., Sung, S. I., Ahn, S. Y., Yoo, H. S., Sung, D. K., Im, G. H., et al. (2015). Hypothermia augments neuroprotective activity of mesenchymal stem cells for neonatal hypoxic-ischemic encephalopathy. *PLoS One* 10:e0120893. doi: 10.1371/journal.pone.0120893

Pendharkar, A. V., Chua, J. Y., Andres, R. H., Wang, N., Gaeta, X., Wang, H., et al. (2010). Biodistribution of neural stem cells after intravascular therapy for hypoxic-ischemia. *Stroke* 41, 2064–2070. doi: 10.1161/STROKEAHA.109.575993

Perlman, J. M. (2006). Intervention strategies for neonatal hypoxic-ischemic cerebral injury. *Clin. Ther.* 28, 1353–1365. doi: 10.1016/j.clinthera.2006.09.005

Pirianov, G., Brywe, K. G., Mallard, C., Edwards, A. D., Flavell, R. A., Hagberg, H., et al. (2007). Deletion of the c-Jun N-terminal kinase 3 gene protects neonatal mice against cerebral hypoxic-ischaemic injury. *J. Cereb. Blood Flow Metab.* 27, 1022–1032. doi: 10.1038/sj.jcbfm.9600413

Prockop, D. J., and Oh, J. Y. (2012). Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. *Mol. Ther.* 20, 14–20. doi: 10.1038/mt.2011.211

Ranghino, A., Cantaluppi, V., Grange, C., Vitillo, L., Fop, F., Biancone, L., et al. (2012). Endothelial progenitor cell-derived microvesicles improve neovascularization in a murine model of hindlimb ischemia. *Int. J. Immunopathol. Pharmacol.* 25, 75–85. doi: 10.1177/039463201202500110

Robbins, P. D., and Morelli, A. E. (2014). Regulation of immune responses by extracellular vesicles. *Nat. Rev. Immunol.* 14, 195–208. doi: 10.1038/nri3622

Rocha-Ferreira, E., and Hristova, M. (2016). Plasticity in the neonatal brain following hypoxic-ischaemic injury. *Neural Plast.* 2016:4901014. doi: 10.1155/2016/4901014

Rocha-Ferreira, E., Phillips, E., Francesch-Domenech, E., Thei, L., Peebles, D. M., Raivich, G., et al. (2015). The role of different strain backgrounds in bacterial endotoxin-mediated sensitization to neonatal hypoxic-ischemic brain damage. *Neuroscience* 311, 292–307. doi: 10.1016/j.neuroscience.2015.10.035

Rocha-Ferreira, E., Vincent, A., Bright, S., Peebles, D. M., and Hristova, M. (2018). The duration of hypothermia affects short-term neuroprotection in a mouse model of neonatal hypoxic ischaemic injury. *PLoS One* 13:e0199890. doi: 10.1371/journal.pone.0199890

Rong, L.-J., Chi, Y., Yang, S.-G., Chen, D.-D., Chen, F., Xu, S.-X., et al. (2012). Effects of interferon-γ on biological characteristics and immunomodulatory property of human umbilical cord-derived mesenchymal stem cells. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 20, 421–426.

Sanches, E. F., Arteni, N. S., Nicola, F., Boisserand, L., Willborn, S., and Netto, C. A. (2013). Early hypoxia-ischemia causes hemisphere and sex-dependent cognitive impairment and histological damage. *Neuroscience* 237, 208–215. doi: 10.1016/j.neuroscience.2013.01.066

Schmidt-Kastner, R. (2015). Genomic approach to selective vulnerability of the hippocampus in brain ischemia-hypoxia. *Neuroscience* 309, 259–279. doi: 10.1016/j.neuroscience.2015.08.034

Shankaran, S., Pappas, A., McDonald, S. A., Vohr, B. R., Hintz, S. R., Yolton, K., et al. (2012). Childhood outcomes after hypothermia for neonatal encephalopathy. *N. Engl. J. Med.* 366, 2085–2092. doi: 10.1056/NEJMoa1112066

Tan, C. Y., Lai, R. C., Wong, W., Dan, Y. Y., Lim, S. K., and Ho, H. K. (2014). Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res. Ther.* 5:76. doi: 10.1186/scrt465

Thei, L., Rocha-Ferreira, E., Peebles, D., Raivich, G., and Hristova, M. (2018). Extracellular signal-regulated kinase 2 has duality in function between neuronal and astrocyte expression following neonatal hypoxic-ischaemic cerebral injury. *J. Physiol.* doi: 10.1113/JP275649 [Epub ahead of print].

Tricarico, C., Clancy, J., and D'Souza-Schorey, C. (2017). Biology and biogenesis of shed microvesicles. *Small GTPases* 8, 220–232. doi: 10.1080/21541248.2016.1215283

Uccelli, A., Moretta, L., and Pistoia, V. (2008). Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* 8, 726–736. doi: 10.1038/nri2395

Uria-Avellanal, C., and Robertson, N. J. (2014). Na+/H+ exchangers and intracellular pH in perinatal brain injury. *Transl. Stroke Res.* 5, 79–98. doi: 10.1007/s12975-013-0322-x

Vallabhaneni, K. C., Penfornis, P., Dhule, S., Guillonneau, F., Adams, K. V., Mo, Y. Y., et al. (2015). Extracellular vesicles from bone marrow mesenchymal stem/stromal cells transport tumor regulatory microRNA, proteins, and metabolites. *Oncotarget* 6:4953. doi: 10.18632/oncotarget.3211

van Niel, G., D'Angelo, G., and Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell Biol.* 19, 213–228. doi: 10.1038/nrm.2017.125

Van Petten, C. (2004). Relationship between hippocampal volume and memory ability in healthy individuals across the lifespan: review and meta-analysis. *Neuropsychologia* 42, 1394–1413. doi: 10.1016/j.neuropsychologia.2004.04.006

van Velthoven, C. T. J., Kavelaars, A., and Heijnen, C. J. (2012). Mesenchymal stem cells as a treatment for neonatal ischemic brain damage. *Pediatr. Res.* 71, 474–481. doi: 10.1038/pr.2011.64

van Velthoven, C. T. J., Kavelaars, A., van Bel, F., and Heijnen, C. J. (2010). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain Behav. Immun.* 24, 387–393. doi: 10.1016/j.bbi.2009.10.017

Vannucci, R. C., and Vannucci, S. J. (1997). A model of perinatal hypoxic-ischemic brain damage. *Ann. N. Y. Acad. Sci.* 835, 234–249. doi: 10.1111/j.1749-6632.1997.tb48634.x

Vohr, B. R., Wright, L. L., Dusick, A. M., Mele, L., Verter, J., Steichen, J. J., et al. (2000). Neurodevelopmental and functional outcomes of extremely low birth weight infants in the National Institute of Child Health and Human Development Neonatal Research Network, 1993-1994. *Pediatrics* 105, 1216–1226. doi: 10.1542/peds.105.6.1216

Volpe, J. J. (2001). Perinatal brain injury: from pathogenesis to neuroprotection. *Ment. Retard. Dev. Disabil. Res. Rev.* 7, 56–64. doi: 10.1002/1098-2779(200102)7:1<56::AID-MRDD1008>3.0.CO;2-A

Volpe, J. J. (2012). Neonatal encephalopathy: an inadequate term for hypoxic-ischemic encephalopathy. *Ann. Neurol.* 72, 156–166. doi: 10.1002/ana.23647

Walczak, P., Zhang, J., Gilad, A. A., Kedziorek, D. A., Ruiz-Cabello, J., Young, R. G., et al. (2008). Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. *Stroke* 39, 1569–1574. doi: 10.1161/STROKEAHA.107.502047

Wen, S., Dooner, M., Cheng, Y., Papa, E., Del Tatto, M., Pereira, M., et al. (2016). Mesenchymal stromal cell-derived extracellular vesicles rescue radiation damage to murine marrow hematopoietic cells. *Leukemia* 30, 2221–2231. doi: 10.1038/leu.2016.107

White, N. M., and McDonald, R. J. (2002). Multiple parallel memory systems in the brain of the rat. *Neurobiol. Learn. Mem.* 77, 125–184. doi: 10.1006/nlme.2001.4008

Wu, Y. W., and Colford, J. M. Jr. (2000). Chorioamnionitis as a risk factor for cerebral palsy: a meta analysis. *JAMA* 284, 1417–1424. doi: 10.1001/jama.284.11.1417

Wyatt, J. S., Gluckman, P. D., Liu, P. Y., Azzopardi, D., Ballard, R., Edwards, A. D., et al. (2007). Determinants of outcomes after head cooling for neonatal encephalopathy. *Pediatrics* 119, 912–921. doi: 10.1542/peds.2006-2839

Yeo, R. W. Y., Lai, R. C., Zhang, B., Tan, S. S., Yin, Y., Teh, B. J., et al. (2013). Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv. Drug Deliv. Rev.* 65, 336–341. doi: 10.1016/j.addr.2012.07.001

Yi, J. H., Park, S. W., Kapadia, R., and Vemuganti, R. (2007). Role of transcription factors in mediating post-ischemic cerebral inflammation and brain damage. *Neurochem. Int.* 50, 1014–1027. doi: 10.1016/j.neuint.2007.04.019

Zhang, S., Chu, W. C., Lai, R. C., Lim, S. K., Hui, J. H. P., and Toh, W. S. (2016). Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. *Osteoarthr. Cartil.* 24, 2135–2140. doi: 10.1016/j.joca.2016.06.022

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Sisa, Kholia, Naylor, Herrera Sanchez, Bruno, Deregibus, Camussi, Inal, Lange and Hristova. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 20 March 2019
doi: 10.3389/fphys.2019.00181

Prevention, Reduction and Repair of Brain Injury of the Preterm Infant

Frank van Bel[1], Josine Vaes[2] and Floris Groenendaal[1]*

[1]Department of Neonatology, Wilhelmina Children's Hospital and Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands, [2]Laboratory of Neuroimmunology and Developmental Origins of Disease, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands

Periventricular-intraventricular hemorrhages (PIVH) and (diffuse) white matter injury (WMI) are the most important acquired brain lesions of the very and extremely prematurely born neonate. Both carry a high risk for death or adverse neurodevelopmental outcome. The first part of the review discusses the standard of care and latest insights with respect to prevention and/or reduction of PIVH and WMI, taking into account their etiopathogenesis which is tightly linked to (functional) immaturity of the cerebral vascular bed and nervous system and commonly encountered inflammation. The second part discusses repair of hemorrhagic- ischemic and post-inflammatory brain lesions as it is an increasingly important topic in newborn medicine. In the near future trials of trophic and (autologous or allogenic) cell-therapy in infants at risk of or demonstrating established PIVH and WMI will be started. The focus of these potential trials will be discussed.

Keywords: prematurity, brain hemorrhage, white matter injury in the preterm infant, neuroprotection, neuroregeneration

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Helen B. Stolp,
Royal Veterinary College (RVC),
United Kingdom
Bobbi Fleiss,
RMIT University, Australia
Robert Galinsky,
Ritchie Centre, Australia

****Correspondence:***
Frank van Bel
f.vanBel@umcutrecht.nl

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *20 September 2018*
Accepted: *14 February 2019*
Published: *20 March 2019*

Citation:
van Bel F, Vaes J and Groenendaal F (2019) Prevention, Reduction and Repair of Brain Injury of the Preterm Infant. Front. Physiol. 10:181. doi: 10.3389/fphys.2019.00181

INTRODUCTION

The most important acquired brain injuries in very and extremely preterm infants born in developed countries are periventricular-intraventricular hemorrhages (PIVH) and diffuse white matter injury (dWMI, **Figure 1**; Stoll et al., 2010; Hamilton et al., 2013; Pierrat et al., 2017). This brain injury may lead to cerebral palsy and learning difficulties, and can have major impact on the quality of life (Stoll et al., 2010; Pierrat et al., 2017).

The first aim of this review is to link the etiopathogenesis of PIVH and dWMI to the standard of care and its latest insights with respect to prevention and reduction of these complications.

The second aim is to focus on repair of the sequelae of PIVH and dWMI. There is increasing evidence that repair of perinatal brain injury with trophic and/or stem cell therapy is currently becoming a realistic and exciting option (Fleiss et al., 2014; Fischer et al., 2017; Wagenaar et al., 2017). We discuss this development in relation with repair of the sequelae of severe PIVH and dWMI.

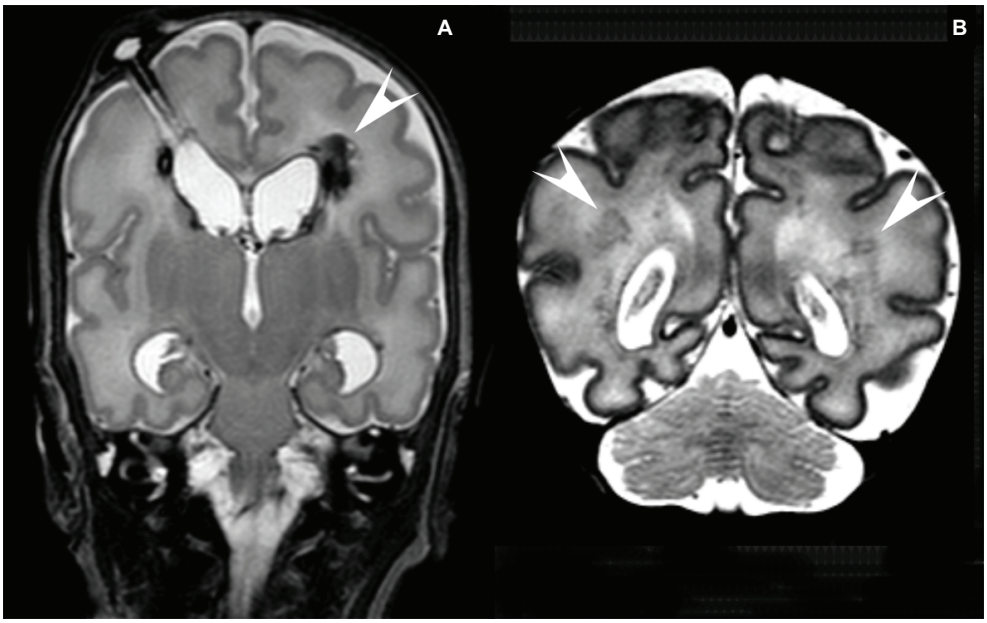


FIGURE 1 | (A) MRI of a preterm infant (gestational age 26 2/7 weeks, birthweight 965 g) with a large IVH and a large left-sided (arrowhead) and small right-sided venous infarct. Post-hemorrhagic ventricular dilatation was treated with CSF removal from a subcutaneous reservoir. T2 weighted coronal image at the age of 8 weeks after birth. **(B)** MRI of a preterm infant (gestational age 32 2/7 weeks, birthweight 1,670 g) with several lesions in the periventricular white matter (arrowheads). T2 weighted coronal image at the age of 6 days after birth.

PERIVENTRICULAR-INTRAVENTRICULAR HEMORRHAGE

PIVH has still a high incidence in the developed world: 25–35% of preterm infants born before 30 weeks of gestation or a birth weight less than 1,500 g develop PIVH. PIVH develops from the fragile vascular network of the germinal matrix mostly within the first 3 days after birth with the highest incidence in extremely low birth weight infants being up to 45% (Jain et al., 2009; Stoll et al., 2010; Mukerji et al., 2015). Although a minority of these infants develop severe PIVH grade III (intraventricular blood in dilated lateral cerebral ventricles) or IV (intraventricular blood with extension into the adjacent parenchymal region, more recently described as venous infarction) according to the Papile grading (Papile et al., 1978), up to 75% develop mild to severe PIVH-related sequelae in later life (Sherlock et al., 2005; Luu et al., 2009). PIVH remains therefore a major health concern.

Although multifactorial, the pathogenesis of PIVH and its extension to more severe stages is firmly linked to pulmonary immaturity. This is clinically represented by the idiopathic respiratory distress syndrome (IRDS), and (functional) immaturity of the cerebral vascular bed (Ozdemir et al., 1997; Krediet et al., 2006; Ballabh, 2014). IRDS may lead to hypoxia and hypercapnia, lack of cerebral autoregulation and the need for blood pressure support often causing fluctuations and hyperperfusion of the immature brain of the extremely and very preterm infant (Perlman et al., 1985; van Bel et al., 1987), although this mechanism may also be operative in the moderate and late preterm neonate with IRDS (Thygesen et al., 2016). Cerebral hemodynamic instability often leads to PIVH, mostly originating in the germinal matrix, which has a dense but fragile vasculature (Ballabh, 2014). Moreover, IRDS has been associated with inflammatory processes and oxidative stress in the immature lung. Several studies showed elevated pro-inflammatory cytokines, chemokines and indicators of oxidative stress in broncho-alveolar lavage fluid and blood in very preterm neonates with IRDS (Beresford and Shaw, 2002; Gitto et al., 2004). A recent study showed that intra-amniotic inflammation and postnatal IRDS markedly increased the risk for PIVH (Oh et al., 2018). PIVHs, which develop within 12 hours of age, inflammation may play an important role as indicated by the strong association between early PIVH and pro-inflammatory cytokines and oxidative stress (Krediet et al., 2006; Chisholm et al., 2016; Chevallier et al., 2017; Villamor-Martinez et al., 2018). Finally genetic factors can be related to the occurrence of PIVH, but this issue is beyond the scope of this review (Bilguvar et al., 2009; Harteman et al., 2011; Ballabh, 2014).

Prevention and Reduction of PIVH: Standard of Care

Prevention and reduction of PIVH starts already in the womb: *maternal corticosteroids* during imminent preterm birth have shown to reduce the occurrence of PIVH and is common practice during preterm labor and imminent preterm birth in most high income countries since the late eighties of the last century (Ment et al., 1995; Ballabh, 2014; Roberts et al., 2017). A recent population study (EPICE Cohort) showed even a risk reduction of up to 50% of severe neonatal injury after

antenatal corticosteroids administered shortly before birth (Norman et al., 2017). Mostly betamethasone or dexamethasone are used although there is an ongoing debate about their superiority (Brownfoot et al., 2013). Besides the well proven effect of antenatal steroids on lung maturation with a positive effect on respiratory and hemodynamic systems (Roberts et al., 2017), a maturational effect of steroids on the germinal matrix microvasculature has been postulated (Xu et al., 2008). This will establish a decrease in permeability of the cerebral vasculature and stabilization of the endothelial basement membrane (Hedley-Whyte and Hsu, 1986; Tokida et al., 1990).

As antenatally administered corticosteroids induce lung maturation and pulmonary stabilization, *exogenous surfactant application via* the trachea does so postnatally (McPherson and Wambach, 2018). Surfactant may add therefore to a hemodynamic stabilization of the systemic and cerebral circulation leading to less disturbances of cerebral autoregulatory ability of the vascular bed (Lemmers et al., 2006).

Several studies indicated a decrease in the incidence in PIVH after the introduction of surfactant therapy, especially regarding more severe PIVHs (Walti et al., 1995; Greenough and Ahmed, 2013). An older meta-analysis, however, showed no clear benefits of surfactant therapy on the incidence of PIVH, although there was a tendency for a reduction of severe PIVH (Rojas-Reyes et al., 2012). A recent systematic review and meta-analysis investigating the use of early surfactant, defined as surfactant administration within one hour after birth, with noninvasive ventilation and stress reduction found a decrease in severe PIVH with this strategy (Anand et al., 1999; Isayama et al., 2015; Ng et al., 2017).

Pharmacologic interventions aiming to prevent or reduce PIVH are numerous. Muscle paralysis was used in order to minimize swings in cerebral perfusion to influence the incidence of PIVH in artificially ventilated preterm infants. PIVH incidence indeed decreased sharply after muscle paralysis (Perlman et al., 1985). More sophisticated ventilation modalities nowadays, including non-invasive ventilation makes muscle paralysis obsolete (McPherson and Inder, 2017). Phenobarbital sedation did not decrease PIVH incidence (Donn et al., 1981; Bedard et al., 1984). Vitamin E, a potent anti-oxidative agent, reduced the incidence of PIVH but routine use was not encouraged because of serious side effects (Brion et al., 2003). Ethamsylate, which has a stabilizing effect on the vascular basement membrane, was widely investigated in the 1980s, but had no positive effect on the PIVH incidence (Benson et al., 1986).

Only prophylactic indomethacin made its way to the clinic. Indomethacin is a (nonselective) cyclo-oxygenase inhibitor which showed a positive effect on PIVH incidence and induced (early) patent ductus arteriosus closure (Vohr and Ment, 1996). Especially in the United States prophylactic indomethacin administration (low dose indomethacin starting within 6 h after birth up to day 3–5) has been utilized in many centers (Nelin et al., 2017). Although, in 2001 the TIPP trial suggested that despite a decreased incidence of (severe) PIVH, long-term developmental outcome did not improve (Schmidt et al., 2001). A recent large study did show improved survival after indomethacin prophylaxis in especially the extremely preterm infants (Nelin et al., 2017). This seemed to be confirmed by a recent meta-analysis which showed a positive effect on mortality of a prophylactic indomethacin regime (Jensen et al., 2018). It has been suggested that indomethacin promotes maturation of the cerebral vasculature (Ment et al., 1992; Ballabh, 2014). We suggest that also an indomethacin-induced stabilization of cerebral perfusion and improvement of cerebral vascular autoregulation plays a role with respect to reduction of PIVH. Earlier studies of our group in preterm fetal and neonatal lambs showed that indomethacin improved the autoregulatory ability of the cerebrovascular bed, probably due to its vasoconstrictive action,

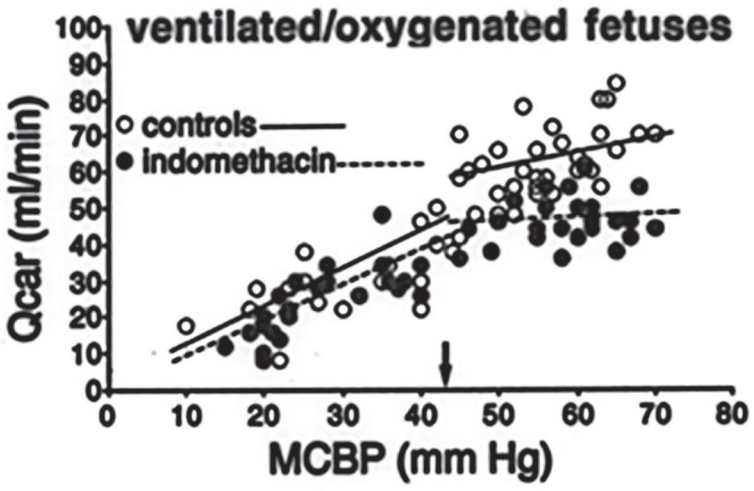


FIGURE 2 | Individual values of Carotid blood flow [Qcar (ml/min)], representing global cerebral blood flow, as a function of (mean) carotid blood pressure (MCBP; mm Hg), representing cerebral perfusion pressure, in pretreated with indomethacin (filled circles) and non-treated ventilated preterm sheep fetuses, representing a perinatal lamb model (van Bel et al., 1993,1994,1995). Note the lower Qcar values and better autoregulatory curve in the indomethacin-treated animals. The small black arrow indicates the lower limit of MCBP where cerebral autoregulation is still operative.

preventing cerebral hyperperfusion as compared to placebo-treated controls (**Figure 2**; van Bel et al., 1993, 1994, 1995).

Head position and especially left or right deviation of the head of very and extremely preterm infants may affect venous drainage by partial occlusion of the jugular vein. This can induce a temporary increase in intracranial pressure. It has been postulated that this may contribute to the occurrence of PIVH (Goldberg et al., 1983).

However, a meta-analysis of relevant studies where the infant was kept supine with the head in the midline position and the bed tilted in 30° to reduce PIVH incidence failed to show a decrease in PIVH incidence as compared to their control counterparts (Romantsik et al., 2017). Additional studies are ongoing.

Prevention and Reduction of PIVH: Emerging Interventions

Suboptimal blood gas values and hypoxia due to pulmonary immaturity and IRDS play a role in the pathogenesis of PIVH (Ballabh, 2014). Experimental studies and clinical studies using near infrared spectroscopy (NIRS) showed that prolonged episodes of cerebral oxygen saturation lower than 40–45% were related to damage in the developing brain (Dent et al., 2005; Hou et al., 2007). With NIRS-derived monitoring of cerebral oxygenation and perfusion it is possible to timely identify and intervene during episodes of suboptimal oxygenation and perfusion of the immature brain (Skov et al., 1991; van Bel et al., 2008; Wintermark et al., 2014; Alderliesten et al., 2016; van Bel and Mintzer, 2018). Recently, a European randomized controlled multicenter intervention trial (the SafeboosC study) focusing on the reduction of hypoxia and/or hyperoxia, provided evidence that *monitoring cerebral oxygenation* with NIRS lowered the hypoxic burden in extremely preterm neonates in the first days after birth (Hyttel-Sorensen et al., 2015), the episode in which most PIVH occur and/or extend. A follow-up study from this SafeboosC cohort showed that the (early) burden of hypoxia was associated with the occurrence of severe PIVH (Plomgaard et al., 2017). To confirm that interventions on basis of NIRS-monitored cerebral oxygenation can decrease PIVH incidence a contemporary randomized controlled trial with adequate patient inclusions is mandatory. In this respect it is also important to emphasize that clinical application of NIRS in the neonatal intensive care unit, to assess (in) adequacy of cerebral oxygenation, requires international consensus with respect to normative values and understanding of cerebral oxygen utilization patterns (van Bel and Mintzer, 2018).

A potentially promising intervention to lower PIVH incidence is *delayed cord clamping or DCC*. The underlying mechanism may be that a greater neonatal blood volume due to DCC gives rise to an improved cardiac preload leading to a stable cardiac output, stable blood pressure and intact cerebral autoregulation with less need for inotropic therapy (Hooper et al., 2015; Perlman et al., 2015; Wyllie et al., 2015). Consequently the stable hemodynamics may ensure an appropriate cerebral perfusion (Baenziger et al., 2007; Ersdal et al., 2014). Especially lack of cerebral autoregulation and use of positive inotropes seem to be related to a higher incidence and extension of PIVH (Alderliesten et al., 2013). Several studies suggest a positive effect of DCC on PIVH incidence (Rabe et al., 2008, 2012). However, a recent meta-analysis did not yet confirm this although there was a strong tendency for a reducing effect of DCC on PIVH incidence (Fogarty et al., 2018). A key issue with respect to the beneficial effects of DCC on PIVH incidence in very and extremely preterm infants to be solved, is the optimal time of DCC. The delay time in the 27 studies included in the meta-analysis of Fogarty et al was very variable, from 30-up to-more than 120 s (Fogarty et al., 2018). It has been suggested by others that an optimal delay time should be 180 s which may optimize the beneficial effects of DCC (Yao et al., 1969).

Preventive treatment with *trophic factors* and especially *Erythropoietin (EPO)* and *Insulin Growth Factor-1 (IGF-1)* and its *binding protein 3 (IGF-1-BP3)* are increasingly recognized to have neuroprotection and PIVH-reducing properties (Juul and Pet, 2015; Hellstrom et al., 2016).

EPO stimulates red cell production, cell survival and differentiation and EPO receptors are detected on endothelial, glial and neuronal cells (van der Kooij et al., 2008; Chateauvieux et al., 2011; Koulnis et al., 2014; Rangarajan and Juul, 2014). EPO has also a modulating effect on glutamate toxicity, stimulating effect on antioxidative ability and anti-inflammatory effect protecting endothelial cells from apoptotic death (Yamaji et al., 1996; Bernaudin et al., 1999; Kawakami et al., 2001). These latter properties of EPO may imply that recombinant human (rh) EPO can also have a positive impact on the PIVH incidence in premature neonates. An older study from Neubauer et al showed indeed a decrease in the incidence of severe PIVH after early rhEPO (Neubauer et al., 2010), although later studies showed conflicting results with respect to PIVH incidence after rhEPO (Ohls et al., 2014; Fauchere et al., 2015). A recent meta-analysis including 3,643 extremely and very preterm infants receiving early EPO therapy reported a reducing effect on PIVH incidence (Fischer et al., 2017; Ohlsson and Aher, 2017).

IGF-1 is an endogenous protein which exerts several actions: its positive effect on proper vascularization (Hellstrom et al., 2001; Bach, 2015) and brain development are important for a normal neurodevelopment (Hellstrom et al., 2016). Following extremely preterm birth, serum IGF-1 levels are much lower than in utero serum concentrations at corresponding gestational ages. Inadequate endogenous postnatal IGF-1 production is regarded to be the result of preterm birth related events such as hypoxia, inflammation and reduced nutrient availability (Hellstrom et al., 2016). The fact that extremely preterm born infants have deficient serum IGF-1 and IGF-1-BP3 concentrations stimulated researchers and clinicians to perform studies in which suppletion of IGF-1 and its IGF-1 bounding protein BP3 were expected to have maturational effects on vascularization of the extremely preterm neonate (Ley et al., 2013). Intranasal IGF-1 reduced germinal matrix hemorrhages in a preterm rat pup model (Lekic et al., 2016). A clinical study of Hellstrom et al on the effects of IGF-1 on ROP, PIVH and bronchopulmonary dysplasia is ongoing (ClinicalTrials.gov: NCT01096784).

In summary, antenatal corticosteroids and the introduction of exogenous surfactant substantially reduced the PIVH incidence

TABLE 1 | Summary of standard care and emerging therapies respectively, for the prevention and reduction of periventricular-intraventricular hemorrhage (PIVH) and (diffuse) white matter injury (dWMI).

PIVH and (d)WMI
Standard care
– Antenatal corticosteroids
– Exogenous surfactant instillation
– Non-invasive ventilation techniques/stress reduction
– Prophylactic early (<6 h) indomethacin
Emerging therapies
– Delayed cord clamping
– Trophic factors i.e. erythropoietin (rhEPO) insulin growth factor-1 and its binding protein 3 (IGF-1/IGF-1BP3)

of the preterm born infant in high income countries. Better and non-invasive ventilation techniques together with exogenous surfactant treatment and stress reduction during patient care had a further reducing effect on PIVH incidence, as did prophylactic indomethacin treatment.

Promising future therapies for PIVH prevention and/or reduction of severity are delayed cord clamping and early and adequate treatment with trophic factors such as erythropoietin and IGF-1. However, further research is mandatory here. **Table 1** shows schematically the above discussed therapeutic considerations.

WHITE MATTER INJURY IN THE VERY AND EXTREMELY PRETERM INFANT

Extremely preterm born infants (or ELGANs) carry a substantial risk of diffuse white matter injury or abnormal white matter development (Volpe, 2009; Chau et al., 2013). In the early days of neonatal intensive care, white matter injury (WMI; or periventricular leukomalacia) was encountered in the form of cystic periventricular leukomalacia (cPVL), as described by Banker and Larroche (1962). cPVL was hard to detect using CT, but could be detected with the use of cranial ultrasound (cUS), in particular when used longitudinally after the first week after birth (de Vries et al., 2004). The cysts of cPVL appear 10–20 days after an insult, and disappear around term equivalent age. Remaining injury can be seen as widening and irregularity of the ventricles on cUS, and loss of white matter and delayed myelination on cranial MRI (Chau et al., 2013; Martinez-Biarge et al., 2016). Later in life gliosis can be seen in the affected areas. The cysts of cPVL occur alongside the ventricles in preterm infants, whereas subcortical cysts are more common in term infants.

Several causes of cPVL have been suggested, including hypoxia-ischemia and inflammation. Fetal inflammation has been reported to be common in preterm birth (reviewed by Hagberg et al., 2015). Furthermore, preterm CSF appears to show a neuroinflammatory response compared to term infants. Although many have reported white matter injury after maternal chorioamnionitis with infection (reviewed by Paton et al., 2017) (O'Shea et al., 2012; Strunk et al., 2014; Paton et al., 2017), a recent study failed to show a detrimental effect of chorioamnionitis (Bierstone et al., 2018). Reactive oxygen species are considered to play a role in the injury of the cerebral white matter of the preterm infant (Hagberg et al., 2015).

Occurrence of cPVL has been demonstrated after severe hypocapnia and subsequent cerebral vasoconstriction (Groenendaal and de Vries, 2001). The incidence of cPVL is decreasing in modern neonatal intensive care to 1.3% of a NICU cohort of very preterm infants (van Haastert et al., 2011). Probably multiple factors may have contributed to the decrease of cPVL, such as monitoring of blood pressure, low carbon dioxide levels, blood glucose, and cerebral oxygenation using NIRS. The role of maternal antibiotics is still unresolved (Shepherd et al., 2017).

Nowadays, diffuse white matter injury (dWMI), and 'punctate white matter lesions' are more commonly seen in extremely preterm infants (Kersbergen et al., 2014a) (**Figure 1**). Diffuse WMI might even be present in more than 50% of extremely and very preterm infants (Hinojosa-Rodríguez et al., 2017).

A recent review by our group (van Tilborg et al., 2018b), summarizing a substantial amount of preclinical studies, suggested that an arrest in maturation of oligodendrocyte precursors is responsible for hypomyelination as seen in experimental models of dWMI (van de Looij et al., 2012; van Tilborg et al., 2018a). As reviewed by Hagberg et al. (2015) pro-inflammatory cytokines, including IL-6, and TNF-alpha will lead to increased activation of microglia with adverse effects on developing oligodendrocyte precursors. Systemic inflammation in common in extremely and very preterm infants. Although beyond the aim of this review it is important to state that also in moderate and late preterm infants inflammation can lead to brain damage and adverse outcome (Gisslen et al., 2016; Musilova et al., 2018).

Preterm white matter can be studied in far more detail using MRI, and longitudinal scans can visualize brain growth, including growth of specific brain regions, cortical folding and white matter development (Kersbergen et al., 2014b), but identification of tissue microstructure is still challenging (Stolp et al., 2018). Scoring systems have been developed to quantify the abnormalities seen at term equivalent age in this population, and the predictive power for neurodevelopment is under investigation (Inder et al., 2005; Kidokoro et al., 2013). At present, MRI might be more informative in hospitals that are dedicated for neonatal MRI than in general.

Prevention and Reduction of (Diffuse) White Matter Injury

Antenatal and perinatal strategies are very important in the prevention of dWMI. *Magnesium sulphate given antenatally* to women at risk of preterm birth substantially reduced the risk of cerebral palsy of the infant (Crowther et al., 2017). The mechanism of this neuroprotection is still unknown. Improved uterine perfusion through vasodilation, and a reduction of neonatal IVH have been proposed mechanisms. Although magnesium reduces EEG activity and the number of seizures in an animal model of preterm asphyxia (Galinsky et al., 2017; Bennet et al., 2018b), blockade of NMDA receptors or other excitotoxic pathways is unlikely. Although plasma concentrations

achieved in mothers and fetuses are increased after maternal administration of magnesium, extracellular magnesium concentrations in the brain are probably lower than those needed for neuroprotection after experimental hypoxia-ischemia. (Crowther et al., 2017; Galinsky et al., 2017).

A recent trial (NCT00724594) tested the pharmacokinetics of maternal and neonatal N-Acetylcysteine. Interestingly, umbilical cord concentrations frequently exceeded maternal concentrations (Wiest et al., 2014). Future studies may aim at the use of N-Acetylcysteine to reduce free radical injury in preterm infants.

Delayed umbilical cord clamping has been advised in 'vigorous' preterm infants. It is associated with significant neonatal benefits, including improved transitional circulation, better establishment of red blood cell volume, decreased need for blood transfusion, and lower incidence of necrotizing enterocolitis, leading to massive systemic inflammation and subsequent white matter injury, and intraventricular hemorrhage (as already discussed above; (Practice, 2017). Thereby it may have an indirect beneficial effect on white matter injury (see also above: emerging therapies for prevention of PIVH (Mercer et al., 2016)).

Reduction of severe IRDS not only reduces IVH (see above), but it may also important in the reduction of severe white matter injury. As through a reduction of severe respiratory illness large fluctuations in oxygen and carbon dioxide levels are avoided, production of reactive oxygen species may be reduced. Furthermore, *stabilization of blood pressure* reduces major swings in cerebral perfusion.

Postnatal pharmacologic interventions for reduction or prevention of dWMI are increasingly recognized as being potentially neuroprotective. Although early *postnatal administration of the corticosteroid dexamethasone* has been reported to be associated with cerebral palsy (Doyle et al., 2017), this may be not the case for *hydrocortisone* (Karemaker et al., 2006). Recently a trial was finished comparing hydrocortisone versus placebo in ventilated preterm infants to reduce chronic lung disease (Onland et al., 2011). Neurodevelopment of these infants will provide information on the benefits (or risks) of postnatal hydrocortisone. Postnatal use of *caffeine* resulted in improved neurodevelopmental outcome (Schmidt et al., 2007). Neonatal caffeine therapy for apnea of prematurity improved visuomotor, visuoperceptual, and visuospatial abilities at age 11 years (Murner-Lavanchy et al., 2018).

It has been suggested that improvement of preterm *nutrition* may contribute to optimizing brain development. In particular the so-called microbiome-gut-brain-*Axis axis* is a proposed mechanism of interaction, including neural, endocrine, and immunological pathways (Cryan and Dinan, 2012). Nutritional components such as fatty acids and protein may stimulate brain growth and neurodevelopment (Uauy and Mena, 2015; Coviello et al., 2018). Also probiotics might be beneficial in reducing the incidence of necrotizing enterocolitis and thereby reduce white matter injury.

Monitoring of cerebral oxygenation with NIRS (as already discussed above in relation with prevention of PIVH) and *of brain function* (amplitude EEG [aEEG]), may also play an important preventing role with relation to dWMI.

Since very low arterial CO_2 levels may contribute to cerebral hypoperfusion and white matter injury (Greisen and Vannucci, 2001). Tools to monitor the neonatal brain oxygenation and function with NIRS and aEEG may contribute to optimize cerebral oxygenation (Hyttel-Sorensen et al., 2015; Plomgaard et al., 2017), and early recognition and treatment of subclinical seizure activity (Glass et al., 2017). Further studies are needed to describe the association with long-term neurodevelopment (Hyttel-Sorensen et al., 2017; Thewissen et al., 2018).

Pain and stress are shown to have negative effects on brain development (Duerden et al., 2018). Avoidance of pain appears to be useful. In very preterm infants on mechanical ventilation, continuous fentanyl infusion might protect the developing brain by relieving pain during the first 72 h of mechanical ventilation (Qiu et al., 2018). In contrast others have demonstrated impaired cerebellar growth in the neonatal period and poorer neurodevelopmental outcomes in early childhood of preterm infants after morphine use (Zwicker et al., 2016).

To find an optimal balance between pain and stress reduction and use of opioids may aid in the reduction of white matter injury. Alternative strategies for stress and pain reduction, such as sucrose, use of pacifiers, or non-sedative analgetics need to be explored further.

Inflammation

Extremely preterm birth is commonly associated with fetal and postnatal systemic inflammation which is likely to contribute to dWMI through adverse effects on oligodendrocyte precursors (Strunk et al., 2014; Hagberg et al., 2015). Novel strategies are explored to counteract these inflammatory pathways to counteract the deleterious effects on preterm white matter (see below).

Prevention and Reduction of (d)WMI: Emerging Pharmacologic Interventions

Many anti-inflammatory interventions have been suggested as a result from animal experiments (reviewed by Hagberg et al., 2015). Almost none of these have been tested in human infants.

Erythropoietin or EPO has been suggested to inhibit glutamate release, reduce accumulation of intracellular calcium, to induce antiapoptotic factors, to reduce inflammation and nitric oxide-mediated injury, and to contribute to regeneration (van der Kooij et al., 2008; Chateauvieux et al., 2011; Rangarajan and Juul, 2014).

In the EpoKids study in Switzerland very preterm infants were randomized to 3 doses of rhEPO (one before birth, 2 after birth) versus placebo. The secondary outcome of MRI at term equivalent age showed less white matter injury in the EPO group compared with the placebo group (Leuchter et al., 2014). A meta-analysis of administration of rhEPO showed an improved the cognitive development of very preterm infants, as assessed by the MDI at a corrected age of 18–24 months, without affecting other neurodevelopmental outcomes (Fischer et al., 2017). Several trials are still ongoing to study neuroprotection by EPO in preterm infants (Juul and Pet, 2015). Given its positive effect on neurogenesis and angiogenesis a more prolonged course of appropriately (high) dosed rhEPO

(up to 2,500 IU/kg daily) may further optimize clinical outcome of the preterm infant (van der Kooij et al., 2008; Chateauvieux et al., 2011; Rangarajan and Juul, 2014).

In animal models *melatonin* has antioxidant properties by influencing several pathways, and reduces (neuro-) inflammation. Through reduction of proinflammatory cytokines pro-oligodendrocyte maturation could be preserved. Administration of *melatonin* to pregnant women with fetal growth restriction or pre-eclampsia is under investigation (NCT02395783 and NCT01695070). Neonatal administration of *melatonin* has been used in preterm newborns with sepsis, surgical procedures or chronic lung disease (Marseglia et al., 2015). However, no beneficial effect on MRI parameters of the preterm brain at term equivalent age could be demonstrated in the relatively low dose administered in this study (Merchant et al., 2014).

IGF-1 plays a crucial role in fetal and postnatal brain development: IGF-1 is shown to stimulate neurogenesis and proliferation, differentiation and survival of brain cells. Regarding white matter development, IGF-1 also stimulates oligodendrocyte maturation and subsequent myelination (Cao et al., 2003; Pang et al., 2010; Cai et al., 2011; Hansen-Pupp et al., 2011; O'Kusky and Ye, 2012). Moreover, genetic studies in mice display lower total brain volumes and severe hypomyelination following IGF-1 knockout (O'Kusky and Ye, 2012). Human studies relating serum IGF-1 levels to brain development show a positive association between postnatal serum IGF-1 concentrations and head circumference, brain volume measures and developmental scores at 2 years of age (Hansen-Pupp et al., 2011). Main focus of previous studies with IGF-1 and its IGF-1- binding protein 3 was the prevention of retinopathy of prematurity, but the incidence of PIVH will be studied in addition (*ClinicalTrials.gov: NCT01096784*). Further studies are needed to explore potential neuroprotective effects of IGF-1 with respect to dWMI.

In summary, Injury to and subnormal development of the periventricular white matter is still very common in extremely preterm born infants. Although improved neonatal intensive care may contribute to improved outcomes, additional strategies to counteract (d) WMI may add to an improved neurodevelopmental outcome.

REPAIR OF SEQUELAE OF PIVH AND dWMI

Increasing experimental evidence shows that regeneration of the injured immature brain with stem cell-based therapies is promising and may serve as an effective treatment strategy. Stem cells have an intrinsic potential for self-renewal and can differentiate into several cellular phenotypes (Fleiss et al., 2014). Given their pluripotent capacity, embryonic stem cells seem the most obvious choice for repair of brain injury, but can induce formation of teratoma after transplantation. Their clinical application raises therefore considerable ethical concerns. This is also true for multipotent neural stem cells: although very attractive given their possibility to derive all neural lineages, their accessibility in humans is limited because they carry also a substantial risk for tumor formation (Comi et al., 2008). Among all progenitor cells, the mesenchymal stem (or stromal) cell (MSC) is at this moment the most optimal choice for near-future use in (preterm) neonates because of the evident neuroregenerative properties and favorable immunological profile and, not for the least, of its favorable safety profile (Uccelli et al., 2008; Fleiss et al., 2014). MSCs are considered to adapt their secretome, after which paracrine signaling results in endogenous brain repair rather than direct cell replacement through MSC differentiation (Qu et al., 2007; van Velthoven et al., 2011). Paracrine effects of MSCs include many growth factors such as insulin-like growth factor (IGF-1), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and vascular endothelial growth factors (VEGF) (Kizil et al., 2015; Ophelders et al., 2016; Bennet et al., 2018a). These factors can promote endogenous repair through brain cell formation in the sub ventricular zone as well as boost neuronal and glial cell proliferation, maturation and survival on other regions, Moreover, MSCs are shown to secrete anti-inflammatory cytokines, involved in reduction of neuroinflammation (**Figure 3**). Upregulation of neoneurogenesis and downregulation of genes involved in inflammation after MSC transplantation has been reported in a review (Wagenaar et al., 2017).

MSCs can be administered to the brain *via* several routes: intravenously, intracranially/intrathecally and nasally. The nasal route is non-invasive and seems more effective without loss of MSCs in other organ systems as compared to intravenous administration (Fischer et al., 2009; Wagenaar et al., 2017). In a neonatal stroke model in mice pups substantial beneficial effects on infarction size, motor function and cognition were demonstrated (Wagenaar et al., 2017). The nasally administered MSC cells were no longer detectable 3 days after the implantation, minimizing the risk for Graft-versus Host Disease and tumor growth (Donega et al., 2014). This is confirmed by a long-term safety study of our group (Donega et al., 2015). Moreover

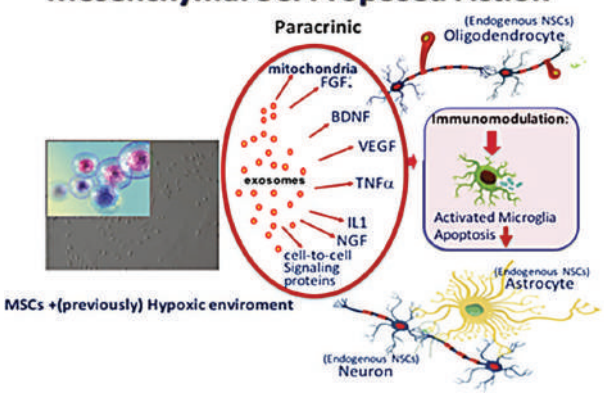


FIGURE 3 | Proposed actions of mesenchymal stem cells (MSC) when present in a (previously) hypoxic–ischemic environment: production of vesicles (exosomes) of various growth factors, (anti-inflammatory) cytokines, signaling proteins and mitochondria which give rise to recovery of affected neurons and to proliferation of endogenous paraventricular-situated neural stem cells to form oligodendrocytes, astrocytes and neurons.

human trials on MSC therapy in adults and children did not provide evidence for serious long-term effects (Lalu et al., 2012). An important advantage of MSC-based cell therapy is that autologous as well as allogeneic transplantation can be applied. Autologous intravenous MSC-transplantations, mostly derived and cultured from MSC-rich umbilical cord tissue or cord blood, as well as allogeneic MSCs (see below) are already reported for clinical use in neonatal medicine (Chang et al., 2014; Cotten et al., 2014). A detailed review concerning stem cell-based therapy in neonatology is beyond the scope of this review but is summarized in several recent reviews (Wagenaar et al., 2017; Gronbach et al., 2018; Niimi and Levison, 2018; Vaes et al., in preparation).

Stem Cell-Therapy and PIVH

Experimental studies reported that cord-derived MSCs substantially attenuated reactive gliosis and cell death which went along with an increase of brain-derived neurotrophic factor (BDNF) (Mukai et al., 2017). Further study showed that MSC-derived BDNF secretion was indeed a critical paracrine factor playing a central role in the attenuation of PIVH-induced brain injury (Ahn et al., 2017). Preclinical data pointed to a repairing effect of MSCs on the sequelae of severe PIVH (Park et al., 2017). Ahn et al showed that in preterm rat pups (P4), in which severe IVH was induced, intraventricularly transplanted human umbilical cord-derived MSCs attenuated posthemorrhagic ventricular dilatation and the area of brain injury (Ahn et al., 2013). They also showed that the window of effective treatment was at least up to 2 days after induction of brain damage (Park et al., 2016).

Clinical experience is still scarce. Some investigators consider DCC as a form of autologous cord blood transplantation since the number of nucleated cord cells in the newborn which also contain pluripotent stem cells increase (Bayer, 2016). A recent small study from Poland in which very preterm infants were given autologous umbilical cord blood showed significantly higher concentrations of growth factors (among them insulin growth factor, epidermal growth factor and stem cell factor), whereas (severe) PIVH incidence seemed lower in the transplanted group as compared to a control group (Kotowski et al., 2017). Although not directly related to the immature brain, a Korean safety and feasibility study in extremely preterm infants to lower the risk of bronchopulmonary dysplasia with allogeneic cord-derived MSCs (endotracheal administration) reported that allogeneic MSC transplantation seemed safe and well-tolerated by the infants (Chang et al., 2014). A safety and efficacy study of the same group is currently including patients with PIVH (*ClinicalTrial.gov: NCT02673788*).

Although MSC transplantation seems very promising, it may be clear that further clinical research is mandatory to proof its efficacy to attenuate the consequences of (severe) PIVH. In particular, optimization of dosing of MSCs, the preferred type of MSCs (cord-derived vs bone marrow-derived; (Chen et al., 2009)) and most optimal route of administration are important pending questions, which have to be elucidated.

Stem Cell-Therapy and Diffuse WMI

Treatment with MSCs in preterm neonates with or at risk for dWMI provides us with an exciting and potentially powerful therapy to reduce or even prevent damage to the vulnerable white matter of the preterm neonate. Experimental studies in which perinatal insults as inflammation and hypoxia-ischemia are used separately or in combination showed us already that the paracrine factors secreted by the MSCs promote oligodendrocyte lineage specification, myelination and maturation (Chen et al., 2010; Jadasz et al., 2013; Jellema et al., 2013; Li et al., 2016; Drommelschmidt et al., 2017). It remains to be proven whether MSC-induced endogenous repair mechanisms also lead to substantial positive effects in diffuse WMI of the preterm infant in whom the interplay of inflammation and hypoxia-ischemia appears to be most relevant. Further research is emerging and mandatory.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Yoo, H. S., Lee, J. H., et al. (2013). Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. *Stroke* 44, 497–504. doi: 10.1161/STROKEAHA.112.679092

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Ahn, J. Y., and Park, W. S. (2017). Pivotal role of brain-derived neurotrophic factor secreted by mesenchymal stem cells in severe intraventricular hemorrhage in newborn rats. *Cell Transplant.* 26, 145–156. doi: 10.3727/096368916X692861

Alderliesten, T., Lemmers, P. M., Smarius, J. J., van de Vosse, R. E., Baerts, W., and van Bel, F. (2013). Cerebral oxygenation, extraction, and autoregulation in very preterm infants who develop peri-intraventricular hemorrhage. *J. Pediatr.* 162, 698–704.e692. doi: 10.1016/j.jpeds.2012.09.038

Alderliesten, T., De Vis, J. B., Lemmers, P. M. A., van Bel, F., Benders, M., Hendrikse, J., et al. (2016). T2-prepared velocity selective labelling: a novel idea for full-brain mapping of oxygen saturation. *NeuroImage* 139, 65–73. doi: 10.1016/j.neuroimage.2016.06.012

Anand, K. J., Barton, B. A., McIntosh, N., Lagercrantz, H., Pelausa, E., Young, T. E., et al. (1999). Analgesia and sedation in preterm neonates who require ventilatory support: results from the NOPAIN trial. Neonatal outcome and prolonged analgesia in neonates. *Arch. Pediatr. Adolesc. Med.* 153, 331–338.

Bach, L. A. (2015). Endothelial cells and the IGF system. *J. Mol. Endocrinol.* 54, R1–R13. doi: 10.1530/JME-14-0215

Baenziger, O., Stolkin, F., Keel, M., von Siebenthal, K., Fauchere, J. C., Das Kundu, S., et al. (2007). The influence of the timing of cord clamping on postnatal cerebral oxygenation in preterm neonates: a randomized, controlled trial. *Pediatrics* 119, 455–459. doi: 10.1542/peds.2006-2725

Ballabh, P. (2014). Pathogenesis and prevention of intraventricular hemorrhage. *Clin. Perinatol.* 41, 47–67. doi: 10.1016/j.clp.2013.09.007

Banker, B. Q., and Larroche, J. C. (1962). Periventricular leukomalacia of infancy. A form of neonatal anoxic encephalopathy. *Arch. Neurol.* 7, 386–410. doi: 10.1001/archneur.1962.04210050022004

Bayer, K. (2016). Delayed umbilical cord clamping in the 21st century: indications for Practice. *Adv. Neonatal Care* 16, 68–73. doi: 10.1097/ANC.0000000000000247

Bedard, M. P., Shankaran, S., Slovis, T. L., Pantoja, A., Dayal, B., and Poland, R. L. (1984). Effect of prophylactic phenobarbital on intraventricular hemorrhage in high-risk infants. *Pediatrics* 73, 435–439.

Bennet, L., Dhillon, S., Lear, C. A., van den Heuij, L., King, V., Dean, J. M., et al. (2018a). Chronic inflammation and impaired development of the preterm brain. *J. Reprod. Immunol.* 125, 45–55. doi: 10.1016/j.jri.2017.11.003

Bennet, L., Galinsky, R., Draghi, V., Lear, C. A., Davidson, J. O., Unsworth, C. P., et al. (2018b). Time and sex dependent effects of magnesium sulphate on post-asphyxial seizures in preterm fetal sheep. *J. Physiol.* 596, 6079–6092. doi: 10.1113/JP275627

Benson, J. W., Drayton, M. R., Hayward, C., Murphy, J. F., Osborne, J. P., Rennie, J. M., et al. (1986). Multicentre trial of ethamsylate for prevention of periventricular haemorrhage in very low birthweight infants. *Lancet* 2, 1297–1300. doi: 10.1016/S0140-6736(86)91432-7

Beresford, M. W., and Shaw, N. J. (2002). Detectable IL-8 and IL-10 in bronchoalveolar lavage fluid from preterm infants ventilated for respiratory distress syndrome. *Pediatr. Res.* 52, 973–978. doi: 10.1203/00006450-200212000-00025

Bernaudin, M., Marti, H. H., Roussel, S., Divoux, D., Nouvelot, A., MacKenzie, E. T., et al. (1999). A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* 19, 643–651.

Bierstone, D., Wagenaar, N., Gano, D. L., Guo, T., Georgio, G., Groenendaal, F., et al. (2018). Association of histologic chorioamnionitis with perinatal brain injury and early childhood neurodevelopmental outcomes among preterm neonates. *JAMA Pediatr.* 172, 534–541. doi: 10.1001/jamapediatrics.2018.0102

Bilguvar, K., DiLuna, M. L., Bizzarro, M. J., Bayri, Y., Schneider, K. C., Lifton, R. P., et al. (2009). COL4A1 mutation in preterm intraventricular hemorrhage. *J. Pediatr.* 155, 743–745. doi: 10.1016/j.jpeds.2009.04.014

Brion, L. P., Bell, E. F., and Raghuveer, T. S. (2003). Vitamin E supplementation for prevention of morbidity and mortality in preterm infants. *Cochrane Database Syst. Rev.* Cd003665. doi: 10.1002/14651858.CD003665

Brownfoot, F. C., Gagliardi, D. I., Bain, E., Middleton, P., and Crowther, C. A. (2013). Different corticosteroids and regimens for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst. Rev.* Cd006764. doi: 10.1002/14651858.CD006764.pub3

Cai, Z., Fan, L. W., Lin, S., Pang, Y., and Rhodes, P. G. (2011). Intranasal administration of insulin-like growth factor-1 protects against lipopolysaccharide-induced injury in the developing rat brain. *Neuroscience* 194, 195–207. doi: 10.1016/j.neuroscience.2011.08.003

Cao, Y., Gunn, A. J., Bennet, L., Wu, D., George, S., Gluckman, P. D., et al. (2003). Insulin-like growth factor (IGF)-1 suppresses oligodendrocyte caspase-3 activation and increases glial proliferation after ischemia in near-term fetal sheep. *J. Cereb. Blood Flow Metab.* 23, 739–747. doi: 10.1097/01.WCB.0000067720.12805.6F

Chang, Y. S., Ahn, S. Y., Yoo, H. S., Sung, S. I., Choi, S. J., Oh, W. I., et al. (2014). Mesenchymal stem cells for bronchopulmonary dysplasia: phase 1 dose-escalation clinical trial. *J. Pediatr.* 164, 966–972.e966. doi: 10.1016/j.jpeds.2013.12.011

Chateauvieux, S., Grigorakaki, C., Morceau, F., Dicato, M., and Diederich, M. (2011). Erythropoietin, erythropoiesis and beyond. *Biochem. Pharmacol.* 82, 1291–1303. doi: 10.1016/j.bcp.2011.06.045

Chau, V., Synnes, A., Grunau, R. E., Poskitt, K. J., Brant, R., and Miller, S. P. (2013). Abnormal brain maturation in preterm neonates associated with adverse developmental outcomes. *Neurology* 81, 2082–2089. doi: 10.1212/01.wnl.0000437298.43688.b9

Chen, M. Y., Lie, P. C., Li, Z. L., and Wei, X. (2009). Endothelial differentiation of Wharton's jelly-derived mesenchymal stem cells in comparison with bone marrow-derived mesenchymal stem cells. *Exp. Hematol.* 37, 629–640. doi: 10.1016/j.exphem.2009.02.003

Chen, A., Siow, B., Blamire, A. M., Lako, M., and Clowry, G. J. (2010). Transplantation of magnetically labeled mesenchymal stem cells in a model of perinatal brain injury. *Stem Cell Res.* 5, 255–266. doi: 10.1016/j.scr.2010.08.004

Chevallier, M., Debillon, T., Pierrat, V., Delorme, P., Kayem, G., Durox, M., et al. (2017). Leading causes of preterm delivery as risk factors for intraventricular hemorrhage in very preterm infants: results of the EPIPAGE 2 cohort study. *Am. J. Obstet. Gynecol.* 216, 518.e511–518.e512. doi: 10.1016/j.ajog.2017.01.002

Chisholm, K. M., Heerema-McKenney, A., Tian, L., Rajani, A. K., Saria, S., Koller, D., et al. (2016). Correlation of preterm infant illness severity with placental histology. *Placenta* 39, 61–69. doi: 10.1016/j.placenta.2016.01.012

Comi, A. M., Cho, E., Mulholland, J. D., Hooper, A., Li, Q., Qu, Y., et al. (2008). Neural stem cells reduce brain injury after unilateral carotid ligation. *Pediatr. Neurol.* 38, 86–92. doi: 10.1016/j.pediatrneurol.2007.10.007

Cotten, C. M., Murtha, A. P., Goldberg, R. N., Grotegut, C. A., Smith, P. B., Goldstein, R. F., et al. (2014). Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *J. Pediatr.* 164, 973–979.e971. doi: 10.1016/j.jpeds.2013.11.036

Coviello, C., Keunen, K., Kersbergen, K. J., Groenendaal, F., Leemans, A., Peels, B., et al. (2018). Effects of early nutrition and growth on brain volumes, white matter microstructure, and neurodevelopmental outcome in preterm newborns. *Pediatr. Res.* 83, 102–110. doi: 10.1038/pr.2017.227

Crowther, C. A., Middleton, P. F., Voysey, M., Askie, L., Duley, L., Pryde, P. G., et al. (2017). Assessing the neuroprotective benefits for babies of antenatal magnesium sulphate: an individual participant data meta-analysis. *PLoS Med.* 14:e1002398. doi: 10.1371/journal.pmed.1002398

Cryan, J. F., and Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat. Rev. Neurosci.* 13, 701–712. doi: 10.1038/nrn3346

de Vries, L. S., van Haastert, I. C., Rademaker, K. J., Koopman, C., and Groenendaal, F. (2004). Ultrasound abnormalities preceding cerebral palsy in high risk preterm infants. *J. Pediatr.* 144, 815–820. doi: 10.1016/j.jpeds.2004.03.034

Dent, C. L., Spaeth, J. P., Jones, B. V., Schwartz, S. M., Glauser, T. A., Hallinan, B.., et al. (2005). Brain magnetic resonance imaging abnormalities after the Norwood procedure using regional cerebral perfusion. *J. Thorac. Cardiovasc. Surg.* 130, 1523–1530. doi: 10.1016/j.jtcvs.2005.10.003

Donega, V., Nijboer, C. H., van Tilborg, G., Dijkhuizen, R. M., Kavelaars, A., and Heijnen, C. J. (2014). Intranasally administered mesenchymal stem cells promote a regenerative niche for repair of neonatal ischemic brain injury. *Exp. Neurol.* 261, 53–64. doi: 10.1016/j.expneurol.2014.06.009

Donega, V., Nijboer, C. H., van Velthoven, C. T., Youssef, S. A., de Bruin, A., van Bel, F., et al. (2015). Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse. *Pediatr. Res.* 78, 520–526. doi: 10.1038/pr.2015.145

Donn, S. M., Roloff, D. W., and Goldstein, G. W. (1981). Prevention of intraventricular haemorrhage in preterm infants by phenobarbitone. A controlled trial. *Lancet* 2, 215–217. doi: 10.1016/S0140-6736(81)90470-0

Doyle, L. W., Cheong, J. L., Ehrenkranz, R. A., and Halliday, H. L. (2017). Early (<8 days) systemic postnatal corticosteroids for prevention of bronchopulmonary dysplasia in preterm infants. *Cochrane Database Syst. Rev.* 10:Cd001146. doi: 10.1002/14651858.CD001145.pub4

Drommelschmidt, K., Serdar, M., Bendix, I., Herz, J., Bertling, F., Prager, S., et al. (2017). Mesenchymal stem cell-derived extracellular vesicles ameliorate inflammation-induced preterm brain injury. *Brain Behav. Immun.* 60, 220–232. doi: 10.1016/j.bbi.2016.11.011

Duerden, E. G., Grunau, R. E., Guo, T., Foong, J., Pearson, A., Au-Young, S., et al. (2018). Early procedural pain is associated with regionally-specific alterations in thalamic development in preterm neonates. *J. Neurosci.* 38, 878–886. doi: 10.1523/JNEUROSCI.0867-17.2017

Ersdal, H. L., Linde, J., Mduma, E., Auestad, B., and Perlman, J. (2014). Neonatal outcome following cord clamping after onset of spontaneous respiration. *Pediatrics* 134, 265–272. doi: 10.1542/peds.2014-0467

Fauchere, J. C., Koller, B. M., Tschopp, A., Dame, C., Ruegger, C., and Bucher, H. U. (2015). Safety of early high-dose recombinant erythropoietin for Neuroprotection in very preterm infants. *J. Pediatr.* 167, 52–57.e51-53. doi: 10.1016/j.jpeds.2015.02.052

Fischer, U. M., Harting, M. T., Jimenez, F., Monzon-Posadas, W. O., Xue, H., Savitz, S. I., et al. (2009). Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev.* 18, 683–692. doi: 10.1089/scd.2008.0253

Fischer, H. S., Reibel, N. J., Buhrer, C., and Dame, C. (2017). Prophylactic early erythropoietin for neuroprotection in preterm infants: a meta-analysis. *Pediatrics* 139:e20164317. doi: 10.1542/peds.2016-4317

Fleiss, B., Guillot, P. V., Titomanlio, L., Baud, O., Hagberg, H., and Gressens, P. (2014). Stem cell therapy for neonatal brain injury. *Clin. Perinatol.* 41, 133–148. doi: 10.1016/j.clp.2013.09.002

Fogarty, M., Osborn, D. A., Askie, L., Seidler, A. L., Hunter, K., Lui, K., et al. (2018). Delayed vs early umbilical cord clamping for preterm infants: a systematic review and meta-analysis. *Am. J. Obstet. Gynecol.* 218, 1–18. doi: 10.1016/j.ajog.2017.10.231

Galinsky, R., Draghi, V., Wassink, G., Davidson, J. O., Drury, P. P., Lear, C. A., et al. (2017). Magnesium sulfate reduces EEG activity but is not neuroprotective

after asphyxia in preterm fetal sheep. *J. Cereb. Blood Flow Metab.* 37, 1362–1373. doi: 10.1177/0271678X16655548

Gisslen, T., Alvarez, M., Wells, C., Soo, M. T., Lambers, D. S., Knox, C. L., et al. (2016). Fetal inflammation associated with minimal acute morbidity in moderate/late preterm infants. *Arch. Dis. Child. Fetal Neonatal Ed.* 101, F513–F519. doi: 10.1136/archdischild-2015-308518

Gitto, E., Reiter, R. J., Cordaro, S. P., La Rosa, M., Chiurazzi, P., Trimarchi, G., et al. (2004). Oxidative and inflammatory parameters in respiratory distress syndrome of preterm newborns: beneficial effects of melatonin. *Am. J. Perinatol.* 21, 209–216. doi: 10.1055/s-2004-828610

Glass, H. C., Shellhaas, R. A., Tsuchida, T. N., Chang, T., Wusthoff, C. J., Chu, C. J., et al. (2017). Seizures in preterm neonates: a multicenter observational cohort study. *Pediatr. Neurol.* 72, 19–24. doi: 10.1016/j.pediatrneurol.2017.04.016

Goldberg, R. N., Joshi, A., Moscoso, P., and Castillo, T. (1983). The effect of head position on intracranial pressure in the neonate. *Crit. Care Med.* 11, 428–430. doi: 10.1097/00003246-198306000-00006

Greenough, A., and Ahmed, N. (2013). Perinatal prevention of bronchopulmonary dysplasia. *J. Perinat. Med.* 41, 119–126. doi: 10.1515/jpm-2012-0084

Greisen, G., and Vannucci, R. C. (2001). Is periventricular leucomalacia a result of hypoxic-ischaemic injury? hypocapnia and the preterm brain. *Biol. Neonate* 79, 194–200. doi: 10.1159/000047090

Groenendaal, F., and de Vries, L. S. (2001). Hypocarbia and white matter echolucencies in newborns < 28 weeks gestation [letter]. *Pediatr. Res.* 50, 772–773. doi: 10.1203/00006450-200112000-00024

Gronbach, J., Shahzad, T., Radajewski, S., Chao, C. M., Bellusci, S., Morty, R. E., et al. (2018). The potentials and caveats of mesenchymal stromal cell-based therapies in the preterm infant. *Stem Cells International.* 2018:9652897. doi: 10.1155/2018/9652897

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Hamilton, B. E., Hoyert, D. L., Martin, J. A., Strobino, D. M., and Guyer, B. (2013). Annual summary of vital statistics: 2010-2011. *Pediatrics* 131, 548–558. doi: 10.1542/peds.2012-3769

Hansen-Pupp, I., Hovel, H., Hellstrom, A., Hellstrom-Westas, L., Lofqvist, C., Larsson, E. M., et al. (2011). Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J. Clin. Endocrinol. Metab.* 96, 1129–1135. doi: 10.1210/jc.2010-2440

Harteman, J. C., Groenendaal, F., van, H. I., Liem, K. D., Stroink, H., Bierings, M. B., et al. (2011). A typical timing and presentation of periventricular haemorrhagic infarction in preterm infants: the role of thrombophilia. *Dev. Med. Child Neurol.* 54, 140–147. doi: 10.1111/j.1469-8749.2011.04135.x

Hedley-Whyte, E. T., and Hsu, D. W. (1986). Effect of dexamethasone on blood-brain barrier in the normal mouse. *Ann. Neurol.* 19, 373–377. doi: 10.1002/ana.410190411

Hellstrom, A., Perruzzi, C., Ju, M., Engstrom, E., Hard, A. L., Liu, J. L., et al. (2001). Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: direct correlation with clinical retinopathy of prematurity. *Proc. Natl. Acad. Sci. U. S. A.* 98, 5804–5808. doi: 10.1073/pnas.101113998

Hellstrom, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Ramenghi, L. A., Lofqvist, C., et al. (2016). Role of insulinlike growth factor 1 in fetal development and in the early postnatal life of premature infants. *Am. J. Perinatol.* 33, 1067–1071. doi: 10.1055/s-0036-1586109

Hinojosa-Rodríguez, M., Harmony, T., Carrillo-Prado, C., Van Horn, J. D., Irimia, A., Torgerson, C., et al. (2017). Clinical neuroimaging in the preterm infant: diagnosis and prognosis. *Neuroimage Clin.* 16, 355–368. doi: 10.1016/j.nicl.2017.08.015

Hooper, S. B., Te Pas, A. B., Lang, J., van Vonderen, J. J., Roehr, C. C., Kluckow, M., et al. (2015). Cardiovascular transition at birth: a physiological sequence. *Pediatr. Res.* 77, 608–614. doi: 10.1038/pr.2015.21

Hou, X., Ding, H., Teng, Y., Zhou, C., Tang, X., Li, S., et al. (2007). Research on the relationship between brain anoxia at different regional oxygen saturations and brain damage using near-infrared spectroscopy. *Physiol. Meas.* 28, 1251–1265. doi: 10.1088/0967-3334/28/10/010

Hyttel-Sorensen, S., Pellicer, A., Alderliesten, T., Austin, T., van Bel, F., Benders, M., et al. (2015). Cerebral near infrared spectroscopy oximetry in extremely preterm infants: phase II randomised clinical trial. *BMJ* 350:g7635. doi: 10.1136/bmj.g7635

Hyttel-Sorensen, S., Greisen, G., Als-Nielsen, B., and Gluud, C. (2017). Cerebral near-infrared spectroscopy monitoring for prevention of brain injury in very preterm infants. *Cochrane Database Syst. Rev.* 9:Cd011506. doi: 10.1002/14651858.CD011506.pub2

Inder, T. E., Warfield, S. K., Wang, H., Huppi, P. S., and Volpe, J. J. (2005). Abnormal cerebral structure is present at term in premature infants. *Pediatrics* 115, 286–294. doi: 10.1542/peds.2004-0326

Isayama, T., Chai-Adisaksopha, C., and McDonald, S. D. (2015). Noninvasive ventilation with vs without early surfactant to prevent chronic lung disease in preterm infants: a systematic review and meta-analysis. *JAMA Pediatr.* 169, 731–739. doi: 10.1001/jamapediatrics.2015.0510

Jadasz, J. J., Kremer, D., Gottle, P., Tzekova, N., Domke, J., Rivera, F. J., et al. (2013). Mesenchymal stem cell conditioning promotes rat oligodendroglial cell maturation. *PLoS One* 8:e71814. doi: 10.1371/journal.pone.0071814

Jain, N. J., Kruse, L. K., Demissie, K., and Khandelwal, M. (2009). Impact of mode of delivery on neonatal complications: trends between 1997 and 2005. *J. Matern. Fetal Neonatal Med.* 22, 491–500. doi: 10.1080/14767050902769982

Jellema, R. K., Wolfs, T. G., Lima Passos, V., Zwanenburg, A., Ophelders, D. R., Kuypers, E., et al. (2013). Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia. *PLoS One* 8:e73031. doi: 10.1371/journal.pone.0073031

Jensen, E. A., Foglia, E. E., and Schmidt, B. (2018). Association between prophylactic indomethacin and death or bronchopulmonary dysplasia: a systematic review and meta-analysis of observational studies. *Semin. Perinatol.* 42, 228–234. doi: 10.1053/j.semperi.2018.05.005

Juul, S. E., and Pet, G. C. (2015). Erythropoietin and neonatal Neuroprotection. *Clin. Perinatol.* 42, 469–481. doi: 10.1016/j.clp.2015.04.004

Karemaker, R., Heijnen, C. J., Veen, S., Baerts, W., Samsom, J., Visser, G. H., et al. (2006). Differences in behavioral outcome and motor development at school age after neonatal treatment for chronic lung disease with dexamethasone versus hydrocortisone. *Pediatr. Res.* 60, 745–750. doi: 10.1203/01.pdr.0000246200.76860.de

Kawakami, M., Sekiguchi, M., Sato, K., Kozaki, S., and Takahashi, M. (2001). Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. *J. Biol. Chem.* 276, 39469–39475. doi: 10.1074/jbc.M105832200

Kersbergen, K. J., Benders, M. J., Groenendaal, F., Koopman-Esseboom, C., Nievelstein, R. A., van Haastert, I. C., et al. (2014a). Different patterns of punctate white matter lesions in serially scanned preterm infants. *PLoS One* e108904. doi: 10.1371/journal.pone.0108904

Kersbergen, K. J., Leemans, A., Groenendaal, F., van der Aa, N. E., Viergever, M. A., de Vries, L. S., et al. (2014b). Microstructural brain development between 30 and 40 weeks corrected age in a longitudinal cohort of extremely preterm infants. *NeuroImage* 103, 214–224. doi: 10.1016/j.neuroimage.2014.09.039

Kidokoro, H., Neil, J. J., and Inder, T. E. (2013). New MR imaging assessment tool to define brain abnormalities in very preterm infants at term. *AJNR Am. J. Neuroradiol.* 34, 2208–2214. doi: 10.3174/ajnr.A3521

Kizil, C., Kyritsis, N., and Brand, M. (2015). Effects of inflammation on stem cells: together they strive? *EMBO Rep.* 16, 416–426. doi: 10.15252/embr.201439702

Kotowski, M., Litwinska, Z., Klos, P., Pius-Sadowska, E., Zagrodnik-Ulan, E., Ustianowski, P., et al. (2017). Autologous cord blood transfusion in preterm infants - could its humoral effect be the key to control prematurity-related complications? A preliminary study. *J. Physiol. Pharmacol.* 68, 921–927.

Koulnis, M., Porpiglia, E., Hidalgo, D., and Socolovsky, M. (2014). Erythropoiesis: from molecular pathways to system properties. *Adv. Exp. Med. Biol.* 844, 37–58. doi: 10.1007/978-1-4939-2095-2_3

Krediet, T. G., Kavelaars, A., Vreman, H. J., Heijnen, C. J., and van Bel, F. (2006). Respiratory distress syndrome-associated inflammation is related to early but not late peri/intraventricular hemorrhage in preterm infants. *J. Pediatr.* 148, 740–746. doi: 10.1016/j.jpeds.2006.01.037

Lalu, M. M., McIntyre, L., Pugliese, C., Fergusson, D., Winston, B. W., Marshall, J. C., et al. (2012). Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One* 7:e47559. doi: 10.1371/journal.pone.0047559

Lekic, T., Flores, J., Klebe, D., Doycheva, D., Rolland, W. B., Tang, J., et al. (2016). Intranasal IGF-1 reduced rat pup germinal matrix hemorrhage. *Acta Neurochir. Suppl.* 121, 209–212. doi: 10.1007/978-3-319-18497-5_38

Lemmers, P. M., Toet, M., van Schelven, L. J., and van Bel, F. (2006). Cerebral oxygenation and cerebral oxygen extraction in the preterm infant: the impact of respiratory distress syndrome. *Exp. Brain Res.* 173, 458–467. doi: 10.1007/s00221-006-0388-8

Leuchter, R. H., Gui, L., Poncet, A., Hagmann, C., Lodygensky, G. A., Martin, E., et al. (2014). Association between early administration of high-dose erythropoietin in preterm infants and brain MRI abnormality at term-equivalent age. *JAMA* 312, 817–824. doi: 10.1001/jama.2014.9645

Ley, D., Hansen-Pupp, I., Niklasson, A., Domellof, M., Friberg, L. E., Borg, J., et al. (2013). Longitudinal infusion of a complex of insulin-like growth factor-I and IGF-binding protein-3 in five preterm infants: pharmacokinetics and short-term safety. *Pediatr. Res.* 73, 68–74. doi: 10.1038/pr.2012.146

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., et al. (2016). Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells. *Exp. Neurol.* 283, 179–187. doi: 10.1016/j.expneurol.2016.06.017

Luu, T. M., Ment, L. R., Schneider, K. C., Katz, K. H., Allan, W. C., and Vohr, B. R. (2009). Lasting effects of preterm birth and neonatal brain hemorrhage at 12 years of age. *Pediatrics* 123, 1037–1044. doi: 10.1542/peds.2008-1162

Marseglia, L., D'Angelo, G., Manti, S., Aversa, S., Reiter, R. J., Antonuccio, P., et al. (2015). Oxidative stress-mediated damage in newborns with necrotizing enterocolitis: a possible role of melatonin. *Am. J. Perinatol.* 32, 905–909. doi: 10.1055/s-0035-1547328

Martinez-Biarge, M., Groenendaal, F., Kersbergen, K. J., Benders, M. J., Foti, F., Cowan, F. M., et al. (2016). MRI based preterm white matter injury classification: the importance of sequential imaging in determining severity of injury. *PLoS One* 11:e0156245. doi: 10.1371/journal.pone.0156245

McPherson, C., and Inder, T. (2017). Perinatal and neonatal use of sedation and analgesia. *Semin. Fetal Neonatal Med.* 22, 314–320. doi: 10.1016/j.siny.2017.07.007

McPherson, C., and Wambach, J. A. (2018). Prevention and treatment of respiratory distress syndrome in preterm neonates. *Neonatal Netw.* 37, 169–177. doi: 10.1891/0730-0832.37.3.169

Ment, L. R., Stewart, W. B., Ardito, T. A., Huang, E., and Madri, J. A. (1992). Indomethacin promotes germinal matrix microvessel maturation in the newborn beagle pup. *Stroke* 23, 1132–1137. doi: 10.1161/01.STR.23.8.1132

Ment, L. R., Oh, W., Ehrenkranz, R. A., Philip, A. G., Duncan, C. C., and Makuch, R. W. (1995). Antenatal steroids, delivery mode, and intraventricular hemorrhage in preterm infants. *Am. J. Obstet. Gynecol.* 172, 795–800. doi: 10.1016/0002-9378(95)90001-2

Mercer, J. S., Erickson-Owens, D. A., Vohr, B. R., Tucker, R. J., Parker, A. B., Oh, W., et al. (2016). Effects of placental transfusion on neonatal and 18 month outcomes in preterm infants: a randomized controlled trial. *J. Pediatr.* 168, 50–55.e51. doi: 10.1016/j.jpeds.2015.09.068

Merchant, N., Azzopardi, D., Counsell, S., Gressens, P., Dierl, A., Gozar, I., et al. (2014). Melatonin as a novel neuroprotectant in preterm infants – a double blinded randomised controlled trial (MINT study). *Arch. Dis. Child.* 99:A43.

Mukai, T., Mori, Y., Shimazu, T., Takahashi, A., Tsunoda, H., Yamaguchi, S., et al. (2017). Intravenous injection of umbilical cord-derived mesenchymal stromal cells attenuates reactive gliosis and hypomyelination in a neonatal intraventricular hemorrhage model. *Neuroscience* 355, 175–187. doi: 10.1016/j.neuroscience.2017.05.006

Mukerji, A., Shah, V., and Shah, P. S. (2015). Periventricular/intraventricular hemorrhage and neurodevelopmental outcomes: a meta-analysis. *Pediatrics* 136, 1132–1143. doi: 10.1542/peds.2015-0944

Murner-Lavanchy, I. M., Doyle, L. W., Schmidt, B., Roberts, R. S., Asztalos, E. V., Costantini, L., et al. (2018). Neurobehavioral outcomes 11 years after neonatal caffeine therapy for apnea of prematurity. *Pediatrics* 141:e20174047. doi: 10.1542/peds.2017-4047

Musilova, I., Andrys, C., Drahosova, M., Zednikova, B., Hornychova, H., Pliskova, L., et al. (2018). Late preterm prelabor rupture of fetal membranes: fetal inflammatory response and neonatal outcome. *Pediatr. Res.* 83, 630–637. doi: 10.1038/pr.2017.300

Nelin, T. D., Pena, E., Giacomazzi, T., Lee, S., Logan, J. W., Moallem, M., et al. (2017). Outcomes following indomethacin prophylaxis in extremely preterm infants in an all-referral NICU. *J. Perinatol.* 37, 932–937. doi: 10.1038/jp.2017.71

Neubauer, A. P., Voss, W., Wachtendorf, M., and Jungmann, T. (2010). Erythropoietin improves neurodevelopmental outcome of extremely preterm infants. *Ann. Neurol.* 67, 657–666. doi: 10.1002/ana.21977

Ng, E., Taddio, A., and Ohlsson, A. (2017). Intravenous midazolam infusion for sedation of infants in the neonatal intensive care unit. *Cochrane Database Syst. Rev.* 1:Cd002052. doi: 10.1002/14651858.CD002052.pub3

Niimi, Y., and Levison, S. W. (2018). Pediatric brain repair from endogenous neural stem cells of the subventricular zone. *Pediatr. Res.* 83, 385–396. doi: 10.1038/pr.2017.261

Norman, M., Piedvache, A., Borch, K., Huusom, L. D., Bonamy, A. E., Howell, E. A., et al. (2017). Association of short antenatal corticosteroid administration-to-birth intervals with survival and morbidity among very preterm infants: results from the EPICE cohort. *JAMA Pediatr.* 171, 678–686. doi: 10.1001/jamapediatrics.2017.0602

Oh, K. J., Park, J. Y., Lee, J., Hong, J. S., Romero, R., and Yoon, B. H. (2018). The combined exposure to intra-amniotic inflammation and neonatal respiratory distress syndrome increases the risk of intraventricular hemorrhage in preterm neonates. *J. Perinat. Med.* 46, 9–20. doi: 10.1515/jpm-2016-0348

Ohls, R. K., Kamath-Rayne, B. D., Christensen, R. D., Wiedmeier, S. E., Rosenberg, A., Fuller, J., et al. (2014). Cognitive outcomes of preterm infants randomized to darbepoetin, erythropoietin, or placebo. *Pediatrics* 133, 1023–1030. doi: 10.1542/peds.2013-4307

Ohlsson, A., and Aher, S. M. (2017). Early erythropoiesis-stimulating agents in preterm or low birth weight infants. *Cochrane Database Syst. Rev.* 11:Cd004863. doi: 10.1002/14651858.CD004863.pub5

O'Kusky, J., and Ye, P. (2012). Neurodevelopmental effects of insulin-like growth factor signaling. *Front. Neuroendocrinol.* 33, 230–251. doi: 10.1016/j.yfrne.2012.06.002

Onland, W., Offringa, M., Cools, F., De Jaegere, A. P., Rademaker, K., BlomH., et al. (2011). Systemic hydrocortisone to prevent bronchopulmonary dysplasia in preterm infants (the SToP-BPD study); a multicenter randomized placebo controlled trial. *BMC Pediatr.* 11:102. doi: 10.1186/1471-2431-11-102

Ophelders, D. R., Wolfs, T. G., Jellema, R. K., Zwanenburg, A., Andriessen, P., Delhaas, T., et al. (2016). Mesenchymal stromal cell-derived extracellular vesicles protect the fetal brain after hypoxia-ischemia. *Stem Cells Transl. Med.* 5, 754–763. doi: 10.5966/sctm.2015-0197

O'Shea, T. M., Allred, E. N., Kuban, K. C., Dammann, O., Paneth, N., Fichorova, R., et al. (2012). Elevated concentrations of inflammation-related proteins in postnatal blood predict severe developmental delay at 2 years of age in extremely preterm infants. *J. Pediatr.* 160, 395–401.e394. doi: 10.1016/j.jpeds.2011.08.069

Ozdemir, A., Brown, M. A., and Morgan, W. J. (1997). Markers and mediators of inflammation in neonatal lung disease. *Pediatr. Pulmonol.* 23, 292–306. doi: 10.1002/(SICI)1099-0496(199704)23:4<292::AID-PPUL7>3.0.CO;2-O

Pang, Y., Zheng, B., Campbell, L. R., Fan, L. W., Cai, Z., and Rhodes, P. G. (2010). IGF-1 can either protect against or increase LPS-induced damage in the developing rat brain. *Pediatr. Res.* 67, 579–584. doi: 10.1203/PDR.0b013e3181dc240f

Papile, L. A., Burstein, J., Burstein, R., and Koffler, H. (1978). Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. *J. Pediatr.* 92, 529–534. doi: 10.1016/S0022-3476(78)80282-0

Park, W. S., Sung, S. I., Ahn, S. Y., Sung, D. K., Im, G. H., Yoo, H. S., et al. (2016). Optimal timing of mesenchymal stem cell therapy for neonatal intraventricular hemorrhage. *Cell Transplant.* 25, 1131–1144. doi: 10.3727/096368915X689640

Park, W. S., Ahn, S. Y., Sung, S. I., Ahn, J. Y., and Chang, Y. S. (2017). Mesenchymal stem cells: the magic cure for intraventricular hemorrhage? *Cell Transplant.* 26, 439–448. doi: 10.3727/096368916X694193

Paton, M. C. B., McDonald, C. A., Allison, B. J., Fahey, M. C., Jenkin, G., and Miller, S. L. (2017). Perinatal brain injury as a consequence of preterm birth and intrauterine inflammation: designing targeted stem cell therapies. *Front. Neurosci.* 11:200. doi: 10.3389/fnins.2017.00200

Perlman, J. M., Goodman, S., Kreusser, K. L., and Volpe, J. J. (1985). Reduction in intraventricular hemorrhage by elimination of fluctuating cerebral blood-flow velocity in preterm infants with respiratory distress syndrome. *N. Engl. J. Med.* 312, 1353–1357. doi: 10.1056/NEJM198505233122104

Perlman, J. M., Wyllie, J., Kattwinkel, J., Wyckoff, M. H., Aziz, K., Guinsburg, R., et al. (2015). Part 7: neonatal resuscitation: 2015 international consensus on cardiopulmonary resuscitation and emergency cardiovascular care science with treatment recommendations (reprint). *Pediatrics* 136(Suppl. 2), S120–S166. doi: 10.1542/peds.2015-3373D

Pierrat, V., Marchand-Martin, L., Arnaud, C., Kaminski, M., Resche-Rigon, M., Lebeaux, C., et al. (2017). Neurodevelopmental outcome at 2 years for preterm children born at 22 to 34 weeks' gestation in France in 2011: EPIPAGE-2 cohort study. *BMJ* 358:j3448. doi: 10.1136/bmj.j3448

Plomgaard, A. M., Alderliesten, T., Austin, T., van Bel, F., Benders, M., Claris, O., et al. (2017). Early biomarkers of brain injury and cerebral hypo- and hyperoxia in the SafeBoosC II trial. *PLoS One* 12:e0173440. doi: 10.1371/journal.pone.0173440

Practice, C. O. (2017). Committee opinion no. 684: delayed umbilical cord clamping after birth. *Obstet. Gynecol.* 129, e5–e10. doi: 10.1097/AOG.0000000000001860

Qiu, J., Zhao, L., Yang, Y., Zhang, J. H., Feng, Y., and Cheng, R. (2018). Effects of fentanyl for pain control and neuroprotection in very preterm newborns on mechanical ventilation. *J. Matern. Fetal Neonatal Med.* 15, 1–7. doi: 10.1080/14767058.2018.1471593

Qu, R., Li, Y., Gao, Q., Shen, L., Zhang, J., Liu, Z., et al. (2007). Neurotrophic and growth factor gene expression profiling of mouse bone marrow stromal cells induced by ischemic brain extracts. *Neuropathology* 27, 355–363. doi: 10.1111/j.1440-1789.2007.00792.x

Rabe, H., Reynolds, G., and Diaz-Rossello, J. (2008). A systematic review and meta-analysis of a brief delay in clamping the umbilical cord of preterm infants. *Neonatology* 93, 138–144. doi: 10.1159/000108764

Rabe, H., Diaz-Rossello, J. L., Duley, L., and Dowswell, T. (2012). Effect of timing of umbilical cord clamping and other strategies to influence placental transfusion at preterm birth on maternal and infant outcomes. *Cochrane Database Syst. Rev.* Cd003248. doi: 10.1002/14651858.CD003248.pub3

Rangarajan, V., and Juul, S. E. (2014). Erythropoietin: emerging role of erythropoietin in neonatal neuroprotection. *Pediatr. Neurol.* 51, 481–488. doi: 10.1016/j.pediatrneurol.2014.06.008

Roberts, D., Brown, J., Medley, N., and Dalziel, S. R. (2017). Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst. Rev.* 3:Cd004454. doi: 10.1002/14651858.CD004454.pub3

Rojas-Reyes, M. X., Morley, C. J., and Soll, R. (2012). Prophylactic versus selective use of surfactant in preventing morbidity and mortality in preterm infants. *Cochrane Database Syst. Rev.* 14:Cd000510. doi: 10.1002/14651858.CD000510.pub2

Romantsik, O., Calevo, M. G., and Bruschettini, M. (2017). Head midline position for preventing the occurrence or extension of germinal matrix-intraventricular hemorrhage in preterm infants. *Cochrane Database Syst. Rev.* 7:Cd012362. doi: 10.1002/14651858.CD012362.pub2

Schmidt, B., Davis, P., Moddemann, D., Ohlsson, A., Roberts, R. S., Saigal, S., et al. (2001). Long-term effects of indomethacin prophylaxis in extremely-low-birth-weight infants. *N. Engl. J. Med.* 344, 1966–1972. doi: 10.1056/NEJM200106283442602

Schmidt, B., Roberts, R. S., Davis, P., Doyle, L. W., Barrington, K. J., Ohlsson, A., et al. (2007). Long-term effects of caffeine therapy for apnea of prematurity. *N. Engl. J. Med.* 357, 1893–1902. doi: 10.1056/NEJMoa073679

Shepherd, E., Salam, R. A., Middleton, P., Makrides, M., McIntyre, S., Badawi, N., et al. (2017). Antenatal and intrapartum interventions for preventing cerebral palsy: an overview of Cochrane systematic reviews. *Cochrane Database Syst. Rev.* 8:Cd012077. doi: 10.1002/14651858.CD012077.pub2

Sherlock, R. L., Anderson, P. J., and Doyle, L. W. (2005). Neurodevelopmental sequelae of intraventricular haemorrhage at 8 years of age in a regional cohort of ELBW/very preterm infants. *Early Hum. Dev.* 81, 909–916. doi: 10.1016/j.earlhumdev.2005.07.007

Skov, L., Pryds, O., and Greisen, G. (1991). Estimating cerebral blood flow in newborn infants: comparison of near infrared spectroscopy and 133Xe clearance. *Pediatr. Res.* 30, 570–573. doi: 10.1203/00006450-199112000-00016

Stoll, B. J., Hansen, N. I., Bell, E. F., Shankaran, S., Laptook, A. R., Walsh, M. C., et al. (2010). Neonatal outcomes of extremely preterm infants from the NICHD neonatal research network. *Pediatrics* 126, 443–456. doi: 10.1542/peds.2009-2959

Stolp, H. B., Ball, G., So, P. W., Tournier, J. D., Jones, M., Thornton, C., et al. (2018). Voxel-wise comparisons of cellular microstructure and diffusion-MRI in mouse hippocampus using 3D bridging of optically-clear histology with neuroimaging data (3D-BOND). *Sci. Rep.* 8:4011. doi: 10.1038/s41598-018-22295-9

Strunk, T., Inder, T., Wang, X., Burgner, D., Mallard, C., and Levy, O. (2014). Infection-induced inflammation and cerebral injury in preterm infants. *Lancet Infect. Dis.* 14, 751–762. doi: 10.1016/S1473-3099(14)70710-8

Thewissen, L., Caicedo, A., Lemmers, P., van Bel, F., Van Huffel, S., and Naulaers, G. (2018). Measuring near-infrared spectroscopy derived cerebral autoregulation in neonates: from research tool toward bedside multimodal monitoring. *Front Pediatr.* 6:117. doi: 10.3389/fped.2018.00117

Thygesen, S. K., Olsen, M., Ostergaard, J. R., and Sorensen, H. T. (2016). Respiratory distress syndrome in moderately late and late preterm infants and risk of cerebral palsy: a population-based cohort study. *BMJ Open* 6:e011643. doi: 10.1136/bmjopen-2016-011643

Tokida, Y., Aratani, Y., Morita, A., and Kitagawa, Y. (1990). Production of two variant laminin forms by endothelial cells and shift of their relative levels by angiostatic steroids. *J. Biol. Chem.* 265, 18123–18129.

Uauy, R., and Mena, P. (2015). Long-chain polyunsaturated fatty acids supplementation in preterm infants. *Curr. Opin. Pediatr.* 27, 165–171. doi: 10.1097/MOP.0000000000000203

Uccelli, A., Moretta, L., and Pistoia, V. (2008). Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* 8, 726–736. doi: 10.1038/nri2395

van Bel, F., and Mintzer, J. P. (2018). Monitoring cerebral oxygenation of the immature brain: a neuroprotective strategy? *Pediatr. Res.* 84, 159–164. doi: 10.1038/s41390-018-0026-8

van Bel, F., Van de Bor, M., Stijnen, T., Baan, J., and Ruys, J. H. (1987). Aetiological role of cerebral blood-flow alterations in development and extension of peri-intraventricular haemorrhage. *Dev. Med. Child Neurol.* 29, 601–614. doi: 10.1111/j.1469-8749.1987.tb08502.x

van Bel, F., Klautz, R. J., Steendijk, P., Schipper, I. B., Teitel, D. F., and Baan, J. (1993). The influence of indomethacin on the autoregulatory ability of the cerebral vascular bed in the newborn lamb. *Pediatr. Res.* 34, 178–181. doi: 10.1203/00006450-199308000-00015

van Bel, F., Roman, C., Klautz, R. J., Teitel, D. F., and Rudolph, A. M. (1994). Relationship between brain blood flow and carotid arterial flow in the sheep fetus. *Pediatr. Res.* 35, 329–333. doi: 10.1203/00006450-199403000-00011

van Bel, F., Bartelds, B., Teitel, D. F., and Rudolph, A. M. (1995). Effect of indomethacin on cerebral blood flow and oxygenation in the normal and ventilated fetal lamb. *Pediatr. Res.* 38, 243–250. doi: 10.1203/00006450-199508000-00018

van Bel, F., Lemmers, P., and Naulaers, G. (2008). Monitoring neonatal regional cerebral oxygenation saturation in clinical practice: value and pitfalls. *Neonatology* 94, 237–244. doi: 10.1159/000151642

van de Looij, Y., Lodygensky, G. A., Dean, J., Lazeyras, F., Hagberg, H., Kjellmer, I., et al. (2012). High-field diffusion tensor imaging characterization of cerebral white matter injury in lipopolysaccharide-exposed fetal sheep. *Pediatr. Res.* 72, 285–292. doi: 10.1038/pr.2012.72

van der Kooij, M. A., Groenendaal, F., Kavelaars, A., Heijnen, C. J., and van B. F. (2008). Neuroprotective properties and mechanisms of erythropoietin in in vitro and in vivo experimental models for hypoxia/ischemia. *Brain Res. Rev.* 59, 22–33. doi: 10.1016/j.brainresrev.2008.04.007

van Haastert, I. C., Groenendaal, F., Uiterwaal, C. S., Termote, J. U., van der Heide-Jalving, M., Eijsermans, M. J., et al. (2011). Decreasing incidence and severity of cerebral palsy in prematurely born children. *J. Pediatr.* 159, 86–91. doi: 10.1016/j.jpeds.2010.12.053

van Tilborg, E., Achterberg, E. J. M., van Kammen, C. M., van der Toorn, A., Groenendaal, F., Dijkhuizen, R. M., et al. (2018a). Combined fetal inflammation and postnatal hypoxia causes myelin deficits and autism-like behavior in a rat model of diffuse white matter injury. *Glia* 66, 78–93. doi: 10.1002/glia.23216

van Tilborg, E., de Theije, C. G. M., van Hal, M., Wagenaar, N., de Vries, L. S., Benders, M. J., et al. (2018b). Origin and dynamics of oligodendrocytes in the developing brain: implications for perinatal white matter injury. *Glia* 66, 221–238. doi: 10.1002/glia.23256

van Velthoven, C. T., Kavelaars, A., van Bel, F., and Heijnen, C. J. (2011). Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. *Brain Behav. Immun.* 25, 1342–1348. doi: 10.1016/j.bbi.2011.03.021

Villamor-Martinez, E., Fumagalli, M., Mohammed Rahim, O., Passera, S., Cavallaro, G., Degraeuwe, P., et al. (2018). Chorioamnionitis is a risk factor

for intraventricular hemorrhage in preterm infants: a systematic review and meta-analysis. *Front. Physiol.* 9:1253. doi: 10.3389/fphys.2018.01253

Vohr, B., and Ment, L. R. (1996). Intraventricular hemorrhage in the preterm infant. *Early Hum. Dev.* 44, 1–16. doi: 10.1016/0378-3782(95)01692-9

Volpe, J. J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 8, 110–124. doi: 10.1016/S1474-4422(08)70294-1

Wagenaar, N., Nijboer, C. H., and van Bel, F. (2017). Repair of neonatal brain injury: bringing stem cell-based therapy into clinical practice. *Dev. Med. Child Neurol.* 59, 997–1003. doi: 10.1111/dmcn.13528

Walti, H., Paris-Llado, J., Breart, G., and Couchard, M. (1995). Porcine surfactant replacement therapy in newborns of 25-31 weeks' gestation: a randomized, multicentre trial of prophylaxis versus rescue with multiple low doses. The French collaborative multicentre study group. *Acta Paediatr.* 84, 913–921. doi: 10.1111/j.1651-2227.1995.tb13792.x

Wiest, D. B., Chang, E., Fanning, D., Garner, S., Cox, T., and Jenkins, D. D. (2014). Antenatal pharmacokinetics and placental transfer of N-acetylcysteine in chorioamnionitis for fetal neuroprotection. *J. Pediatr.* 165, 672–677.e672. doi: 10.1016/j.jpeds.2014.06.044

Wintermark, P., Hansen, A., Warfield, S. K., Dukhovny, D., and Soul, J. S. (2014). Near-infrared spectroscopy versus magnetic resonance imaging to study brain perfusion in newborns with hypoxic-ischemic encephalopathy treated with hypothermia. *NeuroImage* 85, 287–293. doi: 10.1016/j.neuroimage.2013.04.072

Wyllie, J., Bruinenberg, J., Roehr, C. C., Rudiger, M., Trevisanuto, D., and Urlesberger, B. (2015). European resuscitation council guidelines for resuscitation 2015: section 7. Resuscitation and support of transition of babies at birth. *Resuscitation* 95, 249–263. doi: 10.1016/j.resuscitation.2015.07.029

Xu, H., Hu, F., Sado, Y., Ninomiya, Y., Borza, D. B., Ungvari, Z., et al. (2008). Maturational changes in laminin, fibronectin, collagen IV, and perlecan in germinal matrix, cortex, and white matter and effect of betamethasone. *J. Neurosci. Res.* 86, 1482–1500. doi: 10.1002/jnr.21618

Yamaji, R., Okada, T., Moriya, M., Naito, M., Tsuruo, T., Miyatake, K., et al. (1996). Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur. J. Biochem.* 239, 494–500. doi: 10.1111/j.1432-1033.1996.0494u.x

Yao, A. C., Moinian, M., and Lind, J. (1969). Distribution of blood between infant and placenta after birth. *Lancet* 2, 871–873.

Zwicker, J. G., Miller, S. P., Grunau, R. E., Chau, V., Brant, R., Studholme, C., et al. (2016). Smaller cerebellar growth and poorer neurodevelopmental outcomes in very preterm infants exposed to neonatal morphine. *J. Pediatr.* 172, 81–87.e82. doi: 10.1016/j.jpeds.2015.12.024

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 van Bel, Vaes and Groenendaal. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 22 March 2019
doi: 10.3389/fphys.2019.00283

Human Umbilical Cord Therapy Improves Long-Term Behavioral Outcomes Following Neonatal Hypoxic Ischemic Brain Injury

*Tayla R. Penny[1,2], Amy E. Sutherland[1], Jamie G. Mihelakis[1], Madison C. B. Paton[1,2], Yen Pham[1], Joohyung Lee[3], Nicole M. Jones[4], Graham Jenkin[1,2], Michael C. Fahey[5], Suzanne L. Miller[1,2] and Courtney A. McDonald[1]**

[1] The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC, Australia, [2] Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia, [3] Centre for Endocrinology and Metabolism, Hudson Institute of Medical Research, Clayton, VIC, Australia, [4] Department of Pharmacology, School of Medical Sciences, University of New South Wales, Sydney, NSW, Australia, [5] Department of Paediatrics, Monash University, Clayton, VIC, Australia

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Helen B. Stolp,
Royal Veterinary College (RVC),
United Kingdom
Joakim Ek,
University of Gothenburg, Sweden

***Correspondence:**
Courtney A. McDonald
courtney.mcdonald@hudson.org.au;
courtney.mcdonald@monash.edu

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: 15 October 2018
Accepted: 04 March 2019
Published: 22 March 2019

Citation:
Penny TR, Sutherland AE, Mihelakis JG, Paton MCB, Pham Y, Lee J, Jones NM, Jenkin G, Fahey MC, Miller SL and McDonald CA (2019) Human Umbilical Cord Therapy Improves Long-Term Behavioral Outcomes Following Neonatal Hypoxic Ischemic Brain Injury. Front. Physiol. 10:283. doi: 10.3389/fphys.2019.00283

Background: Hypoxic ischemic (HI) insult in term babies at labor or birth can cause long-term neurodevelopmental disorders, including cerebral palsy (CP). The current standard treatment for term infants with hypoxic ischemic encephalopathy (HIE) is hypothermia. Because hypothermia is only partially effective, novel therapies are required to improve outcomes further. Human umbilical cord blood cells (UCB) are a rich source of stem and progenitor cells making them a potential treatment for neonatal HI brain injury. Recent clinical trials have shown that UCB therapy is a safe and efficacious treatment for confirmed cerebral palsy. In this study, we assessed whether early administration of UCB to the neonate could improve long-term behavioral outcomes and promote brain repair following neonatal HI brain injury.

Methods: HI brain injury was induced in postnatal day (PND) 7 rat pups via permanent ligation of the left carotid artery, followed by a 90 min hypoxic challenge. UCB was administered intraperitoneally on PND 8. Behavioral tests, including negative geotaxis, forelimb preference and open field test, were performed on PND 14, 30, and 50, following brains were collected for assessment of neuropathology.

Results: Neonatal HI resulted in decreased brain weight, cerebral tissue loss and apoptosis in the somatosensory cortex, as well as compromised behavioral outcomes. UCB administration following HI improved short and long-term behavioral outcomes but did not reduce long-term histological evidence of brain injury compared to HI alone. In addition, UCB following HI increased microglia activation in the somatosensory cortex compared to HI alone.

Conclusion: Administration of a single dose of UCB cells 24 h after HI injury improves behavior, however, a single dose of cells does not modulate pathological evidence of long-term brain injury.

Keywords: umbilical cord blood, behavior, hypoxia ischemia, hypoxic ischemic encephalopathy, cerebral palsy, stem cells

INTRODUCTION

Neonatal brain injury underlies long-term neurological deficits, including cerebral palsy (CP) and other cognitive and behavioral deficits that may become apparent months to years after birth (Vannucci, 2000; Ferrari et al., 2010). A significant cause of neonatal brain injury is severe and/or prolonged hypoxia-ischemia (HI) in utero, at labor or at birth, resulting in a lack of oxygen supply to the brain (Vannucci, 2000). Resulting brain injury is termed hypoxic-ischemic encephalopathy (HIE) which can be diagnosed through routine testing through use of Apgar and Sarnat scores, and MRI, however, the latter is usually performed outside of the early intervention critical treatment window. Because of this, some infants are not diagnosed within the early stages of HIE, thus early intervention therapies cannot be administered (Aridas et al., 2014; Ahearne et al., 2016). Of all neonates with HIE, 10–15% will die shortly after birth, 10–15% will develop cerebral palsy, and 40% will develop other disabilities, such as autism, epilepsy and impairment of cognition, motor abilities and behavioral problems (Cerio et al., 2013). Currently, the only treatment for HIE is therapeutic hypothermia which is effective but also restricted in scope (Cerio et al., 2013) in that treatment needs to commence within 6 h of birth (Chiang et al., 2017), is best applied in well-equipped tertiary hospitals, and is not a suitable treatment for babies born preterm (Bennet et al., 2012). These limitations indicate the need for the development of novel treatment/s, that could be used in conjunction with hypothermia or as a standalone treatment if required. The motor and behavioral impairments associated with HIE may not be diagnosed until many months after birth. For example, cerebral palsy may not be confirmed until 12–24 months of age, when a child is not reaching developmental milestones (Rosenbaum, 2003). It is therefore critical to assess long-term structural and functional outcomes in animal models of early intervention therapies for neonatal HI, with the aim of implementing a therapy that is beneficial for improving long-term neurodevelopmental outcomes.

Human umbilical cord blood cells (UCB) are an abundant source of naïve stem cells that can be easily harvested and feasibly be delivered to newborns within the first day of life, or be banked for later use (Cotten et al., 2014). UCB contains a large population of stem and progenitor cells, including endothelial progenitor cells and haematopoietic stem cells, as well as immunosuppressive cells, such as regulatory T cells and monocyte derived suppressor cells (Torelli et al., 2012; Jaing, 2014). UCB can be administered with little risk of rejection, even when used allogeneically (Maeda et al., 2005). This is partly due to a reduced presence of HLA antigens and, as such, there is a low risk of graft-versus-host disease, when compared to adult-derived cells, such as those derived from bone marrow (Knutsen and Wall, 2000). UCB is already an established treatment option for many conditions and is now under investigation for early intervention (within the first week after injury) in pre-clinical models of neurological injury (Aridas et al., 2016; Li et al., 2016; McDonald et al., 2018). In addition, there are a number of clinical trials underway that are investigating UCB therapy in children and adolescents with established cases of CP, treating months to years after the initial injury. Kang et al. (2015) demonstrated that a high dose of 20 million cells per kg administered to patients between 6 and 20 years old, results in improved motor functions and muscle strength within 6 months of treatment. Min et al. (2013) also showed that patients aged between 10 months and 10 years of age, treated with 30 million cells/kg also showed an improvement in gross motor function and cognitive scores. Data from clinical trials is promising, however, it is still unclear what long-term effects these cells have on behavioral and neuropathological outcomes, particularly following early intervention, when cells are delivered soon after injury.

This study aimed to assess the therapeutic use of human UCB for the treatment of HI brain injury in term-equivalent newborn rats, focused in particular on the analysis of long-term behavioral outcomes, as well as neuropathological outcomes. We hypothesized that a single dose of UCB at 24 h post neonatal HI insult would improve behavioral outcomes, as well as decrease neuronal damage and inflammation in the brain.

MATERIALS AND METHODS

Ethics Statement

All experiments in this project were performed with human ethics approval from Monash Health Human Ethics Committee (12387B) and Animal ethics approval from Monash Medical Centre Animal Ethics Committee A (MMCA/2015/42). Written informed consent was obtained from all participants prior to collection of UCB. All experiments were performed in accordance with the Australian National Health and Medical Research Council guidelines.

Isolation, Cryopreservation and Preparation of Cells

Human umbilical cord blood was collected via the umbilical vein of healthy term cesarean section births (>37 weeks), into a collection bag containing anticoagulant (Macopharma). The patients gave written informed consent for the collection and research use of cord blood. On average, ∼125 ml of blood was collected from each patient. Blood was stored at room temperature on a shaker for <72 h before processing. To separate the mononuclear cell population, the blood was evenly separated into falcon tubes, diluted with equal amounts of phosphate buffered saline (PBS; Gibco Life Technologies) and centrifuged for 12 min at 3100 RPM at room temperature (RT), with no brake. The mononuclear cell layer was collected and washed with 20 ml PBS and centrifuged at 800 g at RT for 5 min. 20 ml of red blood cell lysis buffer [155 Mm ammonium chloride (NH4Cl: Sigma-Aldrich), 10 mM potassium bicarbonate (KHCO3: Sigma-Aldrich) and 0.1 mM EDTA (Sigma-Aldrich) dissolved in distilled water] was added to the cell pellet to lyse any remaining red blood cells in the sample. The lysis was stopped after 3 min with 30 ml media [10% fetal bovine serum (FBS), 1% antibiotic, 1% L-glutamine in DMEM/F12; all from Gibco Life Technologies] and centrifuged at 400 *g* at RT for 5 min. The cells were counted using trypan blue exclusion dye (Gibco) with a haemocytometer. Samples were then cryopreserved at

an approximate density of 20–50 million cells/ml. UCB cells suspended in media were transferred to 2 ml cryopreservation vials and an equal volume of cryopreservation media [80% FBS (Gibco Life Technologies) containing 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich)] was added dropwise, resulting in a final concentration of 10% DMSO. Vials were placed in a freezing container (Mr. Frosty, Thermo Fisher Scientific) and stored at −80°C overnight. Cells were then moved to liquid nitrogen for long-term storage.

Animals

Animal Ethics, Selection and Welfare

Time-mated pregnant Sprague-Dawley (SD) rat dams were sourced from Monash Animal Research Platform and transported to Monash Medical Centre Animal Facility 1 week prior to birth. They were housed in individual boxes in standard housing conditions in rooms with a 12-h light/dark cycle. The dams were allowed to birth naturally and were only disturbed to count the pups on PND 2–3.

A total of 36 rat pups (from 8 dams) were used in this study. Rat pups were randomly assigned to experimental groups, based on surgery times, to control for any neuroprotective effects associated with prolonged isoflurane exposure. 17 animals were exposed to HI (HI, n = 11: 3 males and 8 females; HI + UCB, n = 6: 3 males and 3 females) and 19 animals received sham surgery with no HI (Sham, n = 19: 8 males, 11 females). Sex of the animal was not taken into consideration when performing analysis.

Animal Surgery to Induce HI

A permanent unilateral carotid artery ligation was performed on post-natal day (PND) 7, followed by exposure to hypoxia, as previously described (McDonald et al., 2018). The pups were separated from their mother and placed on a 37°C heat pad before and after surgery. The pups were anesthetized by inhalation of 4% isoflurane which was maintained at 1–2% for the duration of the surgery. A small incision was made in the neck, and the left carotid artery was exteriorised before being occluded with an electrocautery device. The wound was sutured closed using 6-0 polypropylene suture and isoflurane was stopped. Bupivacaine was applied to the surgery site for pain management. The pups were returned to their dam for a 1 h recovery period. Following this, the pups that underwent artery ligation were placed in a humidified hypoxic chamber for 90 min at 8% oxygen, balanced with 92% nitrogen, and the chamber was maintained at 35–36°C. Control pups underwent a sham surgery, in which the artery was not ligated. Following surgery, they were returned to their dam for a 1 h recovery period and were then placed on a 37°C heating pad and exposed to normal room air for the same duration as the injury group that underwent hypoxia. Following the hypoxic treatment, the pups were returned to the dam for recovery. For the remainder of the experiment, the pups were health checked daily and weighed 3–4 times a week.

Preparation and Administration of UCB Cells

Rat pups received UCB cells 24 h post insult (PND8), via intraperitoneal injection. Cells were thawed rapidly in a 37°C water bath. UCB cells from three different donors were pooled and washed with media to remove DMSO within the freeze media. The cells were counted and viability determined using trypan blue exclusion. Before administration, cells were washed in PBS and resuspended in PBS at a final concentration of 5 million cells/ml. Cells were stored on ice until administration to rat pups.

Pups received 1 million UCB cells (equivalent to 61×10^6 cells/kg) via an intraperitoneal injection in a volume of 200 μl of PBS using a 30-gauge insulin syringe. HI control pups received an injection of 200 μl of PBS only, using a 30-guage insulin syringe.

Behavioral Testing

Negative Geotaxis Analysis

As previously described (McDonald et al., 2018), on PND14 the pups were placed head-down on a 45-degree inclined slope covered with a standard laboratory bench pad. The time taken for them to turn 180 degrees was recorded, and then the time taken to walk ∼15 cm up the slope to cross a line was also recorded. This test was performed 3 times per pup. If the pup took longer than 90 s to complete the test, or if they climbed off the board, it was considered a fail and not included in the analysis. No pups were excluded from analysis in this study.

Open Field Test

The open field test was completed on PND 30 and 50 using the Topscan behavioral analysis program to track the movement of the rat. Distance, speed and time moved were recorded for both the inside area and the perimeter of the box. Before testing, the rats were acclimatized to the room for 1–2 h, and the open field boxes were disinfected to remove foreign smells that could influence behavior. For the open field test, the rats were placed in the large open box, approximately 50 cm × 50 cm, for 10 min and allowed to explore the box freely. This test assesses the anxiety behaviors of the rats, as a more anxious rat will spend the time walking the perimeter of the box (Gould et al., 2009).

Cylinder Test

On PND 30 and 50, the rats completed the cylinder test to analyze forelimb preference. The rats were placed in a clear glass beaker and video recorded from overhead for 2 min. The cylinder was disinfected between trials to remove foreign smells that could influence behavior. The videos were then analyzed to assess, upon rearing, how often the right and left foot touched the wall of the cylinder. This test assesses if the HI injury has impaired limb use and caused asymmetric motor control.

Behavioral Composite Z-Score

All behavioral outcomes were converted into a Z-score which was compared to the Z-score generated from the sham group. Z-score was calculated by subtracting the mean test score of the control group from the individual test score, divided by the standard deviation of the control group.

For the negative geotaxis z-score data was transformed from the time to turn and time to cross the line results (**Supplementary Figures S1A,B**) to generate z-scores. For the

open field *z*-score data from average time spent in the center of the box (**Supplementary Figures S1C,D**) was used to generate *z*-scores. For the cylinder test *z*-score data from the percentage of left foot touches (**Supplementary Figures S1E,F**) was used to generate *z*-scores. These *Z*-scores were summed across the included tests (negative geotaxis analysis, open field test and cylinder test) to give a final cumulative score as an overall indication of behavioral deficits across both motor control and anxiety-like behavior.

Post-mortem and Brain Processing

On PND 50, following behavioral testing, the rats were culled by lethal inhalation of carbon dioxide, followed by decapitation. Brains were collected and fixed in 10% formalin (Grale Scientific) for 3–4 days, then processed and embedded in paraffin wax. For histological analysis, the embedded brains were coronally sectioned in 6 μm slices.

Histology

Gross Brain Morphology

Gross brain morphology and tissue area were assessed with Haematoxylin and Eosin (H&E, Amber Scientific, Australia). For each animal, triplicate slides over two regions approximately 0.2 and −3.3 mm Bregma were examined and data averaged across groups. Images were acquired by Aperio digital scanning (Leica Biosystems, Germany) and the volume of the left (ipsilateral to the injury) and right (contralateral to the injury) hemisphere were measured using Aperio ImageScope (Leica Biosystems, Germany). For percentage tissue loss, the difference in volume between the contralateral and ipsilateral hemispheres over the contralateral hemisphere volume was calculated, using the following formula [(volume of contralateral-volume of ipsilateral)/volume of contralateral], as previously described (Teo et al., 2017).

Immunohistochemistry

Microglia were identified using ionized calcium-binding adapter molecule 1 (Iba-1; 1:1000, Wako Pure Chemical Industries, Ltd., Osaka, Japan), apoptotic cell death was assessed using activated caspase 3 (Cas3; 1:800, R&D Systems, Minneapolis, MN, United States), myelin was assessed using Myelin Basic Protein (MBP; 1:250, Merck, Darmstadt, Germany), neuronal cell counts were assessed using NeuN (1:1000, Millipore, Burlington, MA, United States) and astrocytes were assessed using glial fibrillary acidic protein (GFAP; 1:400, Sigma-Aldrich, Castle Hill, NSW, Australia).

Briefly, brain sections were dewaxed in alcohol (xylene and ethanol), followed by antigen retrieval in heated citric acid buffer. Endogenous peroxidases were blocked by incubating sections with hydrogen peroxide in 50% methanol/dH_2O. The sections were blocked with serum [Iba1 and MBP: 10% normal goat serum (NGS); GFAP: 5% NGS; Cas3: 5% NGS + 2% Bovine serum albumin (BSA); NeuN: 5% NGS + 1% BSA]. Slides were incubated overnight at 4°C in the specific primary antibody. Sections were exposed to a secondary antibody for 1 h (Iba1 and Cas3: Goat anti-Rabbit IgG, 1:200; Vector Laboratories, Burlingame, CA, United States; MBP: Goat anti-Rat IgG, 1:200; Vector Laboratories, Burlingame, CA, United States; GFAP and NeuN: Goat anti- Mouse IgG, 1:200; Vector Laboratories, Burlingame, CA, United States). Staining was visualized using 3,3-diaminobenzidine (MP Biomedicals, Santa Ana, CA, United States).

Coronal brain sections were imaged using bright field microscopy on the Olympus BX-41 microscope (Olympus, Tokyo, Japan). For immunohistochemical analysis, images were acquired at 400×, with three random non-overlapping fields of view analyzed per region, on two non-adjacent duplicate slides per brain, averaged for each animal. The regions of interest examined included the somatosensory cortex (0.2 mm bregma) and the motor cortex (+3 mm bregma). Cell counts (Iba-1, NeuN, Caspase-3) and densitometry (MBP, GFAP) were preformed using Image J (NIH, Bethesda, MD, United States). Quantification of microglia cell types was achieved by classing the microglia as either resting (ramified, with branching projections protruding from the cell body) or active (amoeboid, no projections seen, cell body is round) (Kettenmann et al., 2011). Aggregates of microglia were observed as dense patches of microglia cell bodies and branching projections. All assessments were conducted on coded slides and images, with the examiner blinded to the experimental groups.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, United States). Statistical significance was set at a *P*-value of <0.05, and all data was presented as the mean the standard error of the mean (SEM). Parametric data was analyzed using a one-way ANOVA with Tukey's *post hoc* analysis. Non-parametric data was analyzed using a Kruskal–Wallis test with Dunn's multiple comparisons *post hoc* analysis.

RESULTS

The Effect of HI Injury and UCB Administration on Behavioral Outcomes

Behavioral test scores from negative geotaxis analysis, open field test and cylinder test, were combined to form a *Z*-score such that overall effects of HI and UCB treatment could be compared relative to sham. Non-transformed behavioral data is included in **Supplementary Figure S1**. Regarding negative geotaxis analysis, which evaluates both motor strength and the vestibular reflex, the HI group was the only cohort to show a positive *z*-score compared to sham, where a positive score is indicative of worse outcomes, but overall there were no significant differences (**Figure 1A**). For the open field test, which assesses anxiety/exploratory behaviors, the same group relationships were observed, but also with no significant differences (**Figure 1B**). The cylinder test assesses forelimb asymmetry, and a significant increase in the *Z*-score in the HI group ($P < 0.05$, **Figure 1C**) was observed when compared to sham, where the UCB treated group showed no difference to sham (**Figure 1C**).

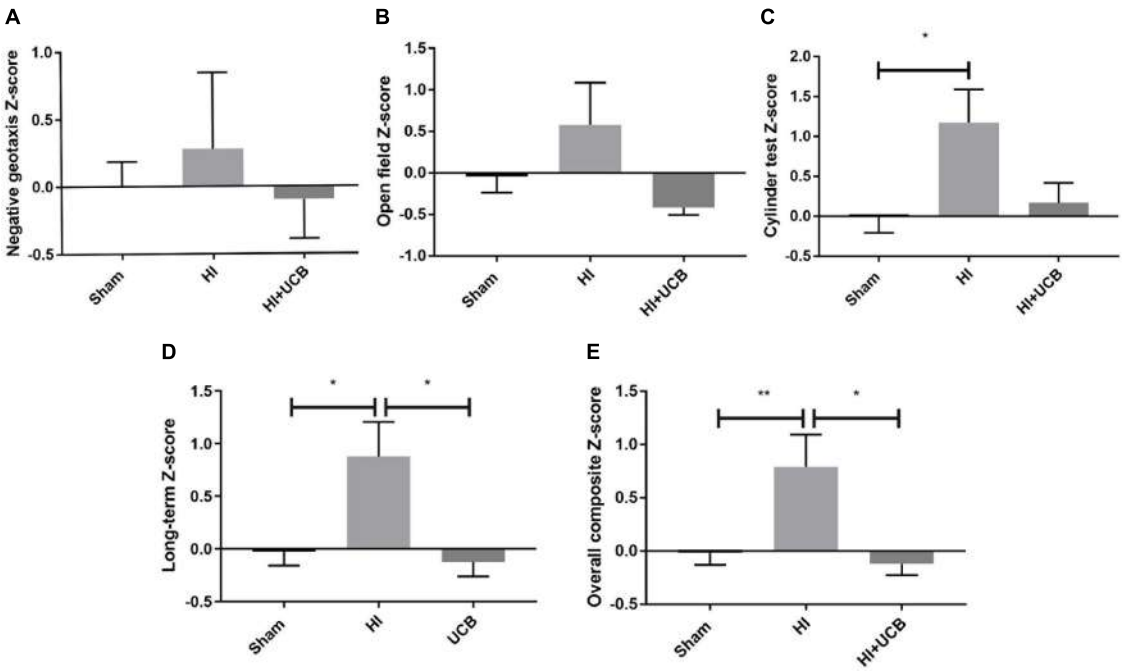


FIGURE 1 | Behavior is impaired by HI injury and is significantly restored following UCB administration. **(A)** *Z*-score of the negative geotaxis analysis at PND 14. **(B)** *Z*-score of overall open field test score from PND 30 and 50. **(C)** *Z*-score of overall cylinder test score from PND 30 and 50. **(D)** A combination of PND 30 and 50 data from the open field test and cylinder test. **(E)** Overall Composite *Z*-score combining data from the negative geotaxis analysis, cylinder test and open field test, from PND 14, 30, and 50 (Data expressed as mean ± SEM, n = 6–19 pups per group, $^{*}P < 0.05$, $^{**}P < 0.01$).

In order to ascertain the long term and overall behavioral burden, a long-term composite behavior score was calculated by combining data from D30 and D50 behavioral tests (**Figure 1D**) and an overall behavioral burden score was calculated by combining each of the individual test *Z*-scores for all behavioral tests (**Figure 1E**). This overall behavioral burden score incorporated motor strength, anxiety/exploratory behaviors and limb asymmetry. For both the long-term composite *z*-score and the overall behavioral burden, we observed a significant impairment following HI injury when compared to sham ($P < 0.05$ **Figure 1D**; $P < 0.01$ **Figure 1E**, respectively). Administration of UCB significantly reduced long-term outcomes ($P < 0.05$, **Figure 1D**) and the overall behavioral burden ($P < 0.05$, **Figure 1E**) compared to HI.

Effect of HI Injury and UCB Administration on Tissue Loss and Apoptotic Cell Death

The HI brain injury and UCB treatment had no effect on body weight over the course of the experiment, compared to sham (data not shown). At PND 50 (43 days post HI) a significant decrease in brain weight in both the HI and UCB groups ($P < 0.05$; **Figure 2A**), was observed when compared to the sham group. This brain injury was further confirmed by the substantial loss of brain tissue in the HI and UCB group, with significant tissue loss in the left hemisphere of the UCB group ($P < 0.05$; **Figures 2B,D,E**), compared to sham (**Figure 2C**). In addition to these morphological changes, we also saw a trend toward an increase in apoptosis in the somatosensory cortex in the HI ($P = 0.06$, **Figures 2F,I**) and a significant increase in the UCB group ($P < 0.05$; **Figures 2F,J**) compared to sham (**Figure 2H**). There was no significant change in apoptosis between groups in the motor cortex (**Figure 2G**). There was no significant change in the number of neurons, seen by NeuN immunohistochemistry, and the white matter density between groups in the somatosensory cortex and motor cortex, seen by MBP immunohistochemistry (data not shown).

Inflammatory Response Following HI Injury and UCB Cell Treatment

The inflammatory response following HI injury involves activation and proliferation of several immune cell types, including microglia (Liu and McCullough, 2013) and astrocytes and while this was thought to occur acutely, there is emerging evidence that the tertiary phase of inflammation plays a role in long-term outcomes (Fleiss and Gressens, 2012). Our results show that administration of UCB cells resulted in a significant increase in activated microglia (Iba1-positive staining) in the somatosensory cortex when compared to sham ($P < 0.05$; **Figure 3A**) and this increase was not seen in the HI group. There was no difference observed in resting microglia in the somatosensory cortex between any groups (**Figure 3B**). When looking at the motor cortex, there was no significant difference in the number of activated or resting microglia in this area (**Figures 3C,D**). In addition, there was no

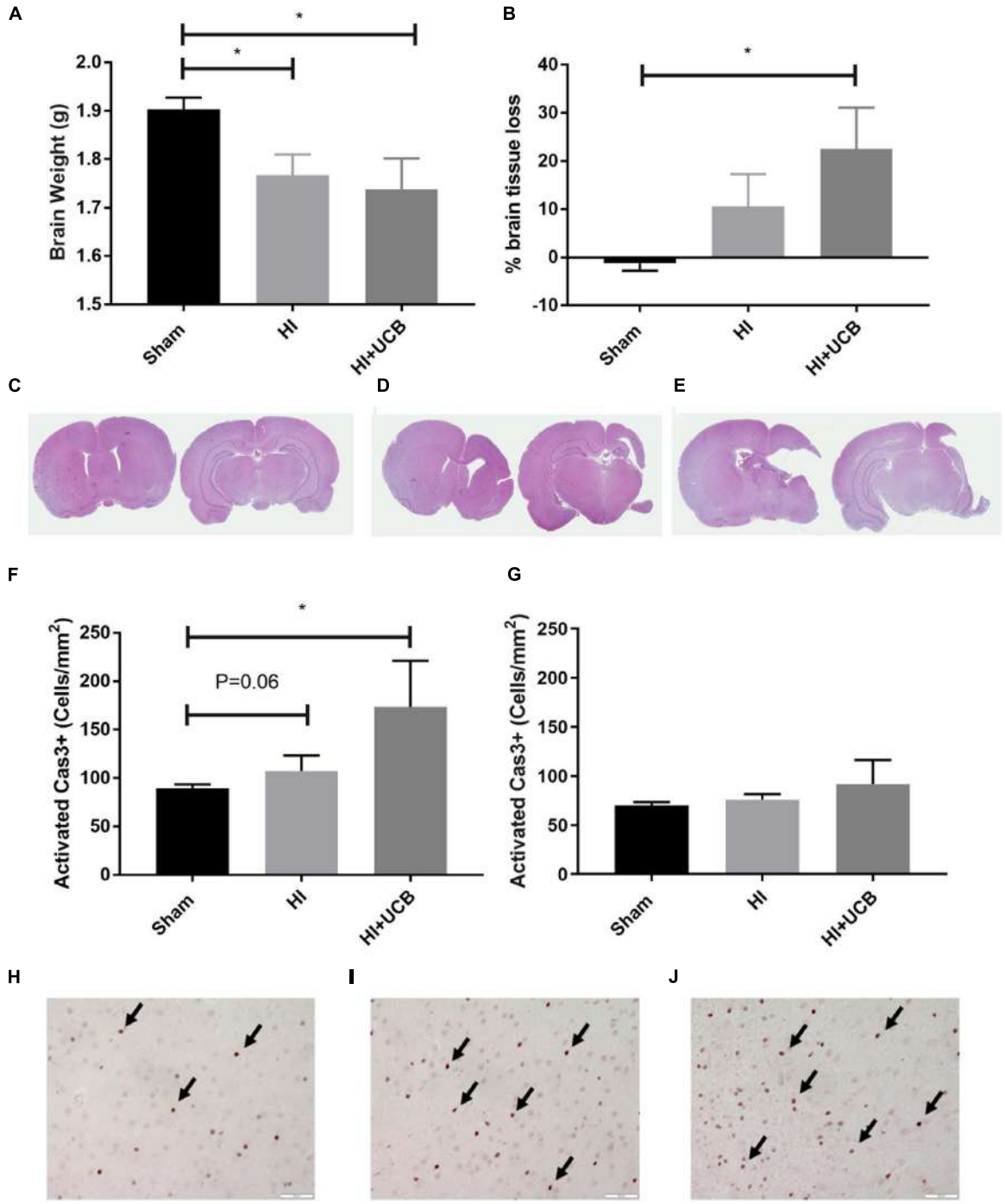


FIGURE 2 | HI injury causes tissue loss and cell death which is exacerbated by UCB administration. **(A)** Brain weights. **(B)** Percentage of left hemisphere volume of the total brain. Representative images of haematoxylin and eosin stained brain sections used for volumetric analysis for sham **(C)**, HI **(D)**, and UCB **(E)** groups. **(F)** Number of activated Caspase 3 positive cells in the somatosensory cortex of the brain. **(G)** Number of activated Caspase 3 positive cells in the motor cortex of the brain. Representative images of Cas3 positive cells (indicated by black arrows) in the somatosensory cortex of the brain in sham **(H)**, HI **(I)**, and UCB **(J)** animals (Data expressed as mean ± SEM, n = 6–19 pups per group, $^*P < 0.05$, Scale bar = 50 μm).

significant difference in the number of microglia aggregates between groups in both the somatosensory and motor cortex (Data not shown).

Following HI injury and UCB treatment, we observed no significant changes in astrogliosis, as indicated by GFAP staining, for either astrocyte cell number or percentage coverage in both

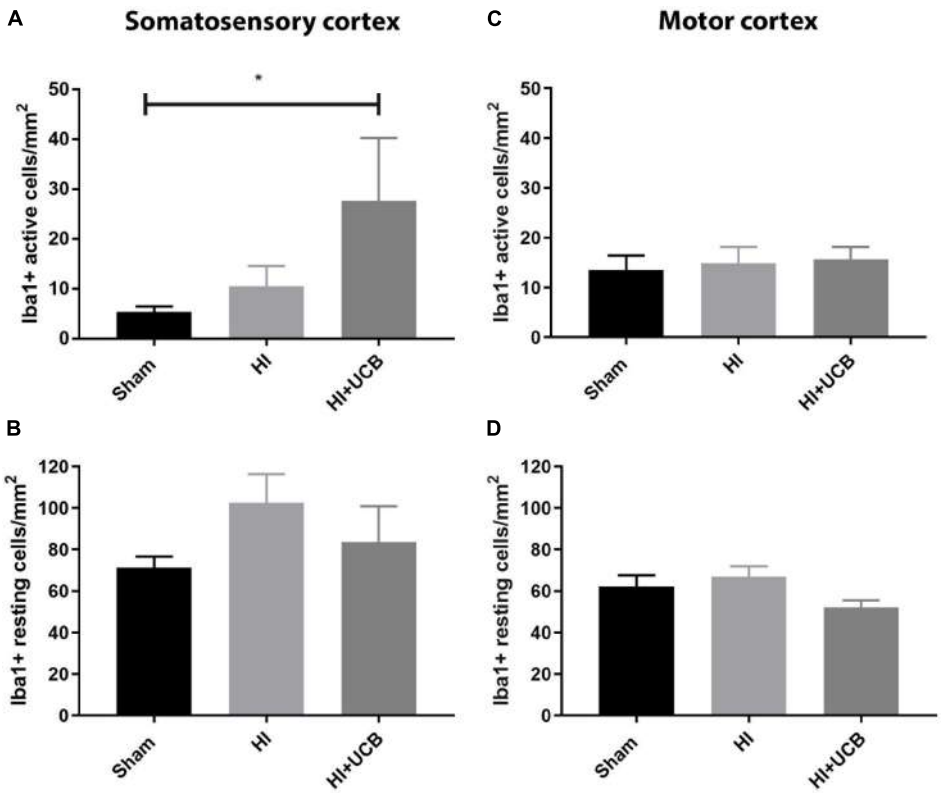


FIGURE 3 | Umbilical cord blood cells delivery increases microglia activation in the somatosensory cortex. **(A)** Number of activated Iba1 positive cells in the somatosensory cortex (SSC) in the brain. **(B)** Number of resting Iba1 positive cells in the SSC. **(C)** Number of activated Iba1 positive cells in the motor cortex (MC) of the brain. **(D)** Number of resting Iba1 positive cells in the MC (Data expressed as mean ± SEM, n = 6–19 pups per group, $^*P < 0.05$).

the somatosensory cortex (**Figures 4A,B**, respectively) and motor cortex (**Figures 4C,D**, respectively).

DISCUSSION

In this study, we investigated the long-term effects of UCB administration for term HI brain injury, particularly regarding behavior, and neuropathology. UCB therapy for HIE and CP has been explored in many preclinical studies and clinical trials and has been shown to improve short-term motor function, muscle strength and cognitive abilities (Kang et al., 2015; Romanov et al., 2015; Jensen and Hamelmann, 2016). In addition, we have previously shown that UCB reduces neuroinflammation and short-term behavioral outcomes in animal models (Aridas et al., 2016; McDonald et al., 2018); however, the long-term effects of UCB administration, particularly regarding behavioral outcomes, have not been widely investigated. It is important that these aspects are investigated to ensure that UCB is not only an effective treatment, but that it is also a sustainable treatment that will show benefits throughout the patient's life.

This study shows for the first time that UCB cell treatment significantly improves behavioral outcomes in the long-term. However, contrary to these positive behavioral effects, a single dose of UCB cells 24 h after a HI insult did not mitigate neuropathology. We provide evidence that UCB cell treatment may even exacerbate injury in particular regions, such as the somatosensory cortex where we observed increased tissue loss, apoptosis and microglial activation compared to the sham group. Nevertheless, this potential exacerbation of injury appeared to be region specific and importantly, was not significantly increased compared to the HI group.

CP is an umbrella term referring to a group of permanent movement disorders with associated deficits in cognition, perception and behavior (Novak et al., 2012). These commonly seen impairments highlight the need to investigate a therapy that will not only target injurious mechanisms, such as inflammation, and repair brain injury but will also improve behavioral outcomes. In this study, we standardized and transformed individual behavior scores into Z-scores, which were then combined to create an overall behavioral burden score. This composite scoring method is standard practice in neurological clinical trials to evaluate changes in motor ability over time in patients with multiple sclerosis (Fischer et al., 1999) and Parkinson's disease (Stocchi et al., 2018). Our previous study analyzed short-term behavioral deficits following HI and a single dose of UCB cells and found that 7 days after injury, UCB cells resolved impairments seen in the negative geotaxis test, indicating improved muscle strength and coordination (McDonald et al., 2018). In this study, we

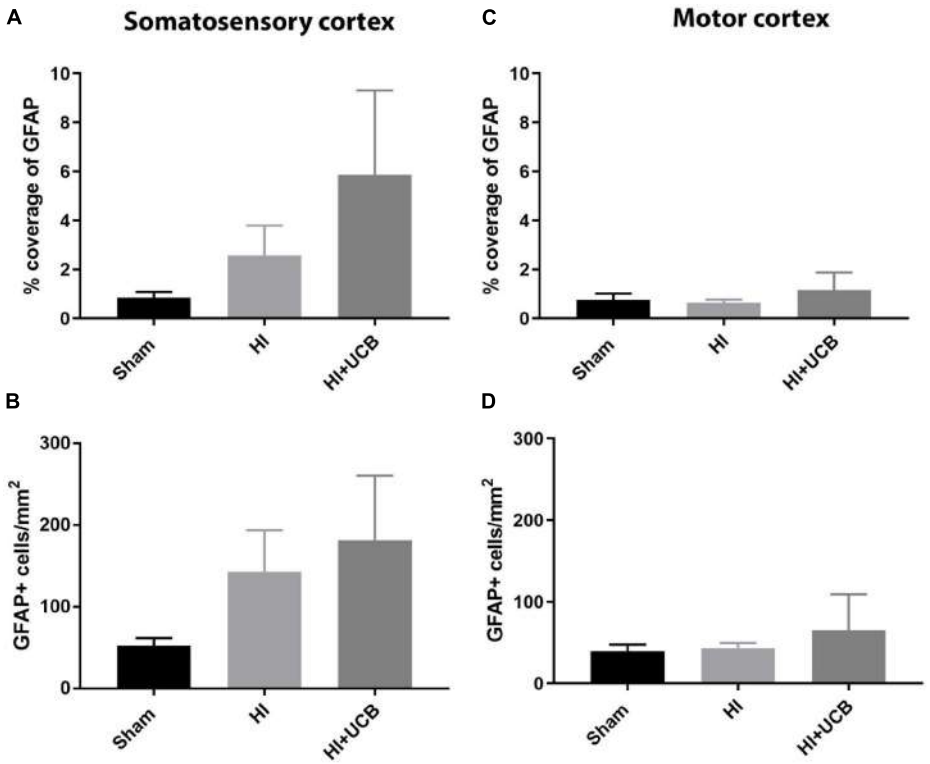


FIGURE 4 | Effects of HI injury and UCB administration on astrogliosis in the somatosensory and motor cortex. **(A)** Percentage coverage of GFAP in the somatosensory cortex (SSC) in the brain. **(B)** Number of GFAP positive cells in the SSC. **(C)** Percentage coverage of GFAP in the motor cortex (MC) of the brain. **(D)** Number of GFAP positive cells in the MC (Data expressed as mean ± SEM, n = 6–19 pups per group).

investigated the overall effects on behavior by examining the functional outcomes at 7, 23, and 43 days post injury and also the long-term subset at 23 and 43 days post injury. These time points were chosen as they correlate to the toddler stage (Colver et al., 2014), pre-pubescent and pubescent stages, respectively, in human development (Bolton et al., 2015). It is important that we look at these timepoints as motor and behavioral deficits associated with CP are often undetected until 12–24 months of age (Rosenbaum, 2003), but persist throughout an individual's life. This study showed that forelimb asymmetry was present after HI injury, which resulted in preference of the left forelimb. UCB administration reduced this asymmetry, where treated animals were mostly supporting on both forelimbs. In addition, the increased composite Z-score for the HI group reveals poor behavioral outcomes and highlights deficits in vestibular reflex, motor strength, coordination, anxiety behaviors, exploratory behaviors and symmetric limb use. UCB administration significantly reversed these behavioral deficits compared to the HI group, thus returning the Z-score to sham level. These results indicate that early administration of a single dose of UCB cells effectively treats motor and behavioral deficits following HI brain injury. These results are consistent with current clinical trial data to show that administration of UCB cells to children with established CP can improve motor function, strength and cognition (Kang et al., 2015; Jensen and Hamelmann, 2016). Given the lack of correlation between our behavioral outcomes and neuropathology, it may be worth considering other neurological tests that may be more relevant in future studies. These could include adhesive removal test and novel object recognition (Patel et al., 2015), however, it has been shown that spontaneous recovery in tests such as adhesive removal test is possible, while deficits in novel object recognition appear to be sustained long-term (Liguz-Lecznar et al., 2014).

Despite these beneficial effects of UCB in restoring motor and behavioral deficits, this improvement was not associated with a decrease in brain injury. We showed that HI significantly reduced brain weight and led to tissue loss and a trend toward increased apoptosis in the somatosensory cortex. Treatment with UCB did not restore brain weight or reduce tissue loss or apoptosis and may even exacerbate injury in specific regions, however, this was not significant compared to HI. Our previous study showed that, when UCB treatment was assessed 6 days after administration, UCB cells recovered tissue loss and reduced apoptosis (McDonald et al., 2018). Together our results indicate a single dose of UCB cells may be able to mediate injury early on, but this effect may not be sustained and as such increased doses and timing of administration needs to be examined. The development of HIE and cell degeneration after a severe asphyxic insult at birth is known to progress over time and is traditionally divided into different phases; the *primary*, *latent*, *secondary*, and

tertiary. The primary phase of injury, which hypothermia targets, describes the period at which the injury is sustained (Gunn and Thoresen, 2006) and is shortly followed by the latent phase at 6–15 h after injury (Gunn and Thoresen, 2006). The secondary phase of injury is the point at which the majority of the damage is thought to occur, due to the acute upregulation of inflammatory pathways, oedema formation, cell death and breakdown of the blood–brain barrier (Gunn and Thoresen, 2006; Kaur et al., 2006; Thornton et al., 2012). This phase has been shown to last as little as 6 h and as long as 3 days in humans, and is a critical window of opportunity for treatment of HIE (Drury et al., 2010). This is the phase that we chose to target in this study by administering UCB cells at 24 h. The tertiary phase of injury can last weeks to years following the initial insult (Fleiss and Gressens, 2012), and is characterized by persisting brain lactic acidosis, epigenetic changes, persistent inflammation and aberrant gliosis, which may inhibit remyelination and regrowth of axons (Thornton et al., 2012; Hassell et al., 2015). One of the key contributing processes to injury during this phase is persistent inflammation. This inflammation has been shown to impede proliferation of cells in the brain, as well as synaptogenesis (Hassell et al., 2015). These prolonged injurious mechanisms could indicate that only targeting the secondary phase of injury with UCB cells is not sufficient for long term repair of the injured brain, and additional doses of UCB cells during the tertiary phase may be required for long-term neuropathological improvements.

Neuroinflammation plays a crucial role in mediating brain injury following a HI event, and is known to persist for many years after the initial injury (Fleiss and Gressens, 2012; Liu and McCullough, 2013). Despite the importance of microglia in the inflammatory response, there have been very few long-term studies looking at long-term microglial activation in any animal models or human cases of HIE (Fleiss and Gressens, 2012), thus we do not know how the microglia act at these long-term time points, or what their morphology might be. In this study, we showed that 43 days after injury, microglial activation remained increased compared to sham in the somatosensory cortex following UCB administration. This increase was not seen in the motor cortex, and there were no differences in resting microglia in either region. The presence of activated microglia for an extended period after injury indicates that there is either ongoing inflammation or repair; thus, microglia may be either in their classically activated M1 state, or in their alternatively activated M2 state. M1 microglia are pro-inflammatory and are activated in response to brain injury or inflammation (Lull and Block, 2010; Cherry et al., 2014). They are classified by their release of pro-inflammatory cytokines, reactive oxygen species and reactive nitrogen species (MacMicking et al., 1997; Cherry et al., 2014). M2 microglia are anti-inflammatory microglia, and express receptors that work to downregulate the inflammatory response and initiate tissue repair (Varin and Gordon, 2009). We hypothesize that the increase in activated microglia in the somatosensory cortex following UCB administration may be contributed by M2 activated microglia which could be working to activate reparative mechanisms. However, given we also observe increased apoptosis and tissue loss in the same region, this may be incorrect. Further investigation is needed to ascertain the long-term role of microglia following UCB cell treatment.

Our results demonstrate that there was an overall improvement in long-term behavioral outcomes following UCB administration, but this was not reflected in improvement in brain pathology, however, it also didn't exacerbate the injury compared to HI. In our study, the infarct was primarily localized to the somatosensory regions of the brain as is common in this model of injury. The damage to the motor cortex region of the brain was less severe, which may reflect improved coordination, motor control and learning abilities (Kawai et al., 2015), that were assessed with our behavioral tests, which could explain the disparity between pathological and behavioral outcomes. Another reason for this difference, may be that a single dose of UCB may have been sufficient to improve neuronal connectivity, which led to improvement in behavior. In a recent human study investigating the potential of UCB cells for Autism (Carpenter et al., 2019), they demonstrated that single infusion of UCB improved white matter connectivity as shown with MRI. In the context of our model, where there is a significant degree of injury, it may be that a single dose can improve behavioral outcomes, possibly through improvements in connectivity, but is not enough to improve neuropathology outcomes. Further optimisation of this UCB cell therapy is needed. In particular, it is important to explore different dosing regimens and test multiple doses of cells in order for this therapy to be appropriate for use in a human clinical cohort. In light of the new evidence for improved connectivity, MRI analysis of connectivity should also be incorporated into these preclinical investigations.

Limitations of this study include the variability that occurs with the HI rodent model. Despite there being clear injury in the HI group, shown by a decrease in brain weight, there was large variation in the size of the infarct between individual pups in both the HI and UCB groups. This variability was also obvious in the UCB group that had large variations for both microglial and astrocyte analysis. This may be overcome by including more animals to examine these indices in future long-term studies. It is understood that cell populations found within UCB are not always consistent between samples with different proportions of stem/progenitor cells. While we did try to reduce the impact of donor variation by pooling cells from multiple UCB donors before administration of UCB, this is an additional limitation that may have added to variation in the UCB treatment group observed in some of our results. In future studies, it is also important to assess adjuvant cell therapies alongside hypothermia, as hypothermia is the current standard of care in cases of term HIE. Studies by Park et al. (2015) have shown that a combination therapy of hypothermia and UCB-derived mesenchymal stem cells was more effective at attenuating injury, as well as improving behavioral function, than either therapy alone.

CONCLUSION

In summary, we show that early administration of a single dose of UCB cells reduces long-term behavioral deficits following HI.

We also show that, when a single dose of human UCB cells are administered 24 h after injury, UCB cells are unable to reduce indices of neuropathology in all regions of the brain, despite improvements in behavioral outcomes. This study supports the use of UCB as a treatment for neonatal HI brain injury and shows that further investigation into long-term outcomes is needed. Given that a single dose of UCB cells does not prevent tissue injury, it is clear that multiple doses of UCB cells should be considered at different time points, particularly the tertiary phase where ingoing inflammation may still be contributing to injury. By doing this, UCB cells will be able to target both the secondary and tertiary phase of injury, thus maximizing the therapeutic efficacy of UCB cells for neonatal HI brain injury.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

TP designed, performed, and analyzed all experiments and was a major contributor in writing the manuscript. AS, JM, MP, and YP performed UCB isolations and animal experiments. JL, NJ, GJ, MF, and SM designed experiments and interpreted the data. CM developed idea, sought funding for project, designed and performed experiments, and interpreted data. All authors contributed to the writing, editing, and final approval of the manuscript.

FUNDING

This work was funded by Inner Wheel Australia and the Victorian Government's Operational Infrastructure Support Program. CM was supported by a National Health and Medical Research Council and Cerebral Palsy Alliance Australia Early Career Fellowship. SM was supported by a National Health and Medical Research Council Senior Research Fellowship.

ACKNOWLEDGMENTS

The authors would like to acknowledge the assistance of the Monash Health and Translation Precinct Histology Platform for their assistance with experiments performed in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00283/full#supplementary-material

REFERENCES

Ahearne, C. E., Boylan, G. B., and Murray, D. M. (2016). Short and long term prognosis in perinatal asphyxia: an update. *World J. Clin. Pediatr.* 5, 67–74. doi: 10.5409/wjcp.v5.i1.67

Aridas, J. D., McDonald, C. A., Paton, M. C., Yawno, T., Sutherland, A. E., Nitsos, I., et al. (2016). Cord blood mononuclear cells prevent neuronal apoptosis in response to perinatal asphyxia in the newborn lamb. *J. Physiol.* 594, 1421–1435. doi: 10.1113/JP271104

Aridas, J. D., Yawno, T., Sutherland, A. E., Nitsos, I., Ditchfield, M., Wong, F. Y., et al. (2014). Detecting brain injury in neonatal hypoxic ischemic encephalopathy: closing the gap between experimental and clinical research. *Exp. Neurol.* 261, 281–290. doi: 10.1016/j.expneurol.2014.07.009

Bennet, L., Tan, S., Van, den Heuij L, Derrick, M., Groenendaal, F., van, Bel F, et al. (2012). Cell therapy for neonatal hypoxia–ischemia and cerebral palsy. *Ann. Neurol.* 71, 589–600. doi: 10.1002/ana.22670

Bolton, M. M., Heaney, C. F., Murtishaw, A. S., Sabbagh, J. J., Magcalas, C. M., and Kinney, J. W. (2015). Postnatal alterations in GABAB receptor tone produce sensorimotor gating deficits and protein level differences in adulthood. *Int. J. Dev. Neurosci.* 41, 17–27. doi: 10.1016/j.ijdevneu.2014.10.001

Carpenter, K. L. H., Samantha, M., Catherine, T., and Lyon, W. C. (2019). White matter tract changes associated with clinical improvement in an open-label trial assessing autologous umbilical cord blood for treatment of young children with autism. *Stem Cells Transl. Med.* 8, 138–147. doi: 10.1002/sctm.18-0251

Cerio, F. G. D., Lara-Celador, I., Antonia, A., and Enrique, H. (2013). Neuroprotective therapies after perinatal hypoxic-ischemic brain injury. *Brain Sci.* 3, 191–214. doi: 10.3390/brainsci3010191

Cherry, J. D., Olschowka, J. A., and O'Banion, M. K. (2014). Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J. Neuroinflamm.* 11, 1–15. doi: 10.1186/1742-2094-11-98

Chiang, M.-C., Jong, Y.-J., and Lin, C.-H. (2017). Therapeutic hypothermia for neonates with hypoxic ischemic encephalopathy. *Pediatr. Neonatol.* 58, 475–483. doi: 10.1016/j.pedneo.2016.11.001

Colver, A., Fairhurst, C., and Pharoah, P. O. D. (2014). Cerebral palsy. *Lancet* 383, 1240–1249. doi: 10.1016/S0140-6736(13)61835-8

Cotten, C. M., Murtha, A. P., Goldberg, R. N., Grotegut, C. A., Smith, P. B., Goldstein, R. F., et al. (2014). Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *J. Pediatr.* 164:973-979.e1. doi: 10.1016/j.jpeds.2013.11.036

Drury, P. P., Bennet, L., and Gunn, A. J. (2010). Mechanisms of hypothermic neuroprotection. *Semin. Fetal Neonatal Med.* 15, 287–292. doi: 10.1016/j.siny.2010.05.005

Ferrari, D. C., Nesic, O., and Perez-polo, J. R. (2010). Perspectives on neonatal hypoxia/ischemia-induced edema formation. *Neurochem. Res.* 35, 1957–1965. doi: 10.1007/s11064-010-0308-y

Fischer, J. S., Richard, R., Gary, R. C., and Reingold, S. C. (1999). The Multiple Sclerosis Functional Composite measure (MSFC): an integrated approach to MS clinical outcome assessment. *Mult. Scler. J.* 5, 244–250. doi: 10.1177/135245859900500409

Fleiss, B., and Gressens, P. (2012). Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? *Lancet Neurol.* 11, 556–566. doi: 10.1016/S1474-4422(12)70058-3

Gould, T. D., Dao, D. T., and Kovacsics, C. E. (2009). "The Open Field Test," in *Mood and Anxiety Related Phenotypes in Mice*, ed. T. D. Gould (Berlin: Springer), 1–20. doi: 10.1007/978-1-60761-303-9

Gunn, A. J., and Thoresen, M. (2006). Hypothermic neuroprotection. *NeuroRx* 3, 154–169. doi: 10.1016/j.nurx.2006.01.007

Hassell, K. J., Ezzati, M., Alonso-Alconada, D., Hausenloy, D. J., and Robertson, N. J. (2015). New horizons for newborn brain protection: enhancing endogenous neuroprotection. *Arch. Dis. Child.* 100, F541–F552. doi: 10.1136/archdischild-2014-306284

Jaing, T.-H. (2014). Umbilical cord blood: a trustworthy source of multipotent stem cells for regenerative medicine. *Cell Transplant.* 23, 493–496. doi: 10.3727/096368914X678300

Jensen, A., and Hamelmann, E. (2016). First autologous cord blood therapy for pediatric ischemic stroke and cerebral palsy caused by cephalic molding during

birth: individual treatment with mononuclear cells. *Case Rep. Transplant.* 2016:1717426. doi: 10.1155/2016/1717426

Kang, M., Min, K., Jang, J., Kim, S. C., Kang, M. S., Jang, S. J., et al. (2015). Involvement of immune responses in the efficacy of cord blood cell therapy for cerebral palsy. *Stem Cells Dev.* 24, 2259–2268. doi: 10.1089/scd.2015.0074

Kaur, C., Viswanathan, S., Lin, S. A., and Alamelu, S. (2006). Hypoxic damage to the periventricular white matter in neonatal brain: role of vascular endothelial growth factor, nitric oxide and excitotoxicity. *J. Neurochem.* 98, 1200–1216. doi: 10.1111/j.1471-4159.2006.03964.x

Kawai, R., Markman, T., Poddar, R., Ko, R., Fantana, A. L., Dhawale, A. K., et al. (2015). Motor cortex is required for learning but not for executing a motor skill. *Neuron* 86, 800–812. doi: 10.1016/j.neuron.2015.03.024

Kettenmann, H., Hanisch, U. K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. *Physiol. Rev.* 91, 461–553. doi: 10.1152/physrev.00011.2010

Knutsen, A. P., and Wall, D. A. (2000). Umbilical cord blood transplantation in severe T-cell immunodeficiency disorders: two-year experience. *J. Clin. Immunol.* 20, 466–476. doi: 10.1023/A:1026463900925

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., et al. (2016). Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells. *Exp. Neurol.* 283, 179–187. doi: 10.1016/j.expneurol.2016.06.017

Liguz-Lecznar, M., Zakrzewska, R., Daniszewska, K., and Kossut, M. (2014). Functional assessment of sensory functions after photothrombotic stroke in the barrel field of mice. *Behav. Brain Res.* 261, 202–209. doi: 10.1016/j.bbr.2013.12.027

Liu, F., and McCullough, L. D. (2013). Inflammatory responses in hypoxic ischemic encephalopathy. *Acta Pharmacol. Sin.* 34, 1121–1130. doi: 10.1038/aps.2013.89

Lull, M. E., and Block, M. L. (2010). Microglial activation and chronic neurodegeneration. *Neurotherapeutics* 7, 354–365. doi: 10.1016/j.nurt.2010.05.014

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15, 323–350. doi: 10.1146/annurev.immunol.15.1.323

Maeda, A., Schwarz, A., Kernebeck, K., Gross, N., Aragane, Y., Peritt, D., et al. (2005). Intravenous infusion of syngeneic apoptotic cells by photopheresis induces antigen-specific regulatory T cells. *J. Immunol.* 174, 5968–5976. doi: 10.4049/jimmunol.174.10.5968

McDonald, C. A., Penny, T. R., Paton, M. C. B., Sutherland, A. E., Nekkanti, L., Yawno, T., et al. (2018). ffects of umbilical cord blood cells, and subtypes, to reduce neuroinflammation following perinatal hypoxic-ischaemic brain injury. *J. Neuroinflamm.* 15:47. doi: 10.1186/s12974-018-1089-5

Min, K., Song, J., Kang, J. Y., Ko, J., Ryu, J. S., Kang, M. S., et al. (2013). Umbilical cord blood therapy potentiated with erythropoietin for children with cerebral palsy: a double-blind, randomized, placebo-controlled trial. *Stem Cells* 31, 581–591. doi: 10.1002/stem.1304

Novak, I., Hines, M., Goldsmith, S., and Barclay, R. (2012). Clinical prognostic messages from a systematic review on cerebral palsy. *Pediatrics* 130, e1285–e1312. doi: 10.1542/peds.2012-0924

Park, W. S., Sung, S. I., Ahn, S. Y., Yoo, H. S., Sung, D. K., Im, G. H., et al. (2015). Hypothermia augments neuroprotective activity of mesenchymal stem cells for neonatal hypoxic-ischemic encephalopathy. *PLoS One* 10:e0120893. doi: 10.1371/journal.pone.0120893

Patel, S. D., Leslie, P., Amber, C., Alexandra, H., Samuel, P., Eric, A., et al. (2015). Therapeutic hypothermia and hypoxia-ischemia in the term-equivalent neonatal rat: characterization of a translational preclinical model. *Pediatr. Res.* 78, 264–271. doi: 10.1038/pr.2015.100

Romanov, Y. A., Tarakanov, O. P., Radaev, S. M., Dugina, T. N., Ryaskina, S. S., Darevskaya, A. N., et al. (2015). Human allogeneic AB0/Rh-identical umbilical cord blood cells in the treatment of juvenile patients with cerebral palsy. *Cytotherapy* 17, 969–978. doi: 10.1016/j.jcyt.2015.02.010

Rosenbaum, P. (2003). Cerebral palsy: what parents and doctors want to know. *Br. Med. J.* 326, 970–974. doi: 10.1136/bmj.326.7396.970

Stocchi, F., Fabiana, G. R., Kallol, R. C., and Anders, J. (2018). The Parkinson's disease composite scale: results of the first validation study. *Eur. J. Neurol.* 25, 503–511. doi: 10.1111/ene.13529

Teo, J. D., Morris, M. J., and Jones, N. M. (2017). Maternal obesity increases inflammation and exacerbates damage following neonatal hypoxic-ischaemic brain injury in rats. *Brain Behav. Immun.* 63, 186–196. doi: 10.1016/j.bbi.2016.10.010

Thornton, C., Catherine, I. R., Anton, K., and Yasuka, M. (2012). Molecular mechanisms of neonatal brain injury. *Neurol. Res. Int.* 2012:16. doi: 10.1155/2012/506320

Torelli, G. F., Maggio, R., Peragine, N., Chiaretti, S., De Propris, M. S., Lucarelli, B., et al. (2012). unctional analysis and gene expression profile of umbilical cord blood regulatory T cells. *Ann. Hematol.* 91, 155–161. doi: 10.1007/s00277-011-1288-y

Vannucci, R. C. (2000). Hypoxic-ischemic encephalopathy. *Am. J. Perinatol.* 17, 113–120. doi: 10.1055/s-2000-9293

Varin, A., and Gordon, S. (2009). Alternative activation of macrophages: immune function and cellular biology. *Immunobiology* 214, 630–641. doi: 10.1016/j.imbio.2008.11.009

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Penny, Sutherland, Mihelakis, Paton, Pham, Lee, Jones, Jenkin, Fahey, Miller and McDonald. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 26 March 2019
doi: 10.3389/fphys.2019.00306

Evidence for Sexual Dimorphism in the Response to TLR3 Activation in the Developing Neonatal Mouse Brain: A Pilot Study

Raul Chavez-Valdez[1], Amin Mottahedin[2], Linnea Stridh[2], Tracylyn R. Yellowhair[1,3], Lauren L. Jantzie[1,3], Frances J. Northington[1] and Carina Mallard[2,4]*

[1] Division of Neonatal-Perinatal Medicine, Department of Pediatrics, Johns Hopkins School of Medicine, Baltimore, MD, United States, [2] Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [3] Department of Pediatrics and Department of Neurosciences, The University of New Mexico, Albuquerque, NM, United States, [4] Henan Key Laboratory of Child Brain Injury, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Charles Evans Wood, University of Florida, United States

Reviewed by:
Gavin John Clowry, Newcastle University, United Kingdom
Alistair Jan Gunn, The University of Auckland, New Zealand
Clyde Jason Wright, University of Colorado Denver, United States

***Correspondence:**
Raul Chavez-Valdez
rchavez2@jhmi.edu

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *20 November 2018*
Accepted: *07 March 2019*
Published: *26 March 2019*

Citation:
Chavez-Valdez R, Mottahedin A, Stridh L, Yellowhair TR, Jantzie LL, Northington FJ and Mallard C (2019) Evidence for Sexual Dimorphism in the Response to TLR3 Activation in the Developing Neonatal Mouse Brain: A Pilot Study. Front. Physiol. 10:306. doi: 10.3389/fphys.2019.00306

Toll-like receptor (TLR)3 activation during the neonatal period produces responses linked to the origins of neuropsychiatric disorders. Although there is sexual dimorphism in neuropsychiatric disorders, it is unknown if brain responses to TLR3 activation are sex-specific. We hypothesized that poly I:C in a post-natal day (P)8 model induces a sexually dimorphic inflammatory responses. C57BL6 mice received intraperitoneal injection of poly I:C (10 mg/kg) or vehicle [normal saline (NS)] at P8. Pups were killed at 6 or 14 h for caspase 3 and 8 activity assays, NFkB ELISA, IRF3, AP1, and GFAP western blotting and cytokines/chemokines gene expression real time qRT-PCR (4–6/group). A second group of pups were killed at 24 h (P9) or 7 days (P15) after poly I:C to assess astrocytic (GFAP) and microglia (Iba1) activation in the hippocampus, thalamus and cortex using immunohistochemistry, and gene and protein expression of cytokines/chemokines using real time RT-PCR and MSD, respectively (4–6/group). Non-parametric analysis was applied. Six hours after poly I:C, caspase-3 and -8 activities in cytosolic fractions were 1.6 and 2.8-fold higher in poly I:C-treated than in NS-treated female mice, respectively, while gene expressions of pro-inflammatory cytokines were upregulated in both sexes. After poly I:C, IRF3 nuclear translocation occurred earlier (6 h) in female mice and later (14 h) in male mice. At 14 h after poly I:C, only male mice also had increased nuclear NFκB levels (88%, $p < 0.001$) and GFAP expression coinciding with persistent IL-6 and FAS gene upregulation (110 and 77%, respectively; $p < 0.001$) and IL-10 gene downregulation (−42%, $p < 0.05$). At 24 h after poly I:C, IL-1β, CXCL-10, TNF-α, and MCP-1 were similarly increased in both sexes but at 7 days after exposure, CXCL-10 and INFγ were increased and IL-10 was decreased only in female mice. Accordingly, microglial activation persisted at 7 days after poly I:C in the hippocampus, thalamus and cortex of female mice. This preliminary study suggests that TLR3 activation may produce in the developing neonatal mouse brain a sexually

dimorphic response with early activation of caspase-dependent pathways in female mice, activation of inflammatory cascades in both sexes, which then persists in female mice. Further well-powered studies are essential to confirm these sex-specific findings.

Keywords: poly I:C, inflammation, caspase, cytokines, astroglia, microglia, endoplasmic reticulum stress

INTRODUCTION

Toll-like receptors (TLRs) are a family of innate immune system receptors that react to both microbial stimulation and to molecules released upon tissue injury. TLR 3 modulates neuronal proliferation, differentiation, and axonal growth but also cell death in the developing brain (Cameron et al., 2007; Lathia et al., 2008; Sun et al., 2011). Neurons and glia cells express a broad variety of intracellular TLRs, including TLR3 in astrocytes and microglia (Bsibsi et al., 2002). In post-mortem specimens of preterm infants suffering periventricular white matter injury secondary to infectious or non-infectious inflammation, perinuclear TLR3 expression predominates in neurons and astrocytes (Vontell et al., 2013). Additionally, TLR3 activation increases the vulnerability to perinatal brain injury in neonatal mice (Stridh et al., 2013). In pathological conditions, TLR3 is activated by viral double stranded RNA or by endogenous ligands such as danger-associated molecular patterns (DAMPs) released from dying cells (Beg, 2002; Kariko et al., 2004). Following TLR3 activation, neuronal death may proceed via: (i) apoptosis with release of apoptotic bodies containing DNA fragments, or (ii) necrosis (or necrosis-like) with release of free suspended dead cell materials rich in DAMPs (Blasius and Beutler, 2010). In the injured developing brain, activated astrocytes and microglia engulf DAMPs forming intracellular vesicles, which fuse with TLR3-cointaing acidic endosomes (Rossi and Volterra, 2009; Ransohoff and Brown, 2012; Wakida et al., 2018). In astrocytes, TLR3 activation initiates a cytosolic TRIF-mediated cascade leading to translocation of NFκB, IRF3 and/or AP-1 to the nucleus upregulating transcription of pro-inflammatory mediators, which may perpetuate microglia activation and extend neuronal injury (Fitzgerald et al., 2003; Pobezinskaya et al., 2008).

Like most intracellular TLRs, TLR3 is a resident of the endoplasmic reticulum (ER) and traffics after uncoupling from chaperone proteins (e.g., GRP94) to the endosomal membrane (Blasius and Beutler, 2010). While chaperone proteins are essential for proper folding of TLR3 (Randow and Seed, 2001; Yang et al., 2007), other ER resident proteins are needed for translocation to the endosome (Tabeta et al., 2006). Consequently, persistent ER stress may modulate the response to many intracellular TLR ligands, including the TLR3 ligand polyinosine-polycytidylic acid (poly I:C) (Randow and Seed, 2001; Tabeta et al., 2006; Yang et al., 2007).

Activation of intracellular TLRs early in life is proposed as a mechanism leading to many neurodevelopmental and neuropsychiatric disorders (Brown, 2006; Arroyo et al., 2011; Kneeland and Fatemi, 2013), such as autism and schizophrenia (Fatemi et al., 1999). Although there is a significant male predominance in these disorders (McGrath, 2006; Loomes et al., 2017), sex differences in the mechanisms of TLR3-mediated brain injury early in life has not been reported. Since other models of neonatal brain injury suggest a greater proclivity to apoptosis in female mice and to necrosis-like cell death in male mice (Hagberg et al., 2004; Zhu et al., 2004, 2007; Northington et al., 2011; Chavez-Valdez et al., 2012; Chavez-Valdez et al., 2014), we hypothesized that poly I:C in the P8 mouse model will also induce greater caspase activation in female mice, and more prominent pro-inflammatory profile, astrogliosis, and injury in male mice. Additionally, we also studied the potential role of ER stress in sex-specific responses to TLR3 activation.

MATERIALS AND METHODS

Mice

Experiments were performed with approval by the Ethical Committee of the Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden (No. 18-2015; 663/17) where the handling of animals was carried out. Handling was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, Council of Europe (ETS 123). All efforts were made to minimize the number of animals used. Both male and female mice were used for these experiments.

Wild type C57BL6J mice (The Jackson Laboratory, Scanbur, Denmark) were kept in a 12 h light/dark cycle at the animal facility at University of Gothenburg (Gothenburg, Sweden). Culling was performed in the last litter to equalize the sample size for each treatment group. In total 92 (48 male and 48 female) pups were used for these experiments. Litter size varied from 6 to 10 pups. At P8, pups were assigned to either sex group based on visual inspection of external characteristics. Animal received food and water *ad libitum*. Pups were weighed at P8, P9, and P15 to evaluate differences in growth by group. At P8 mice received intraperitoneal (IP) injection of the TLR3 agonist poly I:C (Poly I:C- Low Molecular Weight; InvivoGen, Toulouse, France) reconstituted in LPS-free normal saline (NS) to 1 mg/mL and injected at 10 mg/kg or vehicle (LPS-free NS). Pups were killed at 6 or 14 h for: (i) caspase 3 and 8 activity assays; (ii) NFkB ELISA; (iii) western blot for IRF3, AP1, and GFAP; and (iv) pro-inflammatory (IL-1β, TNFα, FAS, CXCL-1, CXCL-10, MCP-1, INF-β, INF-γ, and IL-6) and anti-inflammatory (IL-10) cytokines/chemokines gene expression by real time qRT-PCR. A second group of pups were killed at 24 h (P9) or 7 days (P15) after poly I:C exposure to evaluate: (i) microscopic injury using Nissl counterstaining; (ii) astroglia and microglia activation using GFAP and Iba1 IHC,

respectively; and (iii) gene and protein expression of a similar battery of cytokines/chemokines as described above, by real-time RT-PCR and multiplex electrochemiluminescent immunoassay, respectively. All samples were coded and run by laboratory personnel blinded to treatments, sex, and times.

In all cases, mice were anesthetized and perfused intracardially with NS prior to brain dissection. Brains were snap frozen, and stored at −80°C until analysis except for the hemispheres used for histological evaluation, which were fixed in 4% paraformaldehyde (PFA) by immersion for 1 week followed by cryoprotection using 15 and 30% sucrose gradient prior to freezing at −80°C.

Caspase 3 and 8 Activity Assays

Cytoplasmic and nuclear fractions were prepared using a piece of brain from the same brains used for RNA isolation using a Subcellular Protein Fractionation Kit (Thermo Scientific, Rockford, IL, United States). Buffers were part of the kit and supplemented with protease and phosphatase inhibitors. Approximately 150 mg of tissue washed in ice-cold phosphate-buffered saline (PBS, pH 7.2) was homogenized in Cytoplasmic Extraction Buffer at 1:10 (w:v) using a standard tissue grinder and transferred to a tissue strainer. Following centrifugation at 500 × *g* for 5 min, supernatant (cytoplasmic fraction) was used for Caspase 3 and 8 activity assays. The pellet was sequentially resuspended in ice cold Membrane and Nuclear Extraction Buffers at 1:6.5 and 1:2.25 (w:v), respectively. The final supernatant, the nuclear fractions, were stored at −80°C for nuclear factor κB (NFκB) measurement. Twenty percent (w/v) glycerol was added to the fractions and protein concentrations were determined using the Bradford assay (Bradford, 1976). Cross-contamination between nuclear and cytosolic fractions was minimal following immunoblotting for Lamin B1 (nuclear protein) and HSP90 (cytosolic protein), details below. Cytosolic fractions were assayed for caspase 3 or caspase 8 activity using the respective Colorimetric Activity Assay Kits, (APT165 and APT129; Millipore, Billerica, MA, United States). Cytosolic fractions (400 μg) were plated in a 96-well culture plate and reconstituted in cell lysis buffer and diluted (1:1 v:v) in assay buffer provided by the manufacturer prior to incubation for 60 min at 37°C with the substrate Ac-DEVD-pNA (N-Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) for caspase 3 or Ac-IETD- pNA (*N*-acetyl-Ile-Glu-Thr-Asp-ρ-nitroaniline) for caspase 8 activity assay. The chromophore ρ-NA was measured at 405 nm using a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, United States). Semiquantitative measurement of ρNA concentrations in samples was determined indirectly using the linear model of absorbance.

Nuclear Factor-κB Transcription Factor Determination Using ELISA

Nuclear fractions were used to determine concentrations of NFκB bound to the consensus sequence 5′-GGGACTTTCC-3′ in the biotinylated capture probe and detected by exposure to the primary rabbit anti-NFκB p65 antibody (1:1000) followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary detection antibody (1:500) using the EZ-TFA Transcription Factor Assay (Millipore, Temecula, CA, United States). Positive (TNFα-treated HeLa whole-cell extract), specific competitor (NFκB competitor oligonucleotide), and negative controls were used to establish specificity and sensitivity at λ450/ 650 nm using a microplate reader.

Western Blot for IRF3, AP1, GFAP, GRP94

Twenty μg-aliquots of cytosolic (for all protein targets) or nuclear fractions (for IRF3 and AP1) were diluted 3:1 (v:v) in 4X loading buffer under reducing conditions. Samples were loaded in 4–20% mini-protean TGX polyacrylamide precast protein gels (Biorad, Inc., Hercules, CA, United States) and transferred to nitrocellulose membrane using TransBlot Turbo Midi-size (Biorad, Inc.). Blots were stained with Ponceau S and blocked using 2.5% BSA with 0.1% Tween-20 in 50 mM Tris buffered saline (TBST, pH 7.4) before incubation overnight at 4°C in primary antibodies at 1:1000 (GFAP), and 1:500 (IRF3, AP1, and GRP94). After exposure blots were washed with TBST, exposed to secondary antibodies for 1 h and then developed using enhanced chemiluminescence (Clarity Western ECL Substrate, Biorad, Inc.). Loading control at 1:5000 was performed using β-actin for cytosolic fractions and histone H1.4 (both form Sigma). To quantify protein immunoreactivity, optical density (OD) was determined with ImageJ adjusted for background.

Antibodies

IRF3 (Proteintech Group, Inc. 11312-1-AP: RRID: AB_2127004): rabbit polyclonal IgG antibody raised against the IRF3 fusion protein Ag1858 detecting a single band at 49 to 55 kDa (2 μg/ml). AP1 (Proteintech Group, Inc. 22114-1-AP: RRID: AB_2750860): rabbit polyclonal IgG antibody raised against the p39 fusion protein Ag17419 detecting a single band at 40 to 46 kDa (2 μg/ml). GFAP (Proteintech Group, Inc. 16825-1-AP: RRID: AB_ 2109646): rabbit polyclonal IgG antibody raised against the GFAP fusion protein Ag10423 detecting a single band at 45 to 50 kDa (1 μg/ml). GRP94 (Proteintech Group, Inc. 60012-1-Ig: RRID: AB_2119056): mouse monoclonal IgM antibody raised against the GRP94 fusion protein Ag1439 detecting a single band at 95 kDa (2 μg/ml). For evaluation of nuclear-cytosol cross-contamination of the fractions, the following antibodies were used: (i) HSP90 (cytosolic protein, Cell Signaling Technology, Inc. 4875: RRID: AB_2233331): rabbit polyclonal antibody raised against the synthetic peptide surrounding Glu289 of the HSP protein of human origin detecting a single band at 90 kDa without cross-reactivity with other heat-shock proteins (1 μg/ml); and (ii) Lamin B1 (nuclear protein, Cell Signaling Technology, Inc. 13435: RRID: AB_2737428): rabbit monoclonal antibody produced by immunization with a synthetic peptide corresponding to residues surrounding Lys415 of lamin B1 protein of human origin detecting a band at 68 kDa (1 μg/ml).

Gene Expression Using Real Time qRT-PCR

Total RNA was extracted from two combined pieces from anterior and posterior brain from mice pups treated as described above. Tissues were obtained at 6, 14, 24 h (P9)

and 7 days (P15) after normal saline or poly I:C exposure. PureLink™ RNA mini kit purification system (Invitrogen, Carlsbad, CA, United States) was used for RNA extraction and measured using a spectrophotometer at 260 nm absorbance. Total RNA (1 μg) was used for generation of complementary single strand DNA (cDNA) using iScript cDNA synthesis kit (BioRad). Reverse transcription protocol included 5 min at 25°C; 30 min at 42°C and 5 min at 85°C. cDNA was then used for gene amplification. The following SYBR Green Based primers from Qiagen (Germantown, MD, United States) were used: IL-6 (QT00098875), IL-1β (QT01048355), TNF-α (QT00104006), Fas (QT00095333), IFN-β (QT00249662), INF-γ (QT01038821), interferon-induced protein-10 (IP-10/CXCL-10) (QT00093436), CXCL-1 (QT00115647) monocyte chemoattractant protein-1 (MCP-1) (QT00167832), IL-10 (QT00106169), including the housekeeping genes GAPDH (QT01658692), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ; QT00105350). The amplification protocol included 40 cycles of 30 s at 95.0°C, 1 min at 60°C ending with 30 s at 72.0°C. Fold difference in gene expression was then corrected by the geometric mean of GAPDH and YWHAZ gene expression using the using the Pfaffl method (Pfaffl, 2001). Melting curves confirmed amplification of single PCR products.

Cytokine and Chemokine Measurement in Brain Crudes

To measure cytokine and chemokine concentrations in brain, a V-plex multi-plex electrochemiluminescent immunoassay platform (MesoScale Discovery, Gaithersburg, MD, United States) was used consistent with our prior studies (Maxwell et al., 2015; Robinson et al., 2016, 2018; Yellowhair et al., 2018). This system has been validated by traditional ELISA and produces measurements with high content validity and inter-assay variations less than 12%. Specifically, brain lysate (100 μg) or standard was loaded on to a 96-well plate in duplicate per manufacturer's specification. Plates were read on a Quickplex SQ 120 Imager (Mesoscale Discovery, Gaithersburg, MD, United States).

IHC for GFAP and Iba1

After intracardiac perfusion with NS and dissection, the brain hemispheres assigned for histological analysis were fixed by immersion in 4% PFA in 0.1 M PBS for 7 days. Tissues were cryoprotected with graded immersion in 15% and then 30% sucrose in PBS until the tissue sank, then frozen and stored in −80°C until cut at 50 μm on a freezing microtome. Sections obtained from animals killed 24 h or 7 days after normal saline or poly I:C exposure were used to assess astroglia (GFAP) and microglia (Iba1) activation and overall microscopic evidence of injury (Nissl counterstaining for GFAP IHC). Floating IHC was performed as previously described (Chavez-Valdez et al., 2018) with whole rabbit antisera anti-GFAP (DAKO/Agilent Technologies, Santa Clara, CA, United States; 1:1000), or anti-Iba1 (Wako Chemicals USA, Inc., Richmond, VA, United States; 1:500) followed by goat anti-rabbit antibody (1:200) used as the secondary antibody using DAB as the chromogen for GFAP and using Alexa Fluor 568 for immunofluorescence for Iba1. Cresyl-violet (CV) counterstaining was performed in those sections previously immunostained for GFAP to assess histological structure. GFAP IHC slides were imaged using a light microscope (Nikon Eclipse E400, Nikon, Minato, Japan), to produce high resolution photomicrographs (1344 × 1024 pixels). Percent area of GFAP immunostaining in the region of interest (hippocampal CA1 and DG, thalamic ventroposterior nuclei, and cingulate cortex) inserted within the 4 X × 20 X high-resolution photomicrographs was calculated using the color threshold function of ImageJ 1.8.0 software (NIH, Bethesda, MD, United States). Immunofluorescent images for Iba1 were captured at 512 × 512 pixels using a Laser Scanning Confocal Microscope LSM700 from Carl Zeiss AG (Oberkochen, Germany). Total Iba1 immunofluorescence signal was quantified using the histogram function in the Zen 2.3 blue edition (Carl Zeiss Microscopy GmbH, Jena, Germany).

Antibodies

GFAP (DAKO Z0334; RRID:AB_10013382): rabbit polyclonal antibody raised against GFAP isolated from cow spinal cord with no reported cross reactivity (1 μg/ml). *Iba1* (WAKO 019-19741; RRID:AB_839504): rabbit polyclonal antibody raised against a synthetic peptide corresponding to the C-terminus of Iba1 purified by antigen affinity chromatography, with no reported cross-reactivity with neuronal or astrocytic markers (1 μg/ml).

Statistical Analysis

For analysis of two related groups, such as the fold-change in gene expression relative to control (NS group) produced by the Pfaffl method, non-parametric Wilcoxon signed-rank test vs. control stratified by sex was applied. Data were presented as box and whisker plot, where the box was limited by the 25th and 75th percentiles and the solid line represented the median. The discontinued line sitting at 1 represented the relative expression of the comparison group (NS). For within sex and time group analysis of two independent treatments (NS and Poly I:C), Mann–Whitney *U*-test was applied. Significance was assigned by $p \leq 0.05$ in all cases. Analysis was performed using IBM SPSS Statistics 24V (IBM Corporation, Armonk, NY, United States).

RESULTS

Poly I:C Exposure and Growth

The median weight of the pups included in both treatment groups, NS and poly I:C, was similar prior to injection at P8 ($p = 0.76$ for males and $p = 0.84$ for females). By 24 h (P9) or 7 days (P15) after injection the rate of growth was 0.45 to 0.50 mg/day for pups in the NS and those in poly I:C treatment groups. No difference in growth rate by treatment group was documented

in male pups ($p_{(24\ h)} = 0.70$, $p_{(7\ d)} = 0.31$) or female pups ($p_{(24\ h)} = 0.82$, $p_{(7\ d)} = 0.18$).

Sex Differences in Caspase 3 and Caspase 8 Activity in Mice After Poly I:C

Neither caspase 3 (**Figure 1A**), nor caspase 8 (**Figure 1B**) activity increased at 6 or 14 h after poly I:C exposure in male mice (n = 5/group). To the contrary, in female mice poly I:C increased caspase 3 activity by 57% from 0.28 (IQR 0.22–0.30) units to 0.44 (IQR 0.37–0.62) units at 6 h after exposure (p = 0.02 vs. NS exposed female mice, n = 4/group; **Figure 1A**). This increase in caspase-3 activity resolved at 14 h after poly I:C exposure. Accordingly, in female mice poly I:C also increased caspase-8 activity from 0.12 (IQR 0.05–0.22) units to 0.35 (0.32–0.42) units at 6 h after exposure (p = 0.02 vs. NS exposed female mice, n = 4/group; **Figure 1B**). Similar to caspase 3, caspase 8 increase resolved by 14 h after poly I:C exposure.

Sex Differences in NFκB and IRF3 Nuclear Translocation in Response to Poly I:C

While no difference in nuclear NFκB was observed 6 h after poly I:C (data not shown), 14 h after poly I:C exposure, NFκB nuclear levels increased by 28% in all mice (p = 0.04 vs. NS-exposed, n = 10/group, **Figure 1C**). When stratified by sex, no change in nuclear NFκB levels after poly I:C exposure was documented in female mice, while an increase of 76% (p = 0.04 vs. NS group, n = 5/group, **Figure 1C**) was documented in male mice at 14 h after poly I:C. Other pathways downstream of TLR3 activation were also studied. Nuclear translocation of IRF3 was temporally distinct between females and males. While IRF3 translocation to the nucleus occurred 6 h after poly I:C in female mice (p = 0.01 vs. NS, n = 6/group), this change was only observed at 14 h after poly I:C in the male mice (p = 0.01 vs. NS, n = 5–6/group; **Figure 1D**). No significant nuclear translocation of AP1 was observed at either 6 or 14 h after poly I:C in either sex (**Figure 1D**).

Increase Early Astrocytic Activation in Male Mice Exposed to Poly I:C

In activated astrocytes, TLR3 activation initiates a cytosolic cascade leading to nuclear translocation of NFκB, IRF3, and/or AP-1 and downstream transcription of pro-inflammatory mediators, which may perpetuate microglia activation and extend neuronal injury (Fitzgerald et al., 2003; Pobezinskaya et al., 2008). Expression of GFAP, an astrocytic marker, was unchanged 6 h after poly I:C exposure, while it was increased by 42.7% (p = 0.003 vs. NS-exposed mice, n = 8/group) at 14 h after exposure. Temporarily coinciding with their later nuclear translocation of NFκB and IRF3, only male mice responded with a 40% (p = 0.02, n = 4; **Figure 1E**) increase in GFAP expression at 14 h after poly I:C exposure, while female mice did not show any change.

Sexual Dimorphism in the Temporal Patterns of INF-β Gene Expression After Poly I:C

INF-β is the prime final product downstream of TLR3 activation. Six hours after poly I:C exposure, INF-β gene expression was upregulated by 8.8-fold in all mice (p = 0.005 vs. NS, n = 10; data not shown). INF-β gene upregulation derived from the early upregulation in female mice (11.3-fold, p = 0.03, n = 6; **Figure 1F**). Fourteen hours after poly I:C the gene expression of INF-β was upregulated by sevenfold (p = 0.005 vs. NS, n = 12; data not shown) in all mice, at this time point the increase derived from the upregulation in male mice (8.1-fold, p = 0.03, n = 6; **Figure 1F**). This pattern temporally mirrors the sexual dimorphism in IRF3 and NFκB nuclear translocations. By 24 h after poly I:C INF-β gene expression returns to levels similar to those seen in NS-treated mice. However, 7 days after poly I:C, INF-β becomes again upregulated in male mice (5.4-fold, p = 0.03 vs. NS, n = 6; **Figure 1F**).

Prolonged Pro-inflammatory Gene Profile After Poly I:C Exposure

Similar to INF-β, gene expression profile of many cytokines was sexually dimorphic following poly I:C exposure at P8. In male mice, poly I:C upregulated the mRNA level of the following pro-inflammatory genes at 6 h after exposure: IL-1β (5.1-fold, p = 0.04, n = 5; **Figure 2A**); CXCL-10 (345-fold p = 0.02, n = 6; **Figure 2B**); TNF-α (4.3-fold, p = 0.04, n = 6; **Figure 2C**); MCP-1 (14.3-fold, p = 0.04, n = 6; **Figure 2D**); FAS (twofold, p = 0.01, data not shown); and IL-6 (6.6-fold, p = 0.04, n = 5, **Figure 2E**); without changing the expression of the anti-inflammatory cytokine IL-10 (**Figure 2F**). Similarly, an early pro-inflammatory profile was documented 6 h after poly I:C exposure in female mice with upregulation of: IL-1β (sevenfold, p = 0.03, n = 6; **Figure 2A**); CXCL-10 (594-fold, p = 0.04, n = 5; **Figure 2B**); MCP-1 (46-fold, p = 0.03, n = 6; **Figure 2D**); FAS (2.8-fold, p = 0.04, data not shown); and IL-6 (sixfold, p = 0.03, n = 6; **Figure 2E**). In male mice, 14 h after poly I:C exposure all pro-inflammatory markers remained upregulated, including: IL-1β (3.5-fold, p = 0.02, n = 6; **Figure 2A**); CXCL-10 (51-fold, p = 0.02, n = 6; **Figure 2B**); TNF-α (5.3-fold, p = 0.02, n = 6, **Figure 2C**); MCP-1 (22-fold, p = 0.02, n = 6; **Figure 2D**), and FAS (1.8-fold, p = 0.01, n = 6; data not shown);but additionally IL-10 was downregulated by 42% (p = 0.04, n = 5; **Figure 2F**). Unlike male mice, the gene expression of several pro-inflammatory markers returned to normal in female mice at 14 h after poly I:C including, IL-6 and FAS and at 24 h after poly I:C, IL-1β. Additionally, the brains of female mice did not respond to poly I:C with down-regulation of IL-10 14 h after exposure, as documented in male mice (**Figure 2F**). As expected a trajectory to return to levels similar to those in NS-treated mice was observed between 24 h and 7 days after poly I:C. Thus, at 7 days after poly I:C IL-1β, TNF-α, and MCP-1 gene expressions were similar to those in NS-treated mice in both sexes. However, only in female mice, upregulation of the CXCL-10 (p = 0.03, n = 6) and

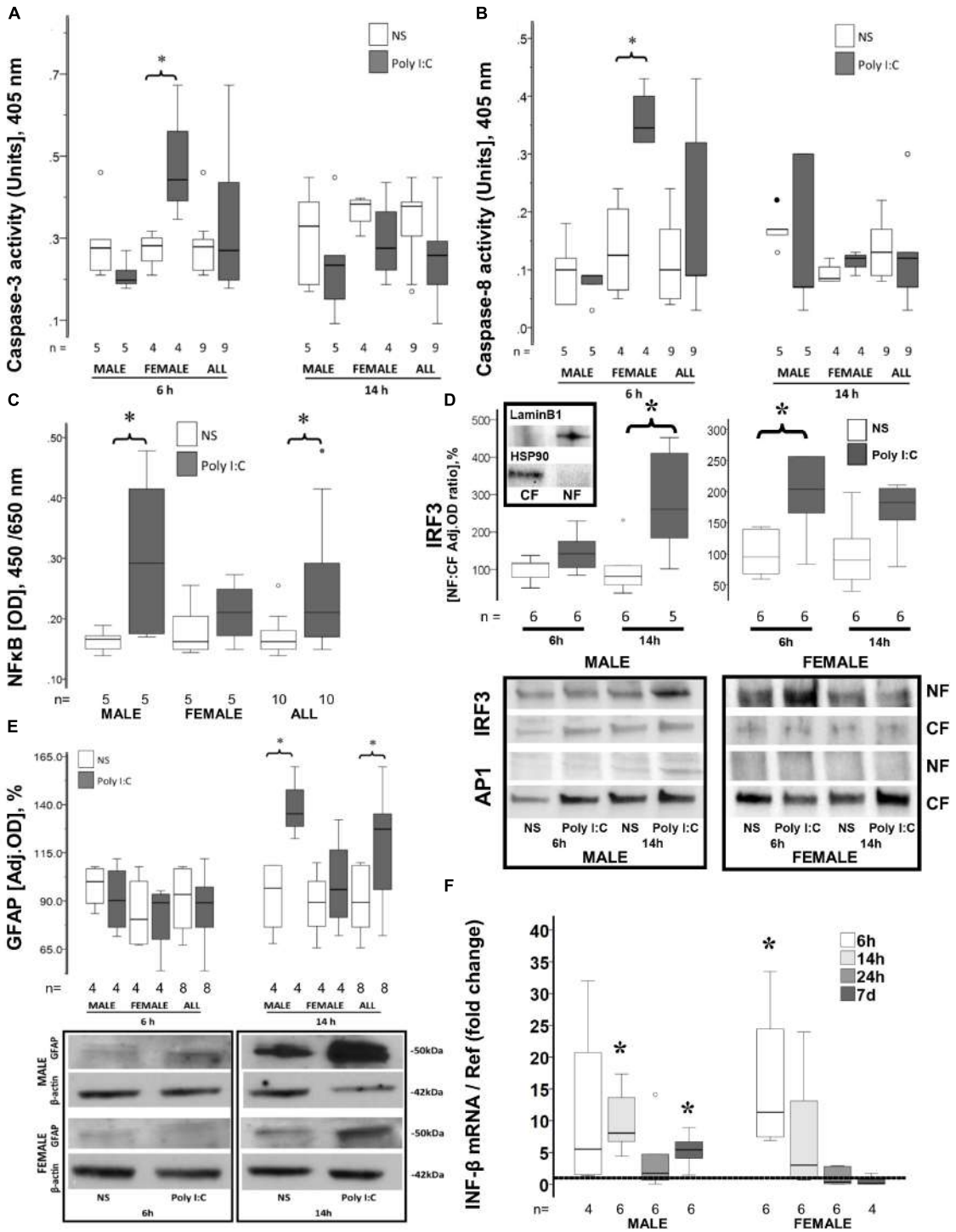


FIGURE 1 | Early sex-specific responses to poly I:C in the brain of P8 mice. Increased caspase-3 **(A)** and caspase-8 activity **(B)**, IRF3 nuclear translocation **(D)**, and INF-β gene upregulation **(F)** occur 6 h after poly I:C in the brain of female mice. Increased NFκB **(C)** and IRF3 **(D)** nuclear translocation, GFAP expression **(E)** and INF-β gene upregulation **(F)** occur 14 h after poly I:C in the brain of male mice. Data are presented using box and whiskers plots, where boxes are limited by the 75th and 25th percentiles interquartile range (IQR)] and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). **(A–E)** White boxes represent NS-treated pups, while gray boxes represent poly I:C-treated pups. **(D)** Representative immunoblots showing translocation of IRF3 and AP1 from the cytosolic fraction (CF) to the nuclear fraction (NF) in males and females at 6 and 14 h after poly I:C. Top immunoblots show purity of the NF and CF using Lamin B1 and HSP90, respectively. **(E)** Representative immunoblots showing GFAP expression in cytosolic fractions. **(F)** Shows the fold change in INF-β mRNA levels in the brain of male and female mice at 6, 14, 24 h and 7 days after poly I:C relative to NS-treated mice (discontinuous line sitting at 1). °, outlier (between 1.5 and 3 times the IQR); •, extreme (> 3 times the IQR); *$p < 0.05$.

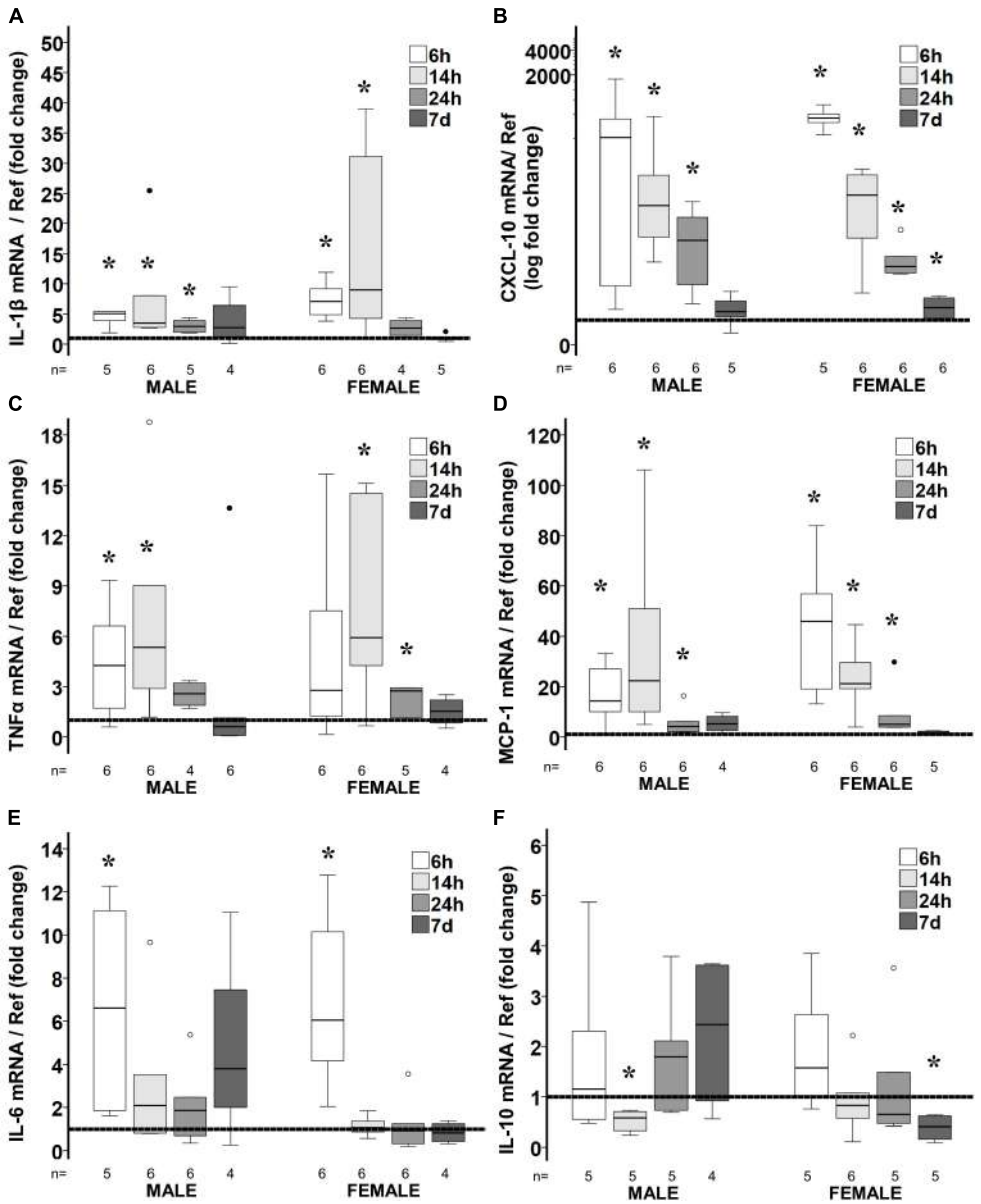


FIGURE 2 | Temporal changes in brain cytokine/ chemokine gene expression in response to poly I:C exposure at P8. Fold change in gene expression of IL-1β **(A)**, CXCL-10 **(B)**, TNFα **(C)**, MCP-1 **(D)**, IL-6 **(E)**, and IL-10 **(F)** in the brain of poly I:C treated mice relative to NS-treated mice. Data are shown using box and whiskers plots, where boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). Data from male and female mice at 6, 14, 24 h and 7 days after poly I:C relative to NS-treated mice (discontinuous line sitting at 1) is shown. °, outlier (between 1.5 and 3 times the IQR); •, extreme (>3 times the IQR); *$p < 0.05$.

downregulation of IL-10 ($p = 0.04$, $n = 5$) genes were present at 7 days after poly I:C (**Figures 2B,F**).

An extensive battery of pro-inflammatory cytokines/chemokine protein levels were measured in poly I:C treated mice at 24 h and 7 days after HI (**Table 1**). Matching the gene expression data, IL-1β, CXCL-10, TNF-α, and MCP-1 were increased at 24 h after poly I:C exposure in both sexes. Also congruent with the gene expression results, 7 days after poly I:C the levels of CXCL-10 (63%, $p = 0.05$), CXCL-1 (19%, $p = 0.01$) and INF-γ (38%, $p = 0.006$) were elevated, while the level of IL-10 (−21%, $p = 0.05$) was decreased only in female mice (**Table 1**).

Astrocyte and Microglia Activation Following Poly I:C

Although by 14 h after poly I:C GFAP protein level was increased only in male mice, at 24 h after poly I:C GFAP expression was equally observed in the hippocampus (CA1 and dentate gyrus subfields) and cingulate cortex of both male and

TABLE 1 | Cytokine expression following poly I:C exposure stratified by sex.

		Males			Females		
		Saline	**Poly I:C**	***p*-value**	**Saline**	**Poly I:C**	***p*-value**
Pro-inflammatory (n = 6/group) (pg/100 μg protein)							
INF-γ Median [IQR]	**24 h**	0.103 [0.08–0.12]	0.119 [0.11–0.15]	0.10	0.091 [0.08–0.09]	0.096 [0.80–0.11]	0.52
	7 days	0.074 [0.06–0.09]	0.063 [0.04–0.07]	0.10	**0.045 [0.03–0.05]**	**0.062 [0.06–0.07]**	**0.006***
IL-1β Median [IQR]	**24 h**	**0.311 [0.27–0.37]**	**0.545 [0.44–0.64]**	**0.006***	**0.367 [0.29–0.45]**	**0.599 [0.47–0.74]**	**0.01***
	7 days	0.395 [0.34–0.43]	0.286 [0.24–0.43]	0.42	0.304 [0.27–0.35]	0.301 [0.26–0.33]	0.87
IL-2 Median [IQR]	**24 h**	0.101 [0.06–0.10]	0.095 [0.07–0.10]	0.56	0.108 [0.05–0.10]	0.106 [0.08–0.24]	0.43
	7 days	ND	ND	–	ND	ND	–
IL-5 Median [IQR]	**24 h**	**0.466 [0.40–0.58]**	**0.605 [0.53–0.64]**	**0.05***	0.483 [0.45–0.55]	0.634 [0.47–0.71]	0.10
	7 days	0.359 [0.29–0.39]	0.358 [0.26–0.38]	1.00	0.349 [0.33–0.37]	0.350 [0.28–0.39]	1.00
IL-12 Median [IQR]	**24 h**	18.16 [13.9–22.6]	23.92 [18.1–31.6]	0.26	20.48 [16.8–26.4]	24.49 [17.5–29.4]	0.42
	7 days	14.55 [9.71–18.9]	15.25 [7.62–19.9]	0.87	15.81 [6.39–18.4]	13.49 [9.62–16.4]	0.72
CXCL-1 Median [IQR]	**24 h**	2.79 [2.65–3.06]	3.19 [2.76–3.61]	0.10	2.66 [2.33–2.77]	3.04 [2.57–3.26]	0.10
	7 days	1.79 [1.39–1.99]	2.14 [1.73–2.34]	0.10	**1.76 [1.57–1.96]**	**2.09 [1.98–2.35]**	**0.01***
CXCL-10 Median [IQR]	**24 h**	**13.34 [10.8–13.3]**	**691.4 [521.1–1043]**	**0.004***	**13.92 [12.4–15.2]**	**480.9 [346.7–1782]**	**0.004***
	7 days	10.34 [9.6–13.7]	11.85 [10.6–12.6]	0.52	**9.45 [7.21–14.7]**	**15.42 [12.6–20.5]**	**0.05***
TNF-α Median [IQR]	**24 h**	**0.243 [0.21–0.31]**	**0.402 [0.37–0.46]**	**0.004***	**0.269 [0.25–0.32]**	**0.406 [0.38–0.43]**	**0.04***
	7 days	0.233 [0.21–0.26]	0.217 [0.18–0.26]	0.42	0.212 [0.18–0.24]	0.176 [0.16–0.23]	0.33
MCP-1 Median [IQR]	**24 h**	**2.14 [1.92–2.46]**	**40.88 [30.9–47.6]**	**0.004***	**1.819 [1.72–2.19]**	**38.58 [30.3–62.5]**	**0.004***
	7 days	1.546 [1.47–1.76]	1.662 [1.52–1.85]	0.42	1.509 [1.23–1.84]	1.706 [1.54–1.89]	0.10
Modulatory (n = 6/group) (pg/100 μg protein)							
IL-6 Median [IQR]	**24 h**	8.72 [8.05–10.5]	10.52 [9.21–10.8]	0.20	8.46 [7.91–10.4]	10.35 [8.53–11.4]	0.20
	7 days	7.62 [6.87–8.44]	7.54 [5.79–8.23]	0.75	7.02 [6.54–7.42]	7.26 [5.68–7.61]	1.00
Anti-inflammatory (n = 6/group) (pg/100 μg protein)							
IL-4 Median [IQR]	**24 h**	0.385 [0.23–0.66]	0.343 [0.28–0.39]	0.52	0.253 [0.18–0.42]	0.266 [0.20–0.36]	0.87
	7 days	0.149 [0.08–0.24]	0.139 [0.13–0.20]	0.87	0.148 [0.08–0.25]	0.132 [0.09–0.18]	0.75
IL-10 Median [IQR]	**24 h**	5.44 [4.52–6.26]	5.07 [4.42–5.91]	0.74	6.55 [5.64–7.09]	6.11 [5.75–6.74]	0.52
	7 days	4.64 [4.12–5.34]	4.63 [4.13–5.61]	0.87	**5.24 [5.05–5.42]**	**4.13 [3.32–5.17]**	**0.05***

Bold font signifies statistical significance.

female mice (**Figure 3** and **Table 2**). Seven days after poly I:C, evidence of astrocytic activation greatly diminished in both sexes in all regions (**Figures 3A,B,D**) with the exception of the ventroposterior nuclei of the thalamus, demonstrating greater number of reactive astrocytes at 7 days than at 24 h after poly I:C (**Figure 3C** and **Table 2**). Similar to GFAP staining, Iba-1

staining peaked at 24 h after poly I:C in the hippocampus and the cingulate cortex (**Figure 4**) but unlike GFAP-staining, residual Iba-1 immunofluorescence at 7 days after exposure show a sex specific pattern. While evidence of microglia activation was almost resolved in the hippocampus (**Figures 4A** [CA1] and **4B** [DG]), thalamus (**Figure 4C**) and cingulate cortex (**Figure 4D**) at 7 days after poly I:C in male mice; significant residual activation was present in all those brain regions in female mice (**Figure 4**). Semi-quantitative analysis of Iba1 immunofluorescence in those regions are shown in **Figure 4** and **Table 3**.

CHOP Expression Following Poly I:C

Both CHOP gene and protein expression were increased 24 h after poly I:C exposure in males and female mice (**Figure 5**). However, only in the male mice, 7 days after poly I:C sexual dimorphism in CHOP gene expression resulted in a threefold increase compared to NS treated mice suggesting persistent ER stress in male mice. Expression of GRP94, an important ER chaperone for TLR3 and a known protective factor against ER stress, was not different between treatment groups in either sex.

The full dataset analyzed and reported in the result section is available to the reader as **Supplementary Material**.

DISCUSSION

Our pilot results suggest that TLR3 activation affects the developing brain differently in male and female mice. Early caspase activation is more prominent in the brain of female mice 6 h after exposure to poly I:C at P8. Here, we also document sexually specific temporal patterns of pro-inflammatory cytokine/chemokine expression. Early IRF-3 nuclear translocation downstream of TLR3 activation leads to INF-β upregulation 6 h after poly I:C in female mice, while delayed NFκB-IRF-3 nuclear translocation leads to INF-β upregulation 14 h after exposure in male mice. Although, several pro-inflammatory cytokines such as IL-1β, CXCL-10, TNF-α, MCP-1, FAS, and IL-6 are increased by 6 h after poly I:C in both sexes, some of them, IL-6 and FAS, remain upregulated at 14 h after poly I:C in conjunction with IL-10 downregulation only in the male mice. Earlier activation of astrocytes after poly I:C may explain the increased pro-inflammatory profile seen in male mice at 14 h after exposure. While most of the pro-inflammatory markers return to levels similar to those seen in NS-treated mice sometime between 24 h and 7 days after poly I:C in male mice; late increase in INF-γ, CXCL-1, and CXCL-10, combined with decrease IL-10, and persistent microglia activation suggest a greater late pro-inflammatory state in female mice 7 days after poly I:C. We speculate that a second "wave" of INF-β upregulation documented in male mice 7 days after poly I:C, may prevent release of INF-γ, microglia activation, and downstream release of CXCL-10 in the male mice (Ottum et al., 2015; Kovarik et al., 2016). Mechanistically, delayed CHOP upregulation in male mice 7 days after poly I:C may prevent cytokine translation in male mice (Moon, 2014). Altogether, TLR3 activation produces early activation of apoptotic pathways predominantly in female mice, early pro-inflammatory cytokine profile in both sexes, which extends at least for 7 days after poly I:C exposure in female mice presumptively secondary to persistent microglia activation. The role of ER-stress in the sexual dimorphism in response to poly I:C is still unclear.

Sex differences in the mechanisms of cell death have been described after brain injury secondary to stroke (Liu et al., 2011) and hypoxia-ischemia (Hagberg et al., 2004; Zhu et al., 2007; Northington et al., 2011; Chavez-Valdez et al., 2012) and here, we provide preliminary evidence that these differences may also occur in response to TLR3 activation in the neonatal brain. In the developing brain, a greater acute proclivity to apoptotic cell death in females and to a pro-inflammatory necrotic-like cell death in males has been documented (Hurn et al., 2005; Vannucci and Hurn, 2009; Northington et al., 2011). Cultured female neurons are more sensitive to etoposide-induced apoptosis, while cultured male neurons are more sensitive to excitotoxic stress (Du et al., 2004). Accordingly, following perinatal hypoxia-ischemia or ischemic brain injury, inhibition of caspases provides protection to female rodents (Renolleau et al., 2007), while inhibition of PARP-1-mediated necrotic pathways provides protection to male rodents (Hagberg et al., 2004). Activation of TLR3 by poly I:C leads to both types of cell death. TLR3-mediated apoptotic cell death occurs via upregulation of death-receptors 4/5 and a transactivating p63 isoform α-mediated and IRF3-mediated initiation of the extrinsic pathway (Sun et al., 2011; Gambara et al., 2015) or via a death receptor devoted RIP1-mediated TLR3/caspase-8 complex formation (Estornes et al., 2012). On the other hand, TLR3-mediated necrotic-like cell death, such as programmed necrosis, occurs in the setting of caspase-8 inhibition via a RIP3 kinase-dependent mechanism that involves TRIF and MLKL, but independent of RIP1 (He et al., 2011; Kaiser et al., 2013; Lotzerich et al., 2018). Indirectly, TNF death receptor activation following exposure to TLR3 ligands may proceed via classic RIP1-RIP3 necroptosis pathway, which is also prominent in male mice in the P7 model of hypoxia-ischemia (Northington et al., 2011; Kaiser et al., 2013; Chavez-Valdez et al., 2014). Our results suggest that similar to stroke and hypoxia-ischemia, the mechanisms of cell death in the developing brain after TLR3 activation diverge by sex. The greater caspase 3 and caspase 8 activity documented in the brain of female mice 6 h after poly I:C suggest a preferential activation of the apoptotic extrinsic pathway compared to male mice. Furthermore, IRF3 mediated initiation of apoptotic extrinsic pathway is suggested by the increased IRF3 nuclear translocation demonstrated 6 h after poly I:C in female mice, and event that coincided with caspase activation.

There is also evidence for intrinsic sex-specific differences in inflammatory responses induced by activation of immune cells in the immature brain (Mallard et al., 2018). Additionally, peripheral inflammatory responses may also have an influence in severity of brain injury in a sex-specific manner. For example, peripheral depletion of circulating myeloid cells reduces brain inflammation and injury in male but not female mice (Smith et al., 2018). Further, in models of non-viral

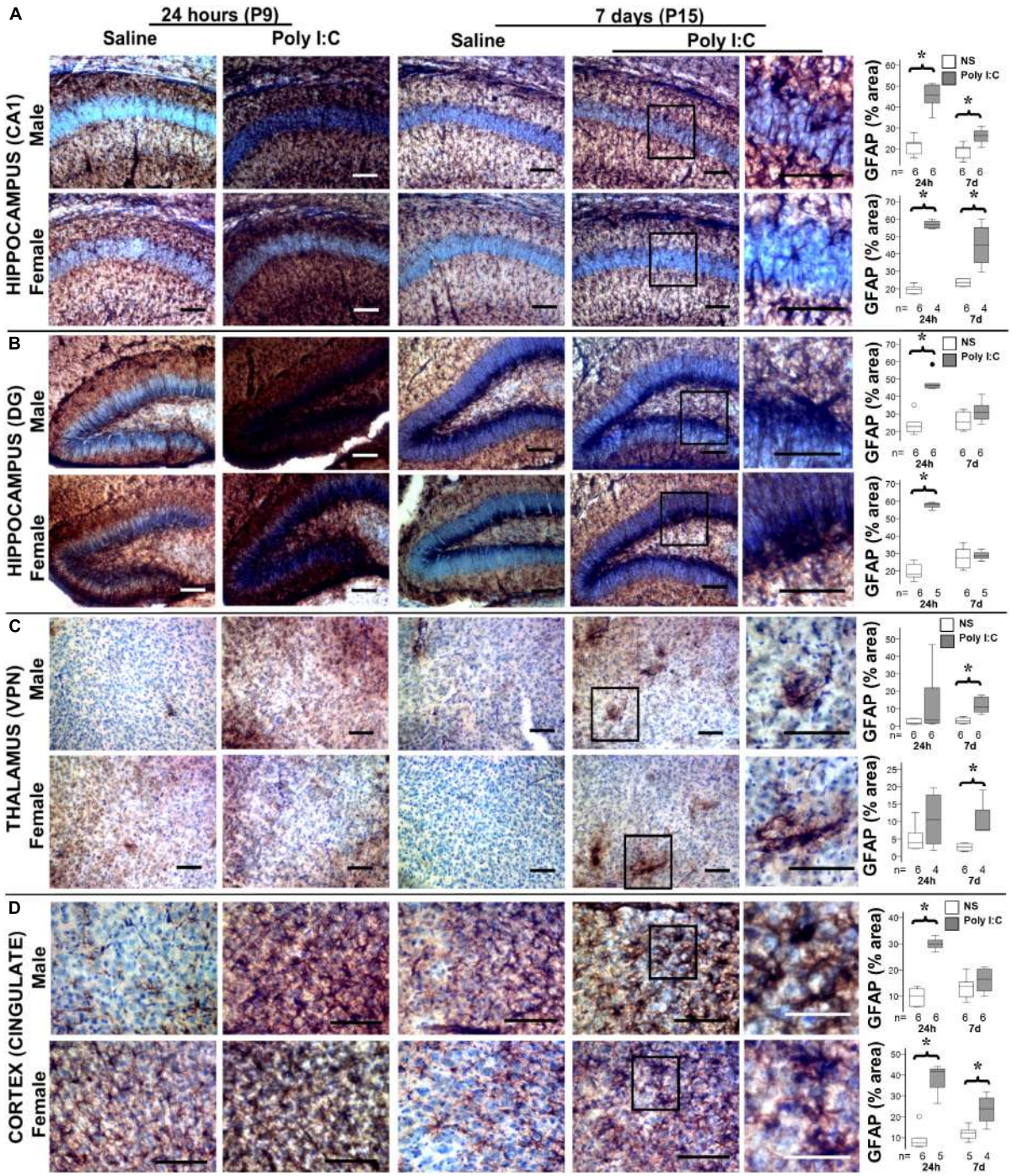


FIGURE 3 | Temporal changes in astrocytic activation after poly I:C exposure at P8. Representative photomicrographs of GFAP immunoreactivity in the CA1 **(A)** and dentate gyrus (DG, **B**) subfields of the hippocampus, ventroposterior nuclei (VPN) of the thalamus **(C)**, and cingulate cortex **(D)** of male and female mice at 24 h and 7 days after treated with normal saline or poly I:C. High magnification details are provided for 7 days after poly I:C. In all panels, bar represents 100 μm, with the exception of the detail higher magnification panel for the cingulate cortex at 7 days after poly I:C, where bar represent 50 μm. Semi-quantitative analyses of percent area of GFAP immunostaining per sex and region of interest are shown using box and whiskers plots, where boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). Data from male (top) and female (bottom) mice at 24 h and 7 days after treatment with NS (white boxes) or poly I:C (gray boxes) are shown. °, outlier (between 1.5 and 3 times the IQR); •, extreme (>3 times the IQR); *$p < 0.05$ (n = 6/ group). Twenty-four hours after poly I:C, significant astrocytic activation was observed in the hippocampus (CA1 and DG subfields) and cingulate cortex. At 7 days after poly I:C, astrocytic activation greatly diminished with the exception of the VPN of the thalamus, demonstrating greater evidence of astrocytosis at 7 days than at 24 h after poly I:C. No sex-specific differences were observed. Detailed information about percent area of GFAP immunoreactivity in each area of interest is provided in **Table 2**.

TABLE 2 | Percent area of GFAP immunostaining per region.

		24 h (P9)			7 days (P15)		
Region	**Sex**	**Saline**	**Poly I:C**	***p*-Value**	**Saline**	**Poly I:C**	***p*-Value**
Hippocampus CA1 Median % [IQR]	**Male**	22.6 [18.9–22.9]	45.9 [42.9–49.5]	**0.002**	20.3 [16.8–20.7]	26.4 [24–28.4]	**0.009**
	Female	19.7 [17.6–20.7]	56.5 [54.9–58.4]	**0.01**	23.6 [21.8–25.2]	45.2 [37.8–52.4]	**0.01**
Hippocampus DG Median % [IQR]	**Male**	22.6 [20.5–24.7]	46.2 [44.9–47.4]	**0.002**	24.4 [21.6–30.2]	30.8 [27.9–33.9]	NS
	Female	18.2 [16.6–22.8]	58.3 [57.1–58.8]	**0.004**	27.3 [22.9–31.1]	28.7 [27.1–30.5]	NS
Thalamus VPN Median % [IQR]	**Male**	1.8 [1–3.9]	3.3 [1.9–17.5]	NS	2.8 [1.9–4.2]	10.8 [8.7–15.2]	**0.002**
	Female	3.9 [2.4–6.3]	10.6 [4.5–16.7]	NS	2.7 [1.9–3.5]	7.6 [7.5–10.5]	**0.01**
Cingulate Cortex Median % [IQR]	**Male**	9.9 [6.8–12.2]	29.9 [28.9–31.1]	**0.002**	13.8 [10.6–15.1]	13.8 [10.6–15.1]	NS
	Female	7.5 [6.3–9.3]	41.7 [34.1–42.9]	**<0.001**	12.4 [9.9–13.6]	23.9 [19.9–27.4]	**0.003**

Data show as percent (%) area of GFAP immunoreactivity in the region of interest within high magnification photomicrograph. n = 4–6/group. Mann–Whitney U-test used for analysis. DG, dentate gyrus; NS, non-significant; VPN, ventroposterior nuclei. Bold font signifies statistical significance.

septic peritonitis and ischemic bowel injury, TLR3 genetic deletion attenuates the perpetuation of cytokines and chemokines release by macrophages, recruitment of neutrophils, systemic inflammation, and multiorgan injury (Cavassani et al., 2008). Similar role of TLR3 activation as an amplifier of the early inflammatory responses have been reported in the kidney and the liver (Patole et al., 2005; Lang et al., 2006). Activation of TLR3 with poly I:C induces a prolonged inflammatory response from cultured astrocytes (Chistyakov et al., 2018), microglia (Dupuis et al., 2016; Yousif et al., 2018), and brain endothelial cells (Johnson et al., 2018). The prolonged inflammatory response induced by TLR3 activation in microglia and astrocytes biologically serves to control or to enhance insidious viral infections such as Chikungunya (Priya et al., 2014) and HIV-1 (Bhargavan and Kanmogne, 2018) and non-viral infections such as Borrelia burgdorferi (Greenmyer et al., 2018). Sex differences in inflammatory responses depend on the specific intracellular TLR ligand used. Male peripheral mononuclear cells (PMNCs) treated with TLR7 ligands produce less INF than females cells, while male PMNCs treated with TLR8 and TLR9 agonists produce more IL-10 than female cells (Torcia et al., 2012). Thus, the activation of TLR7, 8, and 9 may provide a net anti-inflammatory effect in the males. However, the role of sex on the TLR3-mediated prolonged inflammatory response is unknown in the developing brain until now. Our experiments suggest persistent increase in pro-inflammatory cytokines IL-6, FAS and INF-β, and decrease in IL-10 in the brain of male mice up to 14 h after poly I:C, which contrast with the response in the brain of female mice. We speculate that this is the result of more acute/subacute pro-inflammatory necrotic type of cell death with earlier astrocytic activation in male mice. The pro-inflammatory response appears to resolve in the male mice sometime between 24 h and 7 days after poly I:C. In contrast, a pro-inflammatory profile persist at least until 7 days after poly I:C in female mice. The expression of INF-β after poly I:C may explain the lack of INF-γ expression in both males and female pups up to 24 h (Ottum et al., 2015). In contrast, the lack of INF-β upregulation documented in female mice 7 days after poly I:C, may explain the late upregulation of INF-γ, microglia activation and downstream CXCL-10 expression (Kovarik et al., 2016). However, the role of systemic inflammation in the persistent pro-inflammatory profile documented in the brain of female mice after poly I:C is still unclear.

We acknowledge limitations in this study. We have not dissect the few discrepancies between transcription and translation of certain markers, such as IL-1β or CHOP at 24 h, which shows sex dimorphism in gene expression. Further, we have not studied the influence of systemic inflammation in the brain. Knowing that the blood–brain barrier (BBB) integrity is altered by systemic inflammation, disrupting immune cell trafficking to the brain and leading to activation of astrocytes within the BBB (Banks, 2005; Abbott et al., 2006); our exposure paradigm using IP poly I:C will presumably induce both a primary inflammatory response, as well as a systemic-derived response in the brain secondary to glial activation by circulating cytokines released during TLR3 activation of cells in other organs (e.g., macrophages, adipocytes, keratinocytes). Although the complex interactions between systemic and brain inflammatory responses cannot be further elaborated with the reported set of experiments, TLR3 activation by poly I:C appears to produce only a modest systemic inflammatory short lasting response compared to responses to LPS (Packard et al., 2012), thus we speculate that the earlier GFAP peak at 14 h after poly I:C, may be the result of a greater influence of systemic inflammation in the brain of male mice as previously reported (Smith et al., 2018), which may also explain the increase IL-6, FAS and decrease IL-10 seen simultaneously in males. Similarly, we cannot conclude about the cellular source of the sexual dimorphism in cytokine expression documented in female

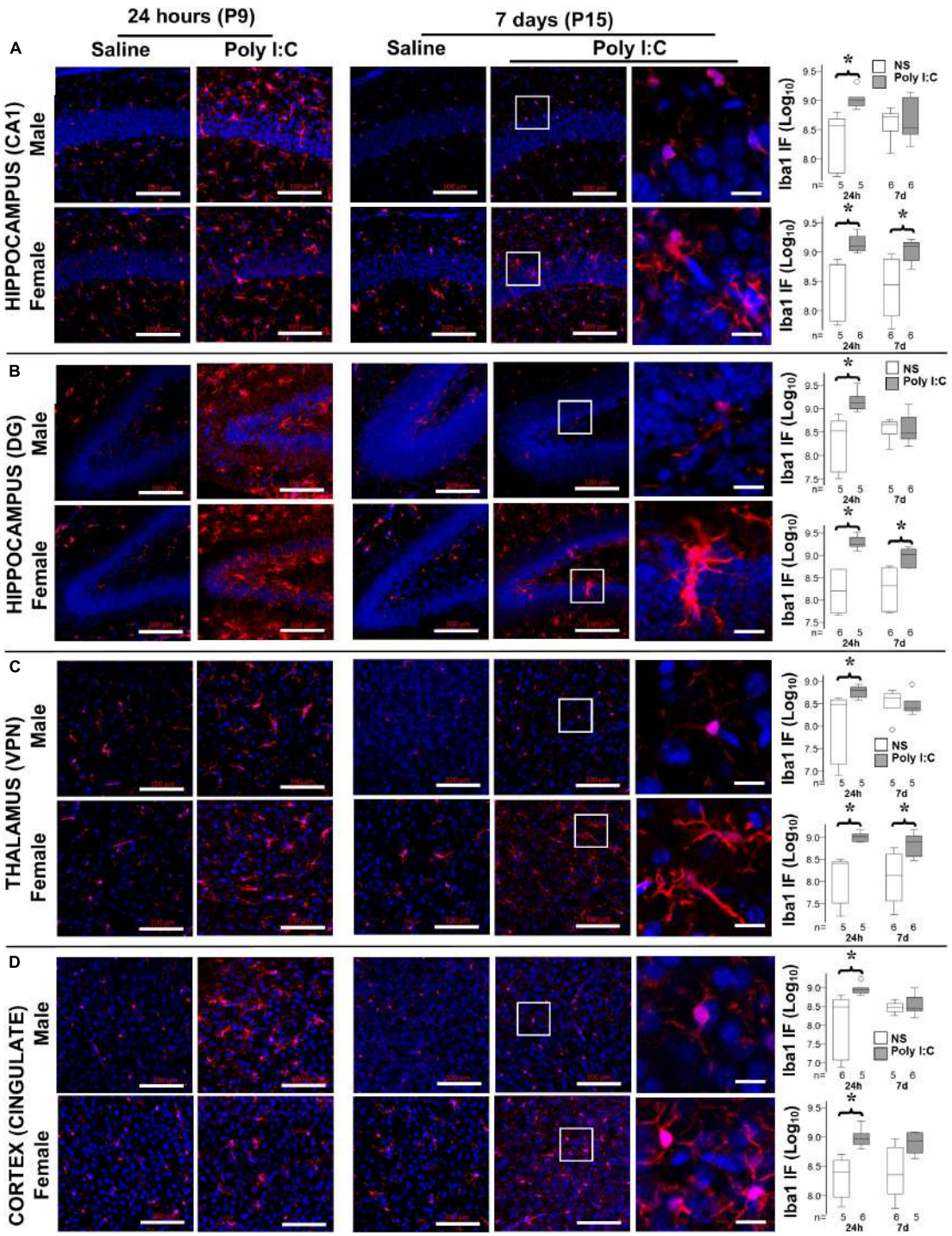


FIGURE 4 | Temporal changes in microglia activation after poly I:C exposure at P8. Representative photomicrographs of Iba1 immunofluorescence in the CA1 **(A)** and dentate gyrus (DG, **B**) subfields of the hippocampus, VPN of the thalamus **(C)**, and cingulate cortex **(D)** of male (top row) and female mice (bottom row) at 24 h and 7 days after treatment with normal saline (NS) or poly I:C. High magnification datails show at 7 d after poly I:C. In all panels, bar represents 100 μm, with the exception of the detail high magnification panels for 7 days after poly I:C, where bar represent 20 μm. Semi-quantitative analyses of Iba1 immunofluorescence per sex and region of interest are shown using box and whiskers plots, where boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). Data from male (top) and female (bottom) mice at 24 h and 7 days after treatment with NS (white boxes) or poly I:C (gray boxes) are shown. Data are presented as the decimal logarithmic transformation of the total immunofluorescence signal in arbitrary units. °, outlier (between 1.5 and 3 times the IQR); •, extreme (>3 times the IQR); *$p < 0.05$ (n = 4–6/group). Twenty-four hours after poly I:C, significant microglia activation was observed in all regions in both sexes. At 7 days after poly I:C, microglia activation significantly diminished in male mice (top), while persisted in all brain regions in female mice (bottom). Detailed information about immunofluorescence provided in **Table 3**.

TABLE 3 | Semi-quantitative analysis of Iba1 immunofluorescence per region.

		24 h (P9)			7 days (P15)		
Region	**Sex**	**Saline**	**Poly I:C**	***p*-Value**	**Saline**	**Poly I:C**	***p*-Value**
Hippocampus CA1 Median [IQR]	**Male**	370 [57–484]	1021 [801–1125]	**0.008**	527 [344–585]	341 [273–929]	NS
	Female	610 [66–617]	1283 [1070–1750]	**0.004**	404 [87–735]	1253 834–1412]	**0.04**
Hippocampus DG Median [IQR]	**Male**	333 [44–538]	1342 [993–1826]	**0.008**	455 [289–518]	296 [243–565]	NS
	Female	260 [52–479]	1748 [1601–2505]	**0.004**	285 [63–511]	1087 [597–1370]	**0.01**
Thalamus VPN Median [IQR]	**Male**	305 [14–388]	635 [434–734]	**0.03**	417 [250–519]	255 [219–365]	NS
	Female	254 [32–279]	1039 [773–1188]	**0.008**	187 [41–390]	824 [426–1059]	**0.04**
Cingulate Cortex Median [IQR]	**Male**	314 [70–458]	903 [728–988]	**0.009**	298 [239–390]	279 [250–482]	NS
	Female	249 [93–397]	899 [741–980]	**0.004**	284 [107–609]	830 [525–1186]	NS

Data show immunofluorescence arbitrary units (millions). n = 5–6/group. Mann–Whitney U-test used for analysis. DG, dentate gyrus; NS, non-significant; VPN, ventroposterior nuclei. Bold font signifies statistical significance.

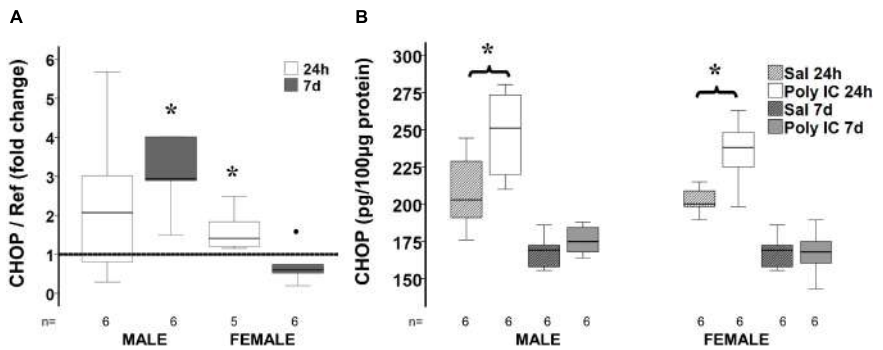


FIGURE 5 | Delayed sex-specific CHOP expression after poly I:C in P8 mice. Fold change in CHOP gene **(A)** and protein **(B)** expression at 24 h and 7 days after poly I:C exposure at P8. Data are shown using box and whiskers plots, where boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). Fold change was calculated relative to NS-treated mice shown as discontinuous line sitting at 1). °, outlier (between 1.5 and 3 times the IQR); •, extreme (>3 times the IQR); *$p < 0.05$.

mice 7 days after poly I:C exposure. However, the simultaneous lack of INF-β gene upregulation, increase INF-γ levels, increase Iba-1 expression across brain regions, and increase CXCL-10 levels, allow us to speculate that persistently activated microglia may be the source of these changes. Other mechanisms by which poly I:C may also produce effects in the brain (e.g., activation of inflammasome) as suggested by others (Rajan et al., 2010; Franchi et al., 2014) need further studies. In rodents, IP injection of poly I:C at less than half of the dose used in our experiments (3–4 instead of 10 mg/kg) produces fever within 5 to 14 h after injection (Hopwood et al., 2009) and also impairs endothelial function (Zimmer et al., 2011). Since a decrease in core temperature may suppress microglial induction and attenuate inflammation, the evaluation of temperature changes in response to poly I:C also needs further investigation. Finally, our experiments are not powered to study differences within sexes and the analysis has been limited to differences between treatments within each sex group. Thus, larger studies, with 20–30 mice per group depending on the primary outcomes used for power calculation, are needed to confirm our preliminary observations between sexes.

Perinatal TLR3 activation is speculated as a mechanism leading to several neurodevelopmental, neurodegenerative, and neuropsychiatric disorders (Brown, 2006; De Miranda et al., 2010; Arroyo et al., 2011; Forrest et al., 2012; Kneeland and Fatemi, 2013). The male predominance in these disorders (McGrath, 2006; Loomes et al., 2017), matches the early sex differences documented within the first 24 h after TLR3 activation in our experiments. We propose that following exposure of the developing brain to TLR3 ligand, female cells acutely die via apoptosis, while male cells die via necrotic-like cell death (i.e., programmed necrosis), as suggested in other models of neonatal brain injury (Hagberg et al., 2004; Zhu et al., 2007; Northington et al., 2011; Chavez-Valdez et al., 2012). The early

release of DAMPs from necrotic cells and the effects of systemic inflammation may intensify astrocytosis in male mice, with downstream nuclear translocation of transcription factors and production of pro-inflammatory mediators within the first 24 h. The role of the late pro-inflammatory response documented in female mice after TLR3 activation in the development of neurological disorders is unclear. However, emergent literature is suggesting a protective role of inflammatory mediators against neurodegenerative diseases (Lemere, 2007; Ottum et al., 2015; Le et al., 2016). Further experiments better powered to confirm our speculations are needed to then study the role of these potential sex differences in the sex dimorphism seen in many of neurological disorders linked to TLR3 activation early in life.

ETHICS STATEMENT

We confirm that any aspect of the work covered in this manuscript involving experimental animals has been conducted with the ethical approval of all relevant bodies.

AUTHOR CONTRIBUTIONS

RC-V, FN, and CM: experimental design. AM and LS: animal works. RC-V, AM, LS, TY, and LJ: tissue processing. RC-V, TY, and LJ: immunohistochemistry, PCR, and biochemical assays. RC-V: statistical analysis and initial draft preparation. All authors: critical reviews of the manuscript and approval of final version.

FUNDING

Experiments and investigators were funded in part by grants from the National Institutes of Health (KO8NS096115 – RC-V; R01HL139492 – LJ; RO1HD070996, RO1HD086058 – FN; R01NS103483 – CM), the Johns Hopkins University-School of Medicine Clinician Scientist Award (RC-V), the Sutland-Pakula Endowment for Neonatal Research (RC-V) and Swedish Research Council (VR-2017-01409 – CM), Swedish state under the agreement between the Swedish government and the county councils, the ALF-agreement (ALFGBG-722491 – CM), Neurobid, HEALTH-F2-2009-241778 (CM), the Leducq foundation (DSRR_P34404 – CM), and the Swedish Brain Foundation (FO2017-0063).

ACKNOWLEDGMENTS

The authors thank Mrs. Deborah Flock, Ms. Elizabeth Krisanda, and Mr. Charles Lechner for their technical support and Mrs. Rosie Silva for her administrative assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00306/full#supplementary-material

TABLE S1 | Manuscript's dataset.

REFERENCES

Abbott, N. J., Ronnback, L., and Hansson, E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* 7, 41–53. doi: 10.1038/nrn1824

Arroyo, D. S., Soria, J. A., Gaviglio, E. A., Rodriguez-Galan, M. C., and Iribarren, P. (2011). Toll-like receptors are key players in neurodegeneration. *Int. Immunopharmacol.* 11, 1415–1421. doi: 10.1016/j.intimp.2011.05.006

Banks, W. A. (2005). Blood-brain barrier transport of cytokines: a mechanism for neuropathology. *Curr. Pharm. Des.* 11, 973–984.

Beg, A. A. (2002). Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. *Trends Immunol.* 23, 509–512.

Bhargavan, B., and Kanmogne, G. D. (2018). Toll-like receptor-3 mediates HIV-1-induced interleukin-6 expression in the human brain endothelium via TAK1 and JNK pathways: implications for viral neuropathogenesis. *Mol. Neurobiol.* 55, 5976–5992. doi: 10.1007/s12035-017-0816-8

Blasius, A. L., and Beutler, B. (2010). Intracellular toll-like receptors. *Immunity* 32, 305–315. doi: 10.1016/j.immuni.2010.03.012

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

Brown, A. S. (2006). Prenatal infection as a risk factor for schizophrenia. *Schizophr. Bull.* 32, 200–202. doi: 10.1093/schbul/sbj052

Bsibsi, M., Ravid, R., Gveric, D., and van Noort, J. M. (2002). Broad expression of Toll-like receptors in the human central nervous system. *J. Neuropathol. Exp. Neurol.* 61, 1013–1021.

Cameron, J. S., Alexopoulou, L., Sloane, J. A., DiBernardo, A. B., Ma, Y., Kosaras, B., et al. (2007). Toll-like receptor 3 is a potent negative regulator of axonal growth in mammals. *J. Neurosci.* 27, 13033–13041. doi: 10.1523/JNEUROSCI.4290-06.2007

Cavassani, K. A., Ishii, M., Wen, H., Schaller, M. A., Lincoln, P. M., Lukacs, N. W., et al. (2008). TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. *J. Exp. Med.* 205, 2609–2621. doi: 10.1084/jem.20081370

Chavez-Valdez, R., Emerson, P., Goffigan-Holmes, J., Kirkwood, A., Martin, L. J., and Northington, F. J. (2018). Delayed injury of hippocampal interneurons after neonatal hypoxia-ischemia and therapeutic hypothermia in a murine model. *Hippocampus* 28, 617–630. doi: 10.1002/hipo.22965

Chavez-Valdez, R., Martin, L. J., and Northington, F. J. (2012). Programmed necrosis: a prominent mechanism of cell death following neonatal brain injury. *Neurol. Res. Int.* 2012:257563. doi: 10.1155/2012/257563

Chavez-Valdez, R., Martin, L. J., Razdan, S., Gauda, E. B., and Northington, F. J. (2014). Sexual dimorphism in BDNF signaling after neonatal hypoxia-ischemia and treatment with necrostatin-1. *Neuroscience* 260, 106–119. doi: 10.1016/j.neuroscience.2013.12.023

Chistyakov, D. V., Azbukina, N. V., Lopachev, A. V., Kulichenkova, K. N., Astakhova, A. A., and Sergeeva, M. G. (2018). Rosiglitazone as a Modulator of TLR4 and TLR3 signaling pathways in rat primary neurons and astrocytes. *Int. J. Mol. Sci.* 19:E113. doi: 10.3390/ijms19010113

De Miranda, J., Yaddanapudi, K., Hornig, M., Villar, G., Serge, R., and Lipkin, W. I. (2010). Induction of Toll-like receptor 3-mediated immunity during gestation inhibits cortical neurogenesis and causes behavioral disturbances. *Mbio* 1, e00176–e00210. doi: 10.1128/mBio.00176-10

Du, L., Bayir, H., Lai, Y., Zhang, X., Kochanek, P. M., Watkins, S. C., et al. (2004). Innate gender-based proclivity in response to cytotoxicity and programmed cell death pathway. *J. Biol. Chem.* 279, 38563–38570. doi: 10.1074/jbc.M405461200

Dupuis, N., Mazarati, A., Desnous, B., Chhor, V., Fleiss, B., Le Charpentier, T., et al. (2016). Pro-epileptogenic effects of viral-like inflammation in both mature and immature brains. *J. Neuroinflamm.* 13:307. doi: 10.1186/s12974-016-0773-6

Estornes, Y., Toscano, F., Virard, F., Jacquemin, G., Pierrot, A., Vanbervliet, B., et al. (2012). dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. *Cell Death Differ.* 19, 1482–1494. doi: 10.1038/cdd.2012.22

Fatemi, S. H., Emamian, E. S., Kist, D., Sidwell, R. W., Nakajima, K., Akhter, P., et al. (1999). Defective corticogenesis and reduction in reelin immunoreactivity in cortex and hippocampus of prenatally infected neonatal mice. *Mol. Psychiatry* 4, 145–154.

Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., et al. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* 4, 491–496. doi: 10.1038/ni921

Forrest, C. M., Khalil, O. S., Pisar, M., Smith, R. A., Darlington, L. G., and Stone, T. W. (2012). Prenatal activation of Toll-like receptors-3 by administration of the viral mimetic poly(I:C) changes synaptic proteins, N-methyl-D-aspartate receptors and neurogenesis markers in offspring. *Mol. Brain* 5:22. doi: 10.1186/1756-6606-5-22

Franchi, L., Eigenbrod, T., Munoz-Planillo, R., Ozkurede, U., Kim, Y. G., Arindam, C., et al. (2014). Cytosolic double-stranded RNA activates the NLRP3 inflammasome via MAVS-induced membrane permeabilization and K+ efflux. *J. Immunol.* 193, 4214–4222. doi: 10.4049/jimmunol.1400582

Gambara, G., Desideri, M., Stoppacciaro, A., Padula, F., De Cesaris, P., Starace, D., et al. (2015). TLR3 engagement induces IRF-3-dependent apoptosis in androgen-sensitive prostate cancer cells and inhibits tumour growth in vivo. *J. Cell Mol. Med.* 19, 327–339. doi: 10.1111/jcmm.12379

Greenmyer, J. R., Gaultney, R. A., Brissette, C. A., and Watt, J. A. (2018). Primary human microglia are phagocytically active and respond to *Borrelia burgdorferi* with upregulation of chemokines and cytokines. *Front. Microbiol.* 9:811. doi: 10.3389/fmicb.2018.00811

Hagberg, H., Wilson, M. A., Matsushita, H., Zhu, C., Lange, M., Gustavsson, M., et al. (2004). PARP-1 gene disruption in mice preferentially protects males from perinatal brain injury. *J. Neurochem.* 90, 1068–1075. doi: 10.1111/j.1471-4159.2004.02547.x

He, S., Liang, Y., Shao, F., and Wang, X. (2011). Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20054–20059. doi: 10.1073/pnas.1116302108

Hopwood, N., Maswanganyi, T., and Harden, L. M. (2009). Comparison of anorexia, lethargy, and fever induced by bacterial and viral mimetics in rats. *Can. J. Physiol. Pharmacol.* 87, 211–220. doi: 10.1139/y09-003

Hurn, P. D., Vannucci, S. J., and Hagberg, H. (2005). Adult or perinatal brain injury: does sex matter? *Stroke* 36, 193–195. doi: 10.1161/01.STR.0000153064.41332.f6

Johnson, R. H., Kho, D. T., O'Carroll, S. J., Angel, C. E., and Graham, E. S. (2018). The functional and inflammatory response of brain endothelial cells to toll-like receptor agonists. *Sci. Rep.* 8:10102. doi: 10.1038/s41598-018-28518-3

Kaiser, W. J., Sridharan, H., Huang, C., Mandal, P., Upton, J. W., Gough, P. J., et al. (2013). Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. *J. Biol. Chem.* 288, 31268–31279. doi: 10.1074/jbc.M113.462341

Kariko, K., Ni, H., Capodici, J., Lamphier, M., and Weissman, D. (2004). mRNA is an endogenous ligand for Toll-like receptor 3. *J. Biol. Chem.* 279, 12542–12550. doi: 10.1074/jbc.M310175200

Kneeland, R. E., and Fatemi, S. H. (2013). Viral infection, inflammation and schizophrenia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 42, 35–48. doi: 10.1016/j.pnpbp.2012.02.001

Kovarik, P., Castiglia, V., Ivin, M., and Ebner, F. (2016). Type I interferons in bacterial infections: a balancing act. *Front. Immunol.* 7:652. doi: 10.3389/fimmu.2016.00652

Lang, K. S., Georgiev, P., Recher, M., Navarini, A. A., Bergthaler, A., Heikenwalder, M., et al. (2006). Immunoprivileged status of the liver is controlled by toll-like receptor 3 signaling. *J. Clin. Invest.* 116, 2456–2463. doi: 10.1172/JCI28349

Lathia, J. D., Okun, E., Tang, S. C., Griffioen, K., Cheng, A., Mughal, M. R., et al. (2008). Toll-like receptor 3 is a negative regulator of embryonic neural progenitor cell proliferation. *J. Neurosci.* 28, 13978–13984. doi: 10.1523/JNEUROSCI.2140-08.2008

Le, W., Wu, J., and Tang, Y. (2016). Protective microglia and their regulation in parkinson's disease. *Front. Mol. Neurosci.* 9:89. doi: 10.3389/fnmol.2016.00089

Lemere, C. A. (2007). A beneficial role for IL-1 beta in Alzheimer disease? *J. Clin. Invest.* 117, 1483–1485. doi: 10.1172/JCI32356

Liu, F., Lang, J., Li, J., Benashski, S. E., Siegel, M., Xu, Y., et al. (2011). Sex differences in the response to poly(ADP-ribose) polymerase-1 deletion and caspase inhibition after stroke. *Stroke* 42, 1090–1096. doi: 10.1161/STROKEAHA.110.594861

Loomes, R., Hull, L., and Mandy, W. P. L. (2017). What is the male-to-female ratio in autism spectrum disorder? a systematic review and meta-analysis. *J. Am. Acad. Child Adolesc. Psychiatry* 56, 466–474. doi: 10.1016/j.jaac.2017.03.013

Lotzerich, M., Roulin, P. S., Boucke, K., Witte, R., Georgiev, O., and Greber, U. F. (2018). Rhinovirus 3C protease suppresses apoptosis and triggers caspase-independent cell death. *Cell Death Dis.* 9:272. doi: 10.1038/s41419-018-0306-6

Mallard, C., Tremblay, M. E., and Vexler, Z. S. (2018). Microglia and neonatal brain injury. *Neuroscience* doi: 10.1016/j.neuroscience.2018.01.023 [Epub ahead of print].

Maxwell, J. R., Denson, J. L., Joste, N. E., Robinson, S., and Jantzie, L. L. (2015). Combined in utero hypoxia-ischemia and lipopolysaccharide administration in rats induces chorioamnionitis and a fetal inflammatory response syndrome. *Placenta* 36, 1378–1384. doi: 10.1016/j.placenta.2015.10.009

McGrath, J. J. (2006). Variations in the incidence of schizophrenia: data versus dogma. *Schizophr. Bull.* 32, 195–197. doi: 10.1093/schbul/sbi052

Moon, Y. (2014). Ribosomal alteration-derived signals for cytokine induction in mucosal and systemic inflammation: noncanonical pathways by ribosomal inactivation. *Med. Inflamm.* 2014:708193. doi: 10.1155/2014/708193

Northington, F. J., Chavez-Valdez, R., Graham, E. M., Razdan, S., Gauda, E. B., and Martin, L. J. (2011). Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI. *J. Cereb. Blood Flow Metab.* 31, 178–189. doi: 10.1038/jcbfm.2010.72

Ottum, P. A., Arellano, G., Reyes, L. I., Iruretagoyena, M., and Naves, R. (2015). Opposing roles of interferon-gamma on cells of the central nervous system in autoimmune neuroinflammation. *Front. Immunol.* 6:539. doi: 10.3389/fimmu.2015.00539

Packard, A. E., Hedges, J. C., Bahjat, F. R., Stevens, S. L., Conlin, M. J., Salazar, A. M., et al. (2012). Poly-IC preconditioning protects against cerebral and renal ischemia-reperfusion injury. *J. Cereb. Blood Flow Metab.* 32, 242–247. doi: 10.1038/jcbfm.2011.160

Patole, P. S., Grone, H. J., Segerer, S., Ciubar, R., Belemezova, E., Henger, A., et al. (2005). Viral double-stranded RNA aggravates lupus nephritis through Toll-like receptor 3 on glomerular mesangial cells and antigen-presenting cells. *J. Am. Soc. Nephrol.* 16, 1326–1338. doi: 10.1681/ASN.2004100820

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.

Pobezinskaya, Y. L., Kim, Y. S., Choksi, S., Morgan, M. J., Li, T., Liu, C., et al. (2008). The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent toll-like receptors. *Nat. Immunol.* 9, 1047–1054. doi: 10.1038/ni.1639

Priya, R., Patro, I. K., and Parida, M. M. (2014). TLR3 mediated innate immune response in mice brain following infection with chikungunya virus. *Virus Res.* 189, 194–205. doi: 10.1016/j.virusres.2014.05.010

Rajan, J. V., Warren, S. E., Miao, E. A., and Aderem, A. (2010). Activation of the NLRP3 inflammasome by intracellular poly I:C. *FEBS Lett.* 584, 4627–4632. doi: 10.1016/j.febslet.2010.10.036

Randow, F., and Seed, B. (2001). Endoplasmic reticulum chaperone gp96 is required for innate immunity but not cell viability. *Nat. Cell Biol.* 3, 891–896. doi: 10.1038/ncb1001-891

Ransohoff, R. M., and Brown, M. A. (2012). Innate immunity in the central nervous system. *J. Clin. Invest.* 122, 1164–1171. doi: 10.1172/JCI58644

Renolleau, S., Fau, S., Goyenvalle, C., Joly, L. M., Chauvier, D., Jacotot, E., et al. (2007). Specific caspase inhibitor Q-VD-OPh prevents neonatal stroke in P7 rat: a role for gender. *J. Neurochem.* 100, 1062–1071. doi: 10.1111/j.1471-4159.2006.04269.x

Robinson, S., Corbett, C. J., Winer, J. L., Chan, L. A. S., Maxwell, J. R., Anstine, C. V., et al. (2018). Neonatal erythropoietin mitigates impaired gait, social interaction and diffusion tensor imaging abnormalities in a rat model of

prenatal brain injury. *Exp. Neurol.* 302, 1–13. doi: 10.1016/j.expneurol.2017.12.010

Robinson, S., Winer, J. L., Berkner, J., Chan, L. A., Denson, J. L., Maxwell, J. R., et al. (2016). Imaging and serum biomarkers reflecting the functional efficacy of extended erythropoietin treatment in rats following infantile traumatic brain injury. *J. Neurosurg. Pediatr.* 17, 739–755. doi: 10.3171/2015.10.PEDS15554

Rossi, D., and Volterra, A. (2009). Astrocytic dysfunction: insights on the role in neurodegeneration. *Brain Res. Bull.* 80, 224–232. doi: 10.1016/j.brainresbull.2009.07.012

Smith, P. L. P., Mottahedin, A., Svedin, P., Mohn, C. J., Hagberg, H., Ek, J., et al. (2018). Peripheral myeloid cells contribute to brain injury in male neonatal mice. *J. Neuroinflamm.* 15:301. doi: 10.1186/s12974-018-1344-9

Stridh, L., Mottahedin, A., Johansson, M. E., Valdez, R. C., Northington, F., Wang, X., et al. (2013). Toll-like receptor-3 activation increases the vulnerability of the neonatal brain to hypoxia-ischemia. *J. Neurosci.* 33, 12041–12051. doi: 10.1523/JNEUROSCI.0673-13.2013

Sun, R., Zhang, Y., Lv, Q., Liu, B., Jin, M., Zhang, W., et al. (2011). Toll-like receptor 3 (TLR3) induces apoptosis via death receptors and mitochondria by up-regulating the transactivating p63 isoform alpha (TAP63alpha). *J. Biol. Chem.* 286, 15918–15928. doi: 10.1074/jbc.M110.178798

Tabeta, K., Hoebe, K., Janssen, E. M., Du, X., Georgel, P., Crozat, K., et al. (2006). The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via toll-like receptors 3, 7 and 9. *Nat. Immunol.* 7, 156–164. doi: 10.1038/ni1297

Torcia, M. G., Nencioni, L., Clemente, A. M., Civitelli, L., Celestino, I., Limongi, D., et al. (2012). Sex differences in the response to viral infections: TLR8 and TLR9 ligand stimulation induce higher IL10 production in males. *PLoS One* 7:e39853. doi: 10.1371/journal.pone.0039853

Vannucci, S. J., and Hurn, P. D. (2009). Gender differences in pediatric stroke: is elevated testosterone a risk factor for boys? *Ann. Neurol.* 66, 713–714. doi: 10.1002/ana.21925

Vontell, R., Supramaniam, V., Thornton, C., Wyatt-Ashmead, J., Mallard, C., Gressens, P., et al. (2013). Toll-like receptor 3 expression in glia and neurons alters in response to white matter injury in preterm infants. *Dev. Neurosci.* 35, 130–139. doi: 10.1159/000346158

Wakida, N. M., Cruz, G. M. S., Ro, C. C., Moncada, E. G., Khatibzadeh, N., Flanagan, L. A., et al. (2018). Phagocytic response of astrocytes to damaged neighboring cells. *PLoS One* 13:e0196153. doi: 10.1371/journal.pone.0196153

Yang, Y., Liu, B., Dai, J., Srivastava, P. K., Zammit, D. J., Lefrancois, L., et al. (2007). Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. *Immunity* 26, 215–226. doi: 10.1016/j.immuni.2006.12.005

Yellowhair, T. R., Noor, S., Maxwell, J. R., Anstine, C. V., Oppong, A. Y., Robinson, S., et al. (2018). Preclinical chorioamnionitis dysregulates CXCL1/CXCR2 signaling throughout the placental-fetal-brain axis. *Exp. Neurol.* 301(Pt B), 110–119. doi: 10.1016/j.expneurol.2017.11.002

Yousif, N. M., de Oliveira, A. C. P., Brioschi, S., Huell, M., Biber, K., and Fiebich, B. L. (2018). Activation of EP2 receptor suppresses poly(I: C) and LPS-mediated inflammation in primary microglia and organotypic hippocampal slice cultures: contributing role for MAPKs. *Glia* 66, 708–724. doi: 10.1002/glia.23276

Zhu, C., Wang, X., Huang, Z., Qiu, L., Xu, F., Vahsen, N., et al. (2007). Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. *Cell Death Differ.* 14, 775–784. doi: 10.1038/sj.cdd.4402053

Zhu, C., Wang, X., Qiu, L., Peeters-Scholte, C., Hagberg, H., and Blomgren, K. (2004). Nitrosylation precedes caspase-3 activation and translocation of apoptosis-inducing factor in neonatal rat cerebral hypoxia-ischaemia. *J. Neurochem.* 90, 462–471. doi: 10.1111/j.1471-4159.2004.02500.x

Zimmer, S., Steinmetz, M., Asdonk, T., Motz, I., Coch, C., Hartmann, E., et al. (2011). Activation of endothelial toll-like receptor 3 impairs endothelial function. *Circ. Res.* 108, 1358–1366. doi: 10.1161/CIRCRESAHA.111.243246

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Chavez-Valdez, Mottahedin, Stridh, Yellowhair, Jantzie, Northington and Mallard. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 28 March 2019
doi: 10.3389/fphys.2019.00227

Emerging Roles of miRNAs in Brain Development and Perinatal Brain Injury

*Kenta Hyeon Tae Cho[1], Bing Xu[1], Cherie Blenkiron[2] and Mhoyra Fraser[1]**

[1] Department of Physiology, Faculty of Medical Health and Sciences, University of Auckland, Auckland, New Zealand,
[2] Departments of Molecular Medicine and Pathology, Faculty of Medical Health and Sciences, University of Auckland, Auckland, New Zealand

In human beings the immature brain is highly plastic and depending on the stage of gestation is particularly vulnerable to a range of insults that if sufficiently severe, can result in long-term motor, cognitive and behavioral impairment. With improved neonatal care, the incidence of major motor deficits such as cerebral palsy has declined with prematurity. Unfortunately, however, milder forms of injury characterized by diffuse non-cystic white matter lesions within the periventricular region and surrounding white matter, involving loss of oligodendrocyte progenitors and subsequent axonal hypomyelination as the brain matures have not. Existing therapeutic options for treatment of preterm infants have proved inadequate, partly owing to an incomplete understanding of underlying post-injury cellular and molecular changes that lead to poor neurodevelopmental outcomes. This has reinforced the need to improve our understanding of brain plasticity, explore novel solutions for the development of protective strategies, and identify biomarkers. Compelling evidence exists supporting the involvement of microRNAs (miRNAs), a class of small non-coding RNAs, as important post-transcriptional regulators of gene expression with functions including cell fate specification and plasticity of synaptic connections. Importantly, miRNAs are differentially expressed following brain injury, and can be packaged within exosomes/extracellular vesicles, which play a pivotal role in assuring their intercellular communication and passage across the blood–brain barrier. Indeed, an increasing number of investigations have examined the roles of specific miRNAs following injury and regeneration and it is apparent that this field of research could potentially identify protective therapeutic strategies to ameliorate perinatal brain injury. In this review, we discuss the most recent findings of some important miRNAs in relation to the development of the brain, their dysregulation, functions and regulatory roles following brain injury, and discuss how these can be targeted either as biomarkers of injury or neuroprotective agents.

Keywords: perinatal, development, brain injury, miRNAs, biomarkers, exosomes, therapies

OPEN ACCESS

Edited by:
Carina Mallard,
University of Gothenburg, Sweden

Reviewed by:
Angela Leigh Cumberland,
RMIT University, Australia
Amin Mottahedin,
University of Cambridge,
United Kingdom

****Correspondence:***
Mhoyra Fraser
m.fraser@auckland.ac.nz

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *19 August 2018*
Accepted: *21 February 2019*
Published: *28 March 2019*

Citation:
Cho KHT, Xu B, Blenkiron C and Fraser M (2019) Emerging Roles of miRNAs in Brain Development and Perinatal Brain Injury. Front. Physiol. 10:227. doi: 10.3389/fphys.2019.00227

INTRODUCTION

MicroRNAs (miRNAs) are a class of endogenous small single-stranded non-protein coding RNA molecules (20–24 nucleotides), often phylogenetically conserved, which play a critical role in the control of gene expression at the post-transcriptional level. Specifically, miRNAs mainly function post-transcriptionally by binding to the 3′ untranslated region (3′UTR) of target messenger RNAs (mRNA) and induce mRNA degradation or translational repression (Bartel, 2009). In addition to their repressive role there is considerable evidence to support post-transcriptional stimulation of gene expression by miRNAs either in specific situations by direct or indirect mechanisms (Vasudevan, 2012).

Given their abundance in the central nervous system (CNS) and their specific patterns of expression within all of the major cell types during development (Sempere et al., 2004; Cao et al., 2006; Cherubini et al., 2006; Narayan et al., 2015), it is unsurprising that a number of miRNAs have emerged as potential regulators of CNS development and homeostatic function and under pathological conditions of hypoxia-ischemia, as mediators of neuroinflammation and neurodegeneration (Bhalala et al., 2013; Moon et al., 2013). Investigation of the possible relationships between miRNAs and their importance to the developing brain, however, remain in its infancy, since the majority of studies have not biologically validated the effects of miRNAs beyond the predicted mRNA targets. Nevertheless, a growing body of studies have demonstrated a critical role of miRNAs in the maturation of oligodendrocytes and myelin formation including the pathophysiology of hypoxia-ischemia-induced brain injury in the developing brain (Barca-Mayo and Lu, 2012; Fitzpatrick et al., 2015; Galloway and Moore, 2016; Su et al., 2016). In relation to the latter, it is presently unknown whether the roles of specific miRNAs or their profiles differ in response to injury with increasing gestational age. However, it is plausible that differences do indeed exist given their importance developmentally and since the neuropathology of brain injury differs as a function of gestational age. Among term infants the spectrum of injuries is dominated by selective necrosis, accompanied by parasagittal cerebral injury involving the paracentral cerebral cortex and associated white matter and represents a watershed injury in a vascular distribution (Ferriero, 2016; Kinney and Volpe, 2018b). In contrast, preterm infants born between 23 and 32 weeks gestation are at greatest risk of injury to the cerebral white matter. Depending on the severity of the insult, the spectrum of white matter injury in the preterm population can differ markedly. In its most severe form, all cell types are affected including oligodendrocytes, glia and axons resulting in focal cystic necrotic lesions (periventricular leukomalacia) forming within regions of the periventricular white matter adjacent to the lateral ventricular wall, which can extend into the centrum semiovale and the subcortical white matter (Back et al., 2002; Kinney, Volpe, 2018a). Milder forms are typically of a diffuse non-cystic variety and are now the most common type of injury observed in the preterm population. Moreover, the predominant pathology underlying diffuse white matter injury in the preterm infant is loss and subsequent arrested differentiation of pre-myelinating oligodendrocyte progenitors, (Volpe et al., 2011; Buser et al., 2012; Back and Miller, 2014; van Tilborg et al., 2016) which results in reduced brain myelination and potentially could be an avenue for miRNA targeted therapy.

In addition to the aforementioned role of miRNAs in the pathophysiology of perinatal brain injury evidence now suggests CNS cells secrete stable miRNAs into the plasma, which are bound to protein, HDL, or packaged within exosomes/microvesicles following stroke (Rao et al., 2013; Chen et al., 2015; Mondello et al., 2018). As their release is intimately related to genomic changes in the brain, they have immense potential as biomarkers of perinatal brain injury and may lead to early diagnosis, thereby allowing early implementation of treatment. This section will review emerging concepts associated with miRNA control of brain development and discuss their connection to perinatal brain injury impacted by inflammation and hypoxia-ischemia and those, which may serve as potential diagnostic biomarkers of injury and therapeutic targets.

miRNAs IN CNS DEVELOPMENT

Development of the mammalian CNS involves a series of intricately coordinated events that requires precise spatial and temporal control of gene expression at both the transcriptional and translational levels (Taverna et al., 2014; Gotz et al., 2016). As previously mentioned, the brain has an abundance of miRNAs; many are specific to a given cell lineage or cell type with some being shown to vary dynamically within the brain both prior to and after birth, suggesting a need for different miRNAs throughout development (Lagos-Quintana et al., 2003; Miska et al., 2004; Bak et al., 2008; Smith et al., 2010; Podolska et al., 2011; Ziats and Rennert, 2014; Chen and Qin, 2015). The interplay between miRNAs and their target mRNAs have a critical regulatory role during neural development, from early neurogenesis to synaptogenesis as well as maintenance of neural function (**Figure 1**) (Davis et al., 2015). miRNAs interact mainly through downregulation of expression of both intrinsic and extrinsic factors and activities of cell-specific signaling mechanisms, and therefore regulate the establishment and maintenance of cell fate specification and differentiation of neural stem cells and neurogenic niches (Shi et al., 2010; Brett et al., 2011; Barca-Mayo and Lu, 2012).

miRNAs in Neuronal Cortical Development

The biological importance of miRNAs in neural development was first demonstrated by conditional knockout experiments of enzymes involved in miRNA biogenesis (Bernstein et al., 2003). The double-stranded RNA (dsRNA) nuclease Dicer is essential to this process (Petri et al., 2014). In mice, targeted ablation of the *Dicer1* gene affects brain development including impaired cortical neuron migration, microcephaly, and precursor differentiation in the spinal cord (Davis et al., 2008; De Pietri Tonelli et al., 2008). However, such studies do not readily assign roles for specific miRNAs since a deficiency in Dicer will affect the full complement of miRNAs in the targeted cells. Moreover, knockouts of specific miRNAs are often complicated, since bioinformatics analyses predict hundreds of targets for

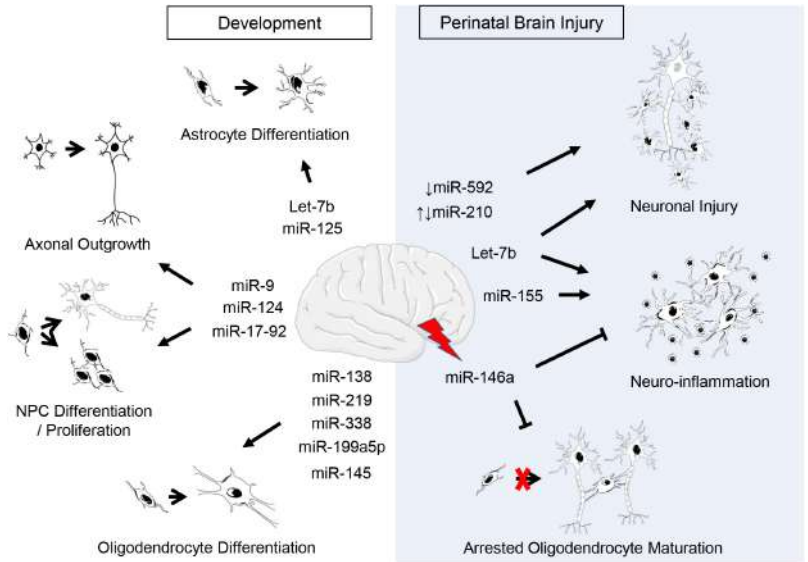


FIGURE 1 | Putative roles of miRNAs in CNS development and perinatal brain injury. Listed are miRNAs covered in this review. *Supporting citations are reviewed in text and summarised below*. **Development:** Let-7b and miR-125 are regulators of astrocyte differentiation (Shenoy et al., 2015). miR-17-92 cluster is involved in neural progenitor cell (NPC) proliferation (Bian et al., 2013) and axonal growth (Zhang et al., 2013). miR-124 regulates neurogenesis (Krichevsky et al., 2006; Makeyev et al., 2007) and axonal growth of retinal ganglion cells (He et al., 2018). miR-9 is implicated in the regulation of NPC differentiation and proliferation (Krichevsky et al., 2006; Radhakrishnan and Alwin Prem Anand, 2016) as well as axonal development and neuronal migration (Dajas-Bailador et al., 2012; Otaegi et al., 2011). miR-219 (Dugas et al., 2010; Shin et al., 2009), miR-338 (Zhao et al., 2010), miR-138 (Dugas et al., 2010), miR-199a-5p (Letzen et al., 2010), miR-145 (Letzen et al., 2010) regulate oligodendrocyte (OL) differentiation. **Perinatal brain injury:** The downregulation of miR-210 is associated with an increase in neuronal apoptosis following hypoxic-ischemic injury (Qiu et al., 2013b) and the upregulation of miR-210 exacerbates cortical injury (Ma et al., 2016; Wang et al., 2017). Post-injury, miR-146a negatively regulates inflammation (Gaudet et al., 2018; Omran et al., 2012) and promotes oligodendrogenesis (Liu et al., 2017). Silencing or inhibition of miR-155 ameliorates inflammation post-injury (Ashhab et al., 2013; Caballero-Garrido et al., 2015; Pena-Philippides et al., 2016; Roitbak, 2018). Let-7b released from neurons and immune cells following injury exacerbates neuronal cell death and induces neuroinflammation (Lehmann et al., 2012; Mueller et al., 2014). The downregulation of miR-592 following hypoxic-ischemic injury induces apoptosis in hippocampal neurons (Sun et al., 2018).

mammalian miRNAs, and it seems likely that many are indeed true targets (Lewis et al., 2003; Lim et al., 2005).

Despite these drawbacks, valuable information has been deemed regarding numerous miRNAs during brain development through loss-of-function and gain-of-function experiments (**Figure 1**). Evidence suggests that miRNAs play an important role in cortical development. For example, the miR-17-92 cluster, together with its paralogs miR-106a-363 and miR-106b-25, is required for appropriate development of embryonic fetal cells (Suh et al., 2004; Ventura et al., 2008). It consists of six miRNAs, processed from a common precursor transcript and grouped in four subfamilies, miR-17, miR-18, miR-19, and miR-92 (Ventura et al., 2008; Bian et al., 2013). A role for miR-17-92 in proliferation has been suggested since phosphatase and tensin homolog (*PTEN*; tumor suppressor gene) is one of its targets (Concepcion et al., 2012). Further, functional role studies have revealed that overexpression of the miR-17-92 cluster in axons of embryonic cortical cells modulates PTEN protein levels and increases axonal growth (Zhang et al., 2013). To confirm additional roles knockout studies of the miR-17-92 cluster and its paralogs have demonstrated an essential role of the miR-17-92 cluster in controlling expansion of neural stem cells and radial glial cells, and transition to intermediate progenitors, which are critical for normal cortical development and function (Bian et al., 2013). Moreover, knockout of miR-17-92 was associated with an upregulation of miR-17-92 target RNAs, *PTEN* and T-box transcription factor Eomes/*Tbr2* (*Tbr2*; a key regulator of neurogenesis in the SVZ), resulting in an increase in intermediate progenitors and suppression of cortical radial glial cells, respectively (Bian et al., 2013).

miR-124, the most abundant miRNA in the brain, is another well-studied regulator of neurogenesis, whose expression increases with commencement of neural differentiation and peaks in mature neurons (Krichevsky et al., 2006; Makeyev et al., 2007; Visvanathan et al., 2007; Cheng et al., 2009; Maiorano and Mallamaci, 2009; Ponomarev et al., 2011; Sanuki et al., 2011; Åkerblom et al., 2012; Sun et al., 2015). Targets of miR-124 include protein jagged-1 (Jag-1), Sry-type high mobility group box 9 (Sox9; involved in adult neurogenesis) and *DLX2* (transcription factor regulating neuronal subtype specification) (Cheng et al., 2009; Liu et al., 2011). Inhibition *in vivo* of miR-124 blocks neurogenesis resulting in a switch to gliogenesis, specifically inducing formation of ectopic astrocytes in the olfactory bulb derived from the subventricular zone (Åkerblom et al., 2012). Furthermore, overexpression experiments both *in vivo* and *in vitro* suggest that miR-124 plays a role in neural fate specification (Smirnova et al., 2005; Krichevsky et al., 2006; Silber et al., 2008; Åkerblom et al., 2012; Xia et al., 2012; Akerblom and Jakobsson, 2014) and most recently promotes

axon growth of retinal ganglion cells differentiated from retinal stem cells (He et al., 2018).

Similarly, miR-9, a neuronal specific miRNA, with a prominent role in development, has also been implicated in the regulation of whether neural precursors will adopt a neuronal or glial fate (Krichevsky et al., 2006; Radhakrishnan and Alwin Prem Anand, 2016). miR-9 is highly expressed within the brain, primarily within neural precursors where it controls neural stem cell numbers (Delaloy et al., 2010; Akerblom et al., 2013; Coolen et al., 2013). Overexpression of miR-9 negatively regulates proliferation and accelerates neural differentiation through suppression of the orphan receptor TLX (human homolog of the tailless gene; also known as nuclear receptor subfamily 2, group E member 1 [Nr2e1]) suggesting that TLX and miR-9 participate in a feedback regulatory loop (Zhao et al., 2009). miR-9 is also involved in cortical axonal development via its target, microtubule-associated protein 1b (*Map1b*) (Dajas-Bailador et al., 2012). Furthermore, neuronal migration and outgrowth is also controlled by miR-9 through its interaction with forkhead transcription factors 1 and 2 (*Foxp1* and *Foxp2*) (Otaegi et al., 2011; Clovis et al., 2012).

miRNAs in Oligodendrocyte Development

Due to the critical roles of miRNAs in neurogenesis, it is unsurprising that miRNAs have also emerged as important regulators of oligodendrocyte development (**Figure 1**). Microarray analysis of miRNA profiles in normal CNS development and *Dicer1* knockout models have identified miR-219 as a crucial regulator of oligodendrocyte differentiation (Shin et al., 2009; Dugas et al., 2010; Zhao et al., 2010). miR-219 is highly expressed in the white matter areas of the brain and expression persists in mature oligodendrocytes (Dugas et al., 2010). Its mechanism of action is via direct repression of expression of its predicted targets, namely platelet-derived growth factor receptor alpha (PDGFRα), SRY-box containing gene 6 (Sox6), forkhead box J3 (FoxJ3), and zinc finger protein 238 (ZFP238), all of which promote oligodendrocyte proliferation and inhibit oligodendrocyte differentiation (Barres et al., 1994; Stolt et al., 2006; Dugas et al., 2010). Transfecting purified oligodendrocytes with miR-219 mimic increases expression levels of early (2′,3′-cyclic nucleotide 3′-phosphodiesterase, CNP; myelin basic protein, MBP) and late (myelin oligodendrocyte glycoprotein, MOG) oligodendrocyte specific differentiation markers (Dugas et al., 2010; Zhao et al., 2010). Furthermore, addition of miR-219 mimic to oligodendrocyte progenitor cells lacking functional *Dicer1* expression and which display deficits in myelin gene expression (CNP, MBP, and MOG), markedly enhanced maturation and restored their expression levels to control transfected cells (Dugas et al., 2010; Zhao et al., 2010). Cumulatively, these data indicate that miR-219 is critical for the coordinated transition of oligodendrocyte progenitor cells to oligodendrocytes and subsequent myelin formation and thus may have potential as a therapeutic strategy to promote myelination following injury.

Other important regulators of oligodendrocyte progenitor differentiation are miR-338 and miR-138 (Lau et al., 2008; Dugas et al., 2010). miR-338 is equally as significant as miR-219 in controlling oligodendrogenesis and shares common targets notably Sox6 and Hes Family BHLH Transcription Factor 5 (Hes5); both of which are negative regulators of myelin gene expression (Liu et al., 2006; Stolt et al., 2006; Dugas et al., 2010; Zhao et al., 2010). Furthermore, miR-338 is upregulated in mature oligodendrocytes (Lau et al., 2008) and its overexpression increases oligodendrocyte differentiation (Zhao et al., 2010). However, the role of miR-138 is somewhat incongruous. While miR-138 expression is also elevated in oligodendrocyte precursors its impact on oligodendrocyte development is less significant than miR-219 and miR-338 (Dugas et al., 2010). In contrast to miR-219, oligodendrocyte progenitors induced to differentiate by miR-138 mimic, only express early oligodendrocyte differentiation markers (CNP, MBP) but not late differentiation markers (Dugas et al., 2010). Moreover, miR-138 inhibits Sox4 transcription factor, a repressor of oligodendrocyte maturation (Potzner et al., 2007; Yeh et al., 2013). Together these findings suggest that miR-138 may play a role in extending the period oligodendrocytes are maintained in the early phase of oligodendrocyte differentiation thereby providing a suitable time frame for terminally differentiating oligodendrocytes to myelinate neighboring axons.

Elegant studies by Letzen et al. (2010), using human embryonic stem cells to investigate miRNA expression profiles have revealed unique patterns of expression during the various stages of oligodendrocyte differentiation and maturation. Specifically, four main clusters of miRNA expression were identified encompassing the breadth of the oligodendrocyte lineage scheme (early, mid, and late progenitors and mature oligodendrocytes). Predicted targets of the top differentially expressed genes included myelin-associated genes namely chromosome 11 open reading frame 9 (*C11Orf9*), myelin gene regulatory factor (*MRF*), claudin-11 (*CLDN11*), myelin transcription factor 1-like (*MYTL1*), myelin-associated oligodendrocyte basic protein (*MOBP*), myelin protein zero-like 2 (*MPZL2*), and discoidin domain receptor tyrosine kinase 1 (*DDR1*). Of interest, the authors showed that within the top 10 differentially expressed miRNAs, spanning early to mid-oligodendrocyte progenitor stages, both miR-199a-5p and miR-145 were strongly biased to *C11Orf9*, a gene considered to play a critical role in oligodendrocyte maturation and myelin production.

Evidence discussed above, thus highlights the need to define the role of miRNAs in normal neurodevelopment since they may lay the foundations for novel miRNA-based therapies for preterm infants at risk of brain injury.

miRNAs in Astroglial and Microglial Development

Within cells of the neural lineage, information on the function of miRNAs is predominately limited to neuronal and oligodendrocyte differentiation. Only a relatively few studies have been conducted to investigate the role of miRNAs in

astrogliogenesis. This is somewhat surprising given astrocytes represent a major glial cell type in the CNS and are powerful homeostatic regulators of brain function (Giaume et al., 2010). Presumably, the difficulty encountered in isolating astrocyte progenitors *in vivo* has been a major constraint when investigating the functions of astrocyte miRNAs. However, in a recent study of glial progenitors induced to differentiate into astrocytes, deletion of all canonical miRNAs by conditional knockout of Dgcr8 (the RNA binding protein involved in processing of all canonical miRNAs) blocked astrocyte differentiation *in vitro* (Shenoy et al., 2015). Such results were also in keeping with Dicer-knockout studies of *in vivo* derived multipotent neural stem cells (Andersson et al., 2010). Furthermore, in the study conducted by Shenoy et al. (2015), let-7 and miR-125, operating through several targets, restored astrocyte differentiation. Additional studies of disruption of both astrogliogenesis and oligodendrogenesis with inhibition of miRNA formation in ventral spinal progenitors from $Olig1^{Cre}$ – mediated Dicer conditional knockout mice provide further support for miRNAs role in gliogenesis (Zheng et al., 2010, 2012). It is also important to note that a recent study has provided unprecedented evidence of miRNA expression profiles of astrocytes isolated by laser capture microdissection from various regions within the human second trimester fetal brain (17–20 weeks gestation) and adult brain (24–76 years) with no discernible pathology (Rao et al., 2016). Regional differences were noted in these studies, as well as lower expression of miRNAs in fetal vs. adult white matter astrocytes and high expression of miRNAs in the fetal germinal matrix, which presumably is of relevance in pathological conditions.

Microglia are another major glial population. Depending on their location within the CNS, microglia can vary in morphology and density and have important functions in immune surveillance, mediating innate immune responses. In recent years there has been an exponential increase in investigations focussing on the function and regulation of microglia by intrinsic and extrinsic factors within the developing and adult brain under both normal and abnormal physiological conditions (Baburamani et al., 2014; Katsumoto et al., 2014; Nayak et al., 2014; Hagberg et al., 2015; Michell-Robinson et al., 2015; Reemst et al., 2016; Li et al., 2017; Tay et al., 2017; Thion et al., 2018). However, to date, Ponomarev et al. (2011, 2013) have performed the only studies thus far on the role of miRNAs in microgliogenesis within the CNS and have demonstrated that miR-124 is highly expressed in normal CNS-resident microglia, but absent in peripheral monocytes and macrophages. As discussed previously (see section "miRNAs in Neuronal Cortical Development"), miR-124 is also highly expressed in other regions of the CNS and is an important regulator of neurogenesis and neuronal differentiation through its regulation of neuronal gene expression.

Ponomarev et al. (2011, 2013) also showed that miR-124 is a key promoter of the quiescent state of microglia. By forced overexpression of miR-124 in macrophages they were able to demonstrate that miR-124 negatively modulates CCAAT/enhancer-binding protein-α (C/EBP-α) transcription factor, and its downstream target PU.1, resulting in their transformation from an activated to a quiescent phenotype (Ponomarev et al., 2011, 2013). Furthermore, knockdown of miR-124 in microglia and macrophages returned both cells into an activated state (Ponomarev et al., 2011). Thus, this supports a role for miR-124 in the maintenance of a resting phenotype through targeting of the CEBPα/PU.1 pathway and possibly is a way to establish an "alternative" activation (M2) phenotype in resident microglia as part of the reparative response to hypoxia-ischemia- or infection-related neuroinflammation (see section "miRNAs and Neuroinflammation"). Finally, it is apparent that there is a need to identify other candidate miRNAs who may participate in developmental regulation of astrocytes and microglia.

ROLE OF miRNAs IN PERINATAL BRAIN INJURY

An extensive body of literature is now available to suggest dysregulation of miRNA biogenesis and their regulatory role is a common theme associated with the development of neurological injury and disorders from adult experimental models and patients (Dharap et al., 2009; Tan et al., 2009; Liu et al., 2010; Yuan et al., 2010; Bhalala et al., 2013; Eacker et al., 2013; Khanna et al., 2013; Moon et al., 2013; Wang and Yang, 2013; Ouyang et al., 2014). Accordingly, given this and evidence of miRNAs regulatory role during all stages of CNS development, there has been an emerging interest into the implications of miRNAs in perinatal brain injury (**Figure 1**).

HypoxamiRs

Insults such as impaired oxygen delivery or hypoxia has the potential to elicit expression of a distinct group of miRNAs known as hypoxamiRs, that according to the miRbase database (Griffiths-Jones, 2006; Kozomara and Griffiths-Jones, 2014) are in excess of a 100. Importantly, the specific hypoxamiR signature in response to hypoxia varies according to cell type affected and physiological response (Kulshreshtha et al., 2007; Nallamshetty et al., 2013).

Hypoxic regulation of miR-210, considered to be the master hypoxamiR, was first identified by miRNA microarray over a decade ago (Kulshreshtha et al., 2007) and has been shown to be consistently upregulated under various hypoxic conditions (Huang et al., 2010; Chan et al., 2012). Indeed, multiple studies involving adult models of ischemic stroke have consistently shown that miR-210 induction is a feature of the hypoxic response (Jeyaseelan et al., 2008; Dharap et al., 2009; Qiu et al., 2013b; Liu et al., 2018; Meng et al., 2018). Furthermore, in terms of neurogenesis, studies are contradictory in relation as to whether miR-210 inhibition increases neurogenesis following ischemia (Zeng et al., 2014; Ma et al., 2016; Voloboueva et al., 2017). Such differences presumably relate to timing of miR-210 inhibition, since evidence points to a reduction in proliferation with early post-ischemic inhibition, whereas later it increases neurogenesis (Voloboueva et al., 2017).

Similar controversy exists in relation to the immature brain, as evidence from various neonatal stroke models suggest miR-210 may play either a protective or a detrimental role

(Ma et al., 2016). For instance, Qiu et al. (2013a), using a PC12 cell model of oxygen glucose deprivation reported that miR-210 reduced PC12 cell death. The same group demonstrated that in PD (postnatal day; day of birth = postnatal day 0) 7 neonatal rats, miR-210 expression is downregulated in response to hypoxia-ischemia in association with increased brain edema (Qiu et al., 2013b) and that pretreatment with miR-210 mimic significantly reduced edema indicating a possible protective role in response to ischemia.

In contrast, Ma et al. (2016) reported that miR-210 is upregulated following a 2.5 h period of hypoxia-ischemia in PD10 neonatal rats. Furthermore, they demonstrated that miR-210 directly targets the 3′UTR region of the glucocorticoid receptor (GR) in the neonatal rat brain and down regulates GR protein following hypoxia-ischemia resulting in increased susceptibility to injury. In the same study, silencing of miR-210 by intracerebroventricular (ICV) administration of complementary locked nucleic oligonucleotides (miR-210-LNA, miR-210 inhibitor), 4 h after hypoxia-ischemia, significantly ameliorated neuronal injury and infarct size in association with a reduction in brain miR-210 levels. Interestingly, intranasal administration of miR-210-LNA under the same conditions resulted in similar effects. Additional studies by Ma et al. (2017), revealed ICV administration of miR-210 mimic in neonatal rats 48 h prior to hypoxic-ischemic injury compromised blood–brain barrier integrity by suppressing junction proteins, thus resulting in increased susceptibility to brain edema and immunoglobulin G (IgG) parenchyma leakage across the blood–brain barrier.

Finally, in a neonatal rat model of perinatal nicotine-sensitized hypoxic-ischemic brain injury, prior treatment with nicotine was associated with increased miR-210 expression, decreased brain-derived neurotrophic factor/tropomyosin receptor kinase B (BDNF/TRKB) protein expression, and increased susceptibility to hypoxic-ischemic injury (Wang et al., 2017). Moreover, ICV administration of miR-210-LNA 48 h before hypoxia-ischemia significantly decreased brain infarct size in both saline control and nicotine-treated cohorts to levels comparable. To conclude, since a number of verified and putative targets have been identified for miR-210 (Chan and Loscalzo, 2010), it is likely that there are contradictions found with respect to miR-210-specific effects as mentioned above. Such putative roles in relation to perinatal brain injury await corroboration that is more definitive.

miRNAs and Oligodendroglial Response to Hypoxia-Ischemia

Studies highlighting miRNAs as key regulators of oligodendrocyte development may have significant clinical implications with respect to further understanding the pathogenesis of preterm hypoxia-ischemia brain injury, since loss and subsequent arrested differentiation of oligodendrocyte progenitors is a hallmark of injury. Presently, there is a paucity of information with regard to how miRNA expression contributes to critical events of oligodendrogenesis occurring in response to hypoxia-ischemia within the developing brain.

Recently, however, the role of miRNAs in perinatal hypoxia-ischemia has been evaluated in NG2 specific *Dicer1* knockout mice (Birch et al., 2014). Loss of Dicer within oligodendrocyte progenitors following hypoxia-ischemia increased both the number of mature oligodendrocytes and MBP expression, which was associated with improved motor co-ordination performance. Furthermore, in the same study, miRNA profiling within lesion sites of wild-type mice, demonstrated delayed but significant increases in miR-138 and miR-338, 7 days following hypoxia-ischemia. These findings are difficult to resolve since *Dicer1* knockout would normally result in myelin loss and since miR-138 and miR-338 increases with oligodendrocyte differentiation, which was shown to be impaired with hypoxia-ischemia. The authors, however, proposed that mature miRNAs upregulated in response to hypoxia-ischemia may increase oligodendrocyte progenitor proliferation rate and thus decrease inversely differentiation. Further studies are required to address the roles of these miRNAs in this model of perinatal hypoxia-ischemia.

miRNAs and Neuroinflammation

Inflammatory responses play key roles in the regulation of neurodevelopment, neurodegeneration and injury. Due to their capacity to regulate simultaneously a cascade of different genes, miRNAs are well placed as key regulators of neuroinflammation and their dysfunction is equally recognized as contributing to adverse neuroinflammatory processes (Su et al., 2016). Depending upon the target mRNAs and stimulant involved, miRNAs can exhibit functions that are either pro-inflammatory, anti-inflammatory, and/or mixed immunomodulatory in nature.

The most notable of these miRNAs are miR-155 and miR-146a. While miR-155 has both pro- and anti-inflammatory functions (Duan et al., 2016), it is widely considered to be the most potent pro-inflammatory miRNA (Gaudet et al., 2018), and recognized as a key regulator of microglial-mediated immune responses (Cardoso et al., 2012; Butovsky et al., 2015). In the context of adult cerebral ischemia, there is substantial evidence that silencing or inhibition of miR-155 ameliorates the damaging effects of neuroinflammation (Liu et al., 2010; Caballero-Garrido et al., 2015; Pena-Philippides et al., 2016; Roitbak, 2018). Importantly, miR-146a, a negative regulator of inflammation, is characteristically upregulated in the pathogenesis of various neurological conditions (Gaudet et al., 2018) and considered to play a key role in the regulation of cell survival responses by negative regulation of Toll-like receptor 4 (TLR4) through targeting tumor necrosis factor (TNF) receptor-associated factor 6 (*TRAF6*) and interleukin-1 receptor-associated kinase 1 (*IRAK1*) genes in innate and adaptive immune cells (Taganov et al., 2006; Baltimore et al., 2008; Mann et al., 2017). In addition, miR-146a is a key of regulator of oligodendrogenesis both in the normal (Galloway and Moore, 2016) and ischemic brain (Liu et al., 2017) and a negative-feedback regulator of astrocyte-mediated inflammation (Taganov et al., 2006; Iyer et al., 2012).

While growing evidence has revealed that several miRNAs including miR-155 and miR-146a regulate the extent and timing of TLR responses and innate immune pathways (Taganov et al., 2006; O'Connell et al., 2007; Nahid et al., 2011; O'Neill et al., 2011; Quinn and O'Neill, 2011; Lehmann et al., 2012), little is known about their roles in modulation of neuroinflammation

within the immature brain following injury. A study to examine the effect of inflammation on epileptogenesis revealed that miR-146a is upregulated in both a PD11 neonatal rat pilocarpine model of mesial temporal lobe epilepsy (MTLE) and children with MTLE and suggest miR-146a modulates the inflammatory response triggered by interleukin-1β (IL-1β) by inhibiting its expression level thus supporting a neuroprotective role for miR-146a (Omran et al., 2012). Further, studies from the same group and animal model revealed that miR-155 and TNF-alpha (TNF-α) is upregulated in seizure-related acute and chronic stages of MTLE (Ashhab et al., 2013). Similar dysregulation was also observed in children with MTLE, thus supporting a role for miR-155 and TNF-α in the development of seizure susceptibility in the immature brain.

Additional support for miR-146a pro-survival functions is also provided by a recent study conducted in PD1 neonatal rats exposed to hypoxia, in conjunction with BV-2 cells (Zhou et al., 2015). The authors showed that treatment with thymosin β4 (Tβ4), a major actin-sequestering protein, known to reduce inflammation and stimulate remyelination after neurological injury (Morris et al., 2010; Xiong et al., 2012), inhibited microglial activation and was associated with *in vitro* upregulation of miR-146a expression (Zhou et al., 2015). Interestingly, Tβ4 upregulation of miR-146a has been shown to promote oligodendrocyte differentiation and suppression of TLR pathways, thus adding to its therapeutic implications (Santra et al., 2014). Furthermore, lipopolysaccharide (LPS) *in vitro* stimulation of newborn cord blood results in upregulation of miR-146a expression in monocytes implicating its involvement in neonatal innate immune responses (Lederhuber et al., 2011). Similarly, studies of miRNA expression profiles from leukocytes isolated from newborn whole cord blood following LPS *in vitro* stimulation show a total of 85 miRNAs are differentially expressed of which several are proposed to modulate TLR inflammatory pathways (Chen J. et al., 2014). As previously discussed (see section "miRNAs in Astroglial and Microglial Development"), miR-124 is another example of a miRNA that regulates CNS inflammation and is highly expressed in microglia and can reduce CNS inflammation through promotion of microglia quiescence via the C/EBP-α-PU.1 pathway. Consequently, overexpression of miR-124 in microglia can induce a switch to M2 polarization, shown by expression of interleukin-10 (IL-10) and transforming growth factor β (TGF-β) (Ponomarev et al., 2011, 2013). Indeed, miR-124 could potentially become a powerful therapeutic strategy for alleviating brain injury in the perinatal period.

Let-7b, a highly abundant miRNA (Pena et al., 2009) and regulator of gene expression in the CNS, released from injured neurons and immune cells, has been demonstrated to exacerbate CNS injury through activation of TLR7 and induce neurodegeneration through neuronal TLR7 (Lehmann et al., 2012). Furthermore, ICV administration of an antagomir to let-7f, another let-7 family member, has been demonstrated to reduce cortical and striatal infarcts in an adult rat stroke model and be preferentially expressed in microglia within the ischemic boundary zone (Selvamani et al., 2012). Recently Mueller et al. (2014), demonstrated that a synthetic peptide analogous to the mammalian preimplantation factor (PIF) secreted by embryos and which is present in the maternal circulation during pregnancy inhibits let-7 miRNA biogenesis in both murine N2a neuroblastoma cells and RAW 264.7 macrophage cell lines. Using a PD3 neonatal rat hypoxic-ischemic brain injury model these authors then showed that subcutaneous administration of synthetic PIF 3 days after injury, significantly abolished the cortical volume reduction, neuronal loss and microgliosis associated with injury in this model 10 days after injury. Although the neuroprotective mechanism remains unclear these authors provided data to suggest that TLR4 may play an important role in synthetic PIF-induced let-7 repression and that KH-type splicing regulatory protein (KSRP) known to be involved with the biogenesis of the let-7 family of miRNAs and a mediator of mRNA decay (Repetto et al., 2012) may be an interacting cofactor involved with this process. Characterization of the specific signaling pathways activated is required to elucidate the significance of this potential pathway in mediating neuroprotection of the developing brain.

miRNAs AS POTENTIAL BIOMARKERS

There are numerous other brain-specific miRNAs known to play potentially crucial roles in the pathological processes of adult brain injury, whose roles in relation in perinatal brain injury have yet to be determined. Nevertheless, recent studies have focussed on identification of several miRNAs as potential biomarkers of perinatal brain injury to enable early diagnosis of the severity of injury (**Table 1**).

Studies, conducted by Looney et al. (2015), involving the analysis of umbilical cord blood miRNA profiles from a cohort of 70 newborn infants [18 controls, 33 with perinatal asphyxia in the absence of hypoxic ischemic encephalopathy (HIE), and 19 infants with HIE analysis], have revealed 70 miRNAs that are differentially expressed with injury. Notably, miR-374a was significantly downregulated in infants with electroencephalographic (EEG) confirmed HIE vs. controls, and further substantiated by quantitative real-time PCR analysis. While no functional mechanism of action and pathways were confirmed, target analysis revealed specific pathways and biological processes associated with neurological injury. Further research from the same group identified several potential downstream targets of this miRNA, namely activin-A receptor type IIb (*ACVR2B*) (Looney et al., 2017). Despite the lack of confirmation of a significant increase in activin-A levels, as previously demonstrated in biological fluids following perinatal asphyxia and HIE (Florio et al., 2004; Florio et al., 2007; Douglas-Escobar and Weiss, 2012), significantly increased levels of ACVR2B were detected in infants with severe HIE. Of interest, however, is the recent demonstration by Dillenburg et al. (2018) that overexpression of Acvr2b in oligodendroglial lineage cells impairs Acvr2a-regulated oligodendrocyte differentiation and myelin formation, thus supporting the possibility of restoration of Acvr2a-mediated signaling as a strategy to combat perinatal white matter injury.

Aside from the above clinical investigation, a recent study of global hypoxia-ischemia in newborn piglets has also provided

TABLE 1 | Potential miRNA biomarkers of perinatal hypoxia-ischemia.

miRNA biomarkers of perinatal HI	Studied cohorts/subjects	Sampling source	Techniques employed for miRNA detection	References
↓ miR-374a	70 newborn infants 18 controls, 33 with perinatal asphyxia in the absence of HIE, 19 infants with HIE	Umbilical cord blood	Microarray, qRT-PCR	Looney et al., 2015
↑ miR-374a ↑ miR-210	13 newborn piglets 5 controls, 8 with transient global HI	Plasma sample	qRT-PCR	Garberg et al., 2016
↑ miR-210	P10 rat pups 4 controls, 4 with right carotid ligation induced HI	Ipsilateral hemisphere	qRT-PCR	Ma et al., 2016
↑ miR-21	78 newborn infants 29 controls, 49 with asphyxia	Serum sample	qRT-PCR	Chen and Yang, 2015
↑ miR-210 ↑ miR-424 ↑ miR-21 ↑ miR-199a ↑ miR-20b	24 infants 12 controls, 12 severely growth restricted preterms	Maternal whole blood sample	qRT-PCR	Whitehead et al., 2013

HIE: hypoxic-ischemic encephalopathy, HI: hypoxia-ischemia, qRT-PCR: quantitative real-time polymerase chain reaction.

evidence to support circulating plasma miR-374a and the hypoxamiR miR-210 as potential biomarkers (Garberg et al., 2016). However, in contrast to Looney et al. (2015), these authors reported a significant upregulation of miR-374a 9.5 h after hypoxia-ischemia and noted that correlations were found between miR-374a and arterial pH, base excess and lactate levels over the study period. Since miR-374a is directly regulated by lactate dehydrogenase A with hypoxia (Wang et al., 2015), the authors concluded that miR-374a might play a role in metabolic adaptive responses to hypoxia-ischemia. Nevertheless, the increase in miR-210 is in congruence with previous studies under a hypoxic environment (Huang et al., 2010; Chan et al., 2012) and those observed by Ma et al. (2016) following hypoxia-ischemia in PD10 neonatal rats.

Other candidate miRNAs have been investigated, namely let7b, miR-29b, miR-124, miR-155, and miR-21 (Ponnusamy et al., 2016). Quantification of these miRNAs in dried blood spots, EDTA-blood, plasma and urine collected from a small cohort of newborns, failed to demonstrate significant differences with injury. However, miR-21, which is expressed in astrocytes, and been shown in adult plasma to be a potential early stage marker of acute cerebral infarction (Zhou and Zhang, 2014), was found to be elevated in serum of 49 neonates with HIE, thus providing support also for an early diagnosis biomarker of neonatal HIE (Chen and Yang, 2015).

Additional studies that warrant mention are preliminary studies conducted by Whitehead et al. (2013). An analysis was made of maternal whole blood expression levels of six miRNAs known to be associated with hypoxia in which fetuses had either experienced acute hypoxia during labor or chronic hypoxia associated with fetal growth retardation. Compared to gestational matched controls there was an upregulation of miR-210, miR-424, miR-21, miR-199a, and miR-20b. Furthermore, correlation with Doppler velocimetry assessments of hypoxia, confirmed the increase was associated with increased severity of hypoxia. The changes observed in miR-210 agree well with that of Ma et al. (2016) and those conducted in the piglet (Garberg et al., 2016) thus supporting the use as a maternally based biomarker of fetal hypoxia.

A caveat: while the field of miRNA research is constantly expanding one must appreciate that the endogenous source of miRNAs within available body fluids including, plasma, serum, urine and saliva can be from a diverse array of peripheral tissue cellular types including those of the brain, thus decreasing in essence the reliability of results. The other point to note is the problem of cell specification of miRNAs; the reality for many miRNAs as biomarkers in body fluids is that they may not be expressed exclusively within one particular cell type. In the last few years, however, since the identification of exosomes [cell-derived vesicles; typically ∼40–100 nm in diameter (Raposo and Stoorvogel, 2013)], as a carrier of protein, lipids, mRNAs and miRNAs, with an important role in cell–cell-communication, there has been intense interest into whether brain-derived exosomes could serve better as biomarkers in the clinical diagnosis and management of brain injury (Valadi et al., 2007; Ludwig and Giebel, 2012; Patz et al., 2013; Taylor and Gercel-Taylor, 2014; Werner and Stevens, 2015). Additionally, unlike free circulating miRNAs, miRNAs are inherently enriched and stable within exosomes (Cheng et al., 2014). In the adult, increased levels of exosomes are released from cells following stroke and traumatic brain injury (Patz et al., 2013; Chiva-Blanch et al., 2016). Based on current information, it is apparent that brain-derived exosomes can traverse across the blood–brain barrier following injury. Their presence in the peripheral circulation places them in an ideal position to provide an informative platform for real-time assessment of newborns who have sustained brain injury or those who are at risk of adverse outcomes and to spearhead therapeutic discovery. However, such enthusiasm must be tempered by the harsh reality that proportionally brain-derived exosomes may represent only a small population of circulating exosomes whereas the contribution from peripheral sources may be relatively high in comparison. Although still in its infancy, platforms that employ

microscale structures (e.g., microfluidics or acoustofluidics) (Contreras-Naranjo et al., 2017; Guo et al., 2018; Hisey et al., 2018; Li et al., 2018; Wu et al., 2018) and high-throughput phenotypic and functional analyses (e.g., advanced imaging flow cytometry) (Mastoridis et al., 2018) could circumvent this problem. If advanced to such a degree as to provide a rapid and effective means to selectively sort and detect brain-derived exosomes at a nanoscale level they would be of significant value for clinical evaluation.

In the adult, only a few clinical studies have been performed to assess the potential of exosomal-derived miRNAs as biomarkers of acute brain injury. Studies by Chen et al. (2017) demonstrated there was an association between increased circulating levels of exosomal miR-223 and acute ischemic stroke occurrence, stroke severity, and short-term outcomes. Furthermore, studies conducted by Ji et al. (2016) showed that serum exosomal miR-9 and miR-124 levels were positively associated with adverse scores of acute stroke injury, infarct volumes, and serum concentrations of the pro-inflammatory cytokine, interleukin-6 (IL-6). Currently, however, little is known of the usefulness of exosomal-derived miRNAs in the diagnosis of perinatal brain injury. Nevertheless, in a recent study conducted to investigate whether exosomal protein biomarkers would be valuable in the diagnosis of brain injury and assessment of the effectiveness of hypothermia, it was shown that neutral or decreasing serum neuronally derived exosomal synaptopodin protein levels occurred in neonates with abnormal neuroimaging scores (Goetzl et al., 2018).

There is also a growing interest as to the regenerative utility of exosomal-derived contents with and without loading with therapeutics (Doeppner et al., 2015; Luarte et al., 2016; Xiong et al., 2017; Kim et al., 2018). Several studies have also documented the neuron healing and protective abilities of stem cell derived exosomes (Lee et al., 2013; Zhang et al., 2015b; Long et al., 2017; Willis et al., 2017). Importantly, studies recently reported highlight the regenerative potential of mesenchymal stem cell (MSC)-derived extracellular vesicles in a preterm fetal sheep model of hypoxia-ischemia (Ophelders et al., 2016). In these studies, systemic administration to the fetus of MSC-extracellular vesicles resulted in improved brain activity namely a reduction in duration and number of seizures. Finally, as discussed later in Section "*In vivo* Evidence," it warrants mentioning that because exosomes can potentially act as a therapeutic delivery system they hold great promise in revolutionizing the way we can effectively treat perinatal brain injury.

miRNAs AS POTENTIAL THERAPEUTIC TARGETS

In alliance with the discovery of miRNAs as functional regulators of cell development, miRNAs have also been shown to orchestrate a variety of critical signaling pathways involved in injury progression and recovery (Shi et al., 2010; Gaudet et al., 2017). Given the recent demonstration that modulation of miRNA expression occurs following a hypoxic-ischemic insult in the developing brain, several therapeutic targets have emerged (**Figure 2**).

In vitro Evidence

In vitro studies have provided insights into the therapeutic potential of miRNAs that regulate reparative processes following a hypoxic-ischemic insult. For example, stroke-induced downregulation of miR-9 and miR-200b expression in the ischemic white matter region mediated serum response factor (SRF) induced differentiation of oligodendrocyte precursor cells (OPCs) into oligodendrocytes (Buller et al., 2012). Accordingly, *in vitro* overexpression of miR-9 and miR-200b suppressed SRF expression and inhibited OPC differentiation (Buller et al., 2012). Upon validation *in vivo*, the inhibition of miR-9 and miR-200b following injury may indicate a potential therapeutic strategy in the future given that myelination disturbances in the cerebral white matter represent a hallmark of perinatal brain injury (Pandit et al., 2013; Back and Miller, 2014).

Chondroitin sulfate proteoglycans (CSPGs) are well-characterized inhibitory extracellular matrix molecules expressed by reactive astrocytes, endothelial and oligodendrocyte progenitor cells that inhibit axonal regeneration after injury and are associated with adverse neurological outcome in preterm infants (McKeon et al., 1999; Jones et al., 2002, 2003; Chow et al., 2005). *In vitro* overexpression of miR29c and miR-17-92 cluster in embryonic cortical neurons has been shown to attenuate the inhibitory effect of CSPG by stimulating intrinsic axonal signals, suppressing Ras homolog gene family, member A (RhoA) and phosphate and tensin homolog (PTEN) protein levels, thereby promoting axonal outgrowth (Park et al., 2008; Zhang et al., 2013, 2015a). Thus, the potential loss or impaired axonal growth observed in focal necrotic white matter injury in the preterm brain could be feasibly targeted (Riddle et al., 2012).

miR-592 was originally suggested as a possible target for promoting cell apoptosis in various cancers (Liu M. et al., 2015; Liu Z. et al., 2015; Fu et al., 2016). Unsurprisingly, recent studies have also supported its regulatory role of cell death following cerebral ischemic injury (Irmady et al., 2014; Sun et al., 2018). In two studies carried out by Irmady et al. (2014) and Sun et al. (2018), both authors observed reduced expression of miR-592 following cerebral ischemic injury in the hippocampus of neonatal and juvenile mice, respectively. Concordantly, overexpression of miR-592 in cultured hippocampal neurons attenuated the activation of pro-apoptotic signaling and cell death (Irmady et al., 2014; Sun et al., 2018). The mechanism underlying this protective mechanism speculates the multi-functional role of miR-592. Sun et al. (2018) demonstrated that miR-592 affords neuroprotection by selectively targeting prostaglandin D2 receptor (PTGDR) and inhibiting prostaglandin D2 (PGD2)-DP signaling, an inflammatory pathway involving the release of glutamate (Weaver-Mikaere et al., 2013). Irmady et al. (2014), on the other hand revealed that vector mediated transfection of miR-592 in embryonic hippocampal neurons attenuated the level of neurotrophin receptor (NTR) p75 induced by ischemic injury and subsequent apoptotic cell death. The NTR p75 is a member

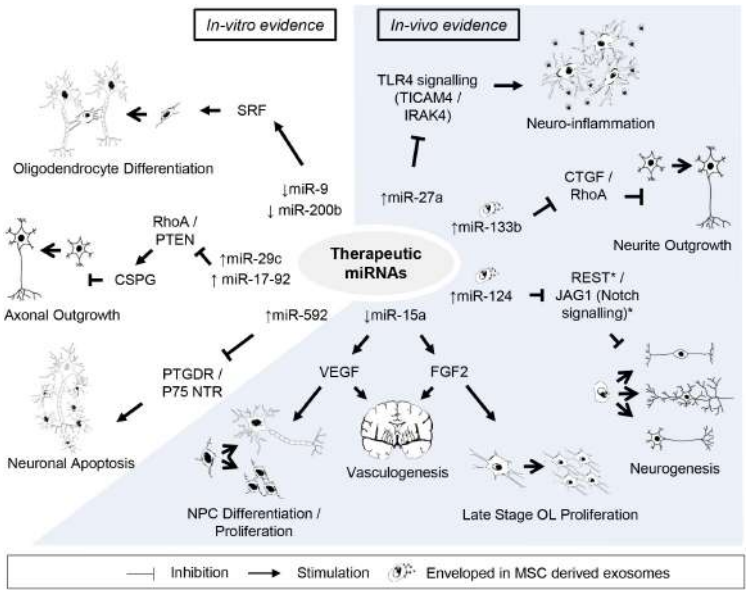


FIGURE 2 | Potential candidates of miRNA-based therapy against perinatal hypoxic-ischemic brain injury. Listed are miRNAs and their downstream targets supporting potentially beneficial outcomes after perinatal hypoxic-ischemic brain injury. *Supporting citations are reviewed in text and summarised below*. ***In-vitro* evidence:** Downregulation of miR-9 and miR-200b mediates serum response factor (SRF) induced oligodendrocyte progenitor cell (OPC) differentiation (Buller et al., 2012). miR-29c (Yi Zhang et al., 2015) and miR-17-92 cluster (Zhang et al., 2013) attenuates the inhibitory effect of chondroitin sulphate proteoglycans (CSPG) on axonal regrowth by stimulating ras homolog family member A (RhoA) and phosphate and tensin homolog (PTEN) protein levels. miR-592 attenuates the activation of pro-apoptotic signalling and neuronal death by targeting the prostaglandin D2 receptor (PTGDR) and neurotrophin receptor (NTR) p75 (Irmady et al., 2014; Sun et al., 2018). ***In-vivo* evidence:** miR-27a reduces TLR4-mediated inflammation (Li et al., 2015). Downregulation of miR-15a expression promote vasculogenesis by stimulating neurotrophic factors, fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) (Yin et al., 2012), which can further support late stage oligodendrocyte (OL) proliferation/migration (Shindo et al., 2016) and neural progenitor cell (NPC) differentiation/proliferation (Teng et al., 2008), respectively. Mesenchymal stem cell (MSC) derived exosomal transfer of miR-133b enhanced neurite outgrowth by inhibiting connective tissue growth factor (CTGF) and RhoA expression (Xin et al., 2012, 2013). Exosome mediated neuronal delivery of miR-124 induces neurogenesis (Yang et al., 2017) speculatively via Usp14-dependent REST degradation (Doeppner et al., 2013) and inhibition of the JAG/Notch signalling pathway (Liu et al., 2011).

of the TNF receptor superfamily closely implicated with neuronal apoptosis following experimental perinatal brain injury (Volosin et al., 2006; Griesmaier et al., 2010). Given the prospective dual anti-apoptotic mechanism of miR-592, results of future *in vivo* studies are eagerly awaited.

In vivo Evidence

miR-27 is a potential regulator of cortical neuronal apoptosis whose expression in embryonic mouse cerebral cortices is attenuated in response to maternal hypoxia (Chen Q. et al., 2014). Furthermore, neuron-specific over-expression of miR-27b in the mouse cortex increased resistance to hypoxia induced apoptosis by inhibiting apoptotic protease-activating factor 1 (Apaf-1) (Chen Q. et al., 2014). Similar observations have been reported in rat primary embryonic hippocampal neuron cultures (Cai et al., 2016) and further potential targets and mechanisms of the miR-27 family have been alluded. For example, miR-27a, directly modulates components of the TLR4 signaling cascade, including TIR domain-containing adaptor molecule-2 (TICAM2) and interleukin-1 receptor-associated kinase 4 (IRAK4), cytoplasmic proteins that link TLR4 and recruit to adaptor protein MyD88 following TLR4 activation, respectively, and coordinates gene transcription and inflammation (Li et al., 2015; Lv et al., 2017). Prophylaxis treatment with miR-27a mimics in an ischemic reperfusion model results in reduced mRNA and protein expression of TICAM2 accompanied by attenuation of TLR4 activation and pro-inflammatory cytokine production, while pretreatment with miR-27a inhibitory oligonucleotides show opposite effects (Li et al., 2015). Comparable anti-inflammatory effects of miR-27a have also been observed in cultured neonatal microglial cells which were achieved by targeting IRAK4 and TLR4 (Lv et al., 2017). Speculatively, miR-27a can target multiple genes and regulate the TLR4 signaling pathway to prevent an excessive inflammatory response to injury. Indeed, various animal models of perinatal brain injury have shown that TLR4 activation and the ensuing inflammatory response can result in cell death and a pattern of injury similar to that seen in human infants, including hypomyelination, glia activation and disruption of thalamocortical function (Dean et al., 2011; Kannan et al., 2011; Dhillon et al., 2015). Therefore, the anti-apoptotic and anti-inflammatory effects of miR-27a/b may prove to be a potential therapeutic target in the future.

Cerebral angiogenesis is a critical reparative process of the microvasculature following hypoxic-ischemic injury, involving cellular cross-talk through neurotropic factors, improving regional blood supply, and facilitating the migration of neurons toward damaged regions (Ohab et al., 2006; Yin et al., 2015). Modulating this reparative process holds promise since

the perinatal brain has the greatest potential for repair and recovery (Dzietko et al., 2013). miR-15a in vascular endothelial cells has emerged as a key regulator of angiogenesis, such that downregulation of miR-15a promotes vasculogenesis by increasing neurotrophic factors, including fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) (Yin et al., 2012). Critically, VEGF released by angiogenic endothelial cells can promote proliferation and differentiation of neural progenitor cells via vascular endothelial growth factor receptor 2 (VEGFR2) (Teng et al., 2008). FGF2 is also an important growth factor involved in neurogenesis and gliogenesis during embryonic and postnatal development (Vaccarino et al., 1999). Crosstalk between cerebral endothelium and oligodendrocytes can promote proliferation and migration of late-stage OPCs through FGF2 (Shindo et al., 2016). Given that delayed treatment with VEGF and FGF2 have proven to be neuroprotective in perinatal models of brain injury (Monfils et al., 2006; Dzietko et al., 2013), miR-15a may be an attractive therapeutic target in the tertiary phase of injury (Fleiss and Gressens, 2012). In fact, it may pose advantages given its ability to target multiple genes in addition to delivering a synergistic effect.

MSCs have been extensively applied in both experimental and clinical settings of CNS diseases owing to their immunomodulatory, regenerative and reparative properties including stroke (Koh et al., 2008; Steinberg et al., 2016), multiple sclerosis (Zhang et al., 2005; Gerdoni et al., 2007; Uccelli et al., 2011) and perinatal brain injury (van Velthoven et al., 2010; Jellema et al., 2013; Drommelschmidt et al., 2017). Currently, it is proposed that MSCs exert their therapeutic potency at least in part through a paracrine mechanism involving the release of extracellular vesicles, which based on their size and intracellular origin include microvesicles (∼100–1000 nm in diameter) and exosomes (∼40–100 nm in diameter) (Hass and Otte, 2012; Mokarizadeh et al., 2012; Xin et al., 2012; Lee et al., 2013; Koniusz et al., 2016). Indeed, MSCs are prolific producers of extracellular vesicles; a feature that is maintained with immortalization of cells to generate permanent cell lines, making them an ideal option for biological tissue replacement regeneration (Yeo et al., 2013).

MSC-derived extracellular vesicles are enriched with a variety of proteins and different RNA species (mainly mRNA and miRNA) as well as trophic factors whose functions are linked to MSCs biological effects. Importantly, evidence now suggests that specific miRNAs are necessary to mediate MSC-derived extracellular vesicles neuroprotective effect (Xin et al., 2012). While miRNAs encapsulated within MSC derived microvesicles are predominantly in their precursor form (pre-miRNAs) (Chen et al., 2009), studies have demonstrated the presence and biological functional roles of exosomal mature miRNAs (Koh et al., 2010; Katakowski et al., 2013; Ono et al., 2014). The transfer of miR-133b from exosomal MSCs directly enhanced neurite outgrowth and functional recovery in adult stroke models (Xin et al., 2012, 2013). Given the putative occurrence of impaired neurite outgrowth in perinatal brain injury (Robinson et al., 2006; Dean et al., 2013), exosomal miR-133b may be important for brain connectivity and function. In an elegant series of studies conducted by Xin et al. (2012), miR-133b was substantially downregulated in the ischemic rat brain and increased following MSC intravenous administration. Connective tissue growth factor (CTGF) and RhoA are both inhibitors of neurite growth and are selective targets of miR-133b (Xin et al., 2014). Critically, administration of MSC-derived exosomes enriched with miR-133b reduced CTGF and RhoA expression and exhibited enhanced axonal plasticity, neurite remodeling and functional recovery compared to naturally occurring MSC-derived exosomes (Xin et al., 2013). These changes were confirmed in primary cultured neurons and astrocytes (Xin et al., 2013). Transfer of miR-133b enriched MSC derived exosomes in cultured neurons, inhibited RhoA expression and stimulated neurite outgrowth, while the transfer in astrocytes, downregulated CTGF expression, a known inhibitor of axonal growth and contributor to glial scar formation in human cerebral infarction (Schwab et al., 2000; Xin et al., 2013).

In a recent conducted study by Yang et al. (2017), rabies virus glycoprotein modified exosomes were employed to achieve neuron-specific delivery of miR-124 across the blood–brain barrier. Previously, invasive cerebral administration of miR-124, a regulator of neurogenesis (Makeyev et al., 2007; Cheng et al., 2009; Åkerblom et al., 2012), was reported to reduce infarct area and improve neuronal survival against ischemic injury in mice (Liu et al., 2011; Doeppner et al., 2013). In the study conducted by Yang et al. (2017), rabies virus glycoprotein exosomes effectively carried miR-124 to neurons of the ischemic region and supported neuronal identity of cortical neural progenitors and reduced ischemic cortical injury by robust neurogenesis. Thus the above evidence supports the therapeutic potential to ameliorate neuronal injury by exploiting the neurodevelopmental function of miRNAs.

Additionally, in concordance with Xin et al. (2012, 2013), MSC derived exosomes provide a therapeutically viable delivery of gene drugs to the brain and possibly specific cells across the blood–brain barrier. Since MSCs produce an abundant source of extracellular vesicles that contain a selection of miRNAs with the potential to elicit neuroprotective biological processes in response to injury, including the ability to modulate the action of neighboring cells, it seems worthwhile to investigate whether MSC-extracellular vesicles would be a promising therapy to promote neurological functional recovery in the developing brain following injury. Indeed, recent *in vivo* investigations using animal models of perinatal and neonatal brain injury support their application (Drommelschmidt et al., 2017); however, further investigations are required to characterize what specific miRNA profiles potentially contribute to protection.

Finally, it is pertinent to mention that exosomes/extracellular vesicles, viewed as potent vehicles by which to deliver potentially therapeutic miRNAs to the brain, can equally participate in the pathophysiological processes of blood-CSF-brain-communication. Recent studies undertaken in both an *in vivo*, *in vitro,* and *ex vivo* mouse model of endotoxemia have shown that miRNA-containing extracellular vesicles originating from the choroid plexus epithelium can enter brain parenchymal cells and via astrocytic and microglial processes induce miRNA target repression and inflammatory gene expression (Balusu et al., 2016). The transfer of potentially adverse proinflammatory driven extracellular vesicle-derived miRNAs to the brain via this route of communication would seem of considerable importance for the advancement of our understanding of the

pathophysiological mechanisms of intrauterine infection-related preterm brain injury (Dammann and Leviton, 1997; Malaeb and Dammann, 2009), including the role of placental vesicle-derived miRNAs (Ilekis et al., 2016; Wei et al., 2017; Salomon et al., 2018) in this process and warrants further investigation.

CONCLUSION AND FUTURE DIRECTIONS

In the past decade, numerous articles have been published on the role of miRNAs within the brain. As post-transcriptional regulators of gene expression, miRNAs most definitely play a crucial role in the development of the brain. Nevertheless, research conducted to define their impact on the developing and injured brain is still in its infancy. Given specific miRNAs can exhibit diverse functional roles throughout development and can act synergistically, identification of their precise functional roles is fraught with difficulties. This is particularly relevant when considering adopting specific miRNAs as biomarkers of perinatal brain injury. Thus, careful interpretation of data is required not only in the context of biomarker potential, but also application as a therapeutic strategy since off-target effects can confound the latter. One attractive possibility to ensuring, at least targeted delivery, is the fast developing field of research involving exosome-based miRNA therapies for neurological injuries and disorders. Exosomes are considered a key carrier of circulating miRNAs. Since they mediate the exchange of miRNAs between cells, readily cross the blood–brain barrier and fuse with cell membranes, they hold promise not only as a miRNA biomarker carrier, but also as a means to deliver miRNA-based therapies to the developing injured brain. Clearly, continued developments in this field of research has the potential to enhance future prospects of effectively treating perinatal brain injury especially those vulnerable to premature injury.

AUTHOR CONTRIBUTIONS

KHTC and MF devised main conceptual ideas and outlines and took the lead in writing the manuscript. All authors contributed to the manuscript and provided feedback and discussed the manuscript.

FUNDING

This work was supported in part by the Neurological Foundation of NZ 1519-PG (MF), Health Research Council of New Zealand 18/183 (MF), Cure Kids 3581 (MF), Auckland Medical Research Foundation 1117009 (MF), and the Barbara Basham Doctoral Scholarship – Auckland Medical Research Foundation 1216004 (KHTC).

ACKNOWLEDGMENTS

We apologize to those authors whose excellent studies we have not dealt with within the scope and limitation of this review.

REFERENCES

Akerblom, M., and Jakobsson, J. (2014). MicroRNAs as neuronal fate determinants. *Neuroscientist* 20, 235–242. doi: 10.1177/1073858413497265

Åkerblom, M., Sachdeva, R., Barde, I., Verp, S., Gentner, B., Trono, D., et al. (2012). MicroRNA-124 is a subventricular zone neuronal fate determinant. *J. Neurosci.* 32, 8879–8889. doi: 10.1523/JNEUROSCI.0558-12.2012

Akerblom, M., Sachdeva, R., Quintino, L., Wettergren, E. E., Chapman, K. Z., Manfre, G., et al. (2013). Visualization and genetic modification of resident brain microglia using lentiviral vectors regulated by microRNA-9. *Nat. Commun.* 4:1770. doi: 10.1038/ncomms2801

Andersson, T., Rahman, S., Sansom, S. N., Alsio, J. M., Kaneda, M., Smith, J., et al. (2010). Reversible block of mouse neural stem cell differentiation in the absence of dicer and microRNAs. *PLoS One* 5:e13453. doi: 10.1371/journal.pone.0013453

Ashhab, M. U., Omran, A., Kong, H., Gan, N., He, F., Peng, J., et al. (2013). Expressions of tumor necrosis factor alpha and microRNA-155 in immature rat model of status epilepticus and children with mesial temporal lobe epilepsy. *J. Mol. Neurosci.* 51, 950–958. doi: 10.1007/s12031-013-0013-9

Baburamani, A. A., Supramaniam, V. G., Hagberg, H., and Mallard, C. (2014). Microglia toxicity in preterm brain injury. *Reprod. Toxicol.* 48, 106–112. doi: 10.1016/j.reprotox.2014.04.002

Back, S. A., Luo, N. L., Borenstein, N. S., Volpe, J. J., and Kinney, H. C. (2002). Arrested oligodendrocyte lineage progression during human cerebral white matter development: dissociation between the timing of progenitor differentiation and myelinogenesis. *J. Neuropathol. Exp. Neurol.* 61, 197–211. doi: 10.1093/jnen/61.2.197

Back, S. A., and Miller, S. P. (2014). Brain injury in premature neonates: a primary cerebral dysmaturation disorder? *Ann. Neurol.* 75, 469–486. doi: 10.1002/ana.24132

Bak, M., Silahtaroglu, A., Moller, M., Christensen, M., Rath, M. F., Skryabin, B., et al. (2008). MicroRNA expression in the adult mouse central nervous system. *RNA* 14, 432–444. doi: 10.1261/rna.783108

Baltimore, D., Boldin, M. P., O'Connell, R. M., Rao, D. S., and Taganov, K. D. (2008). MicroRNAs: new regulators of immune cell development and function. *Nat. Immunol.* 9, 839–845. doi: 10.1038/ni.f.209

Balusu, S., Van Wonterghem, E., De Rycke, R., Raemdonck, K., Stremersch, S., Gevaert, K., et al. (2016). Identification of a novel mechanism of blood-brain communication during peripheral inflammation via choroid plexus-derived extracellular vesicles. *EMBO Mol. Med.* 8, 1162–1183. doi: 10.15252/emmm.201606271

Barca-Mayo, O., and Lu, Q. R. (2012). Fine-tuning oligodendrocyte development by microRNAs. *Front. Neurosci.* 6:13. doi: 10.3389/fnins.2012.00013

Barres, B. A., Lazar, M. A., and Raff, M. C. (1994). A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. *Development* 120, 1097–1108.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233. doi: 10.1016/j.cell.2009.01.002

Bernstein, E., Kim, S. Y., Carmell, M. A., Murchison, E. P., Alcorn, H., Li, M. Z., et al. (2003). Dicer is essential for mouse development. *Nat. Genet.* 35, 215–217. doi: 10.1038/ng1253

Bhalala, O. G., Srikanth, M., and Kessler, J. A. (2013). The emerging roles of microRNAs in CNS injuries. *Nat. Rev. Neurol.* 9, 328–339. doi: 10.1038/nrneurol.2013.67

Bian, S., Hong, J., Li, Q., Schebelle, L., Pollock, A., Knauss, J. L., et al. (2013). MicroRNA cluster miR-17-92 regulates neural stem cell expansion and transition to intermediate progenitors in the developing mouse neocortex. *Cell Rep.* 3, 1398–1406. doi: 10.1016/j.celrep.2013.03.037

Birch, D., Britt, B. C., Dukes, S. C., Kessler, J. A., and Dizon, M. L. (2014). MicroRNAs participate in the murine oligodendroglial response to perinatal hypoxia-ischemia. *Pediatr. Res.* 76, 334–340. doi: 10.1038/pr.2014.104

Brett, J. O., Renault, V. M., Rafalski, V. A., Webb, A. E., and Brunet, A. (2011). The microRNA cluster miR-106b∼25 regulates adult neural stem/progenitor cell proliferation and neuronal differentiation. *Aging* 3, 108–124. doi: 10.18632/aging.100285

Buller, B., Chopp, M., Ueno, Y., Zhang, L., Zhang, R. L., Morris, D., et al. (2012). Regulation of serum response factor by miRNA-200 and miRNA-9 modulates oligodendrocyte progenitor cell differentiation. *Glia* 60, 1906–1914. doi: 10.1002/glia.22406

Buser, J. R., Maire, J., Riddle, A., Gong, X., Nguyen, T., Nelson, K., et al. (2012). Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. *Ann. Neurol.* 71, 93–109. doi: 10.1002/ana.22627

Butovsky, O., Jedrychowski, M. P., Cialic, R., Krasemann, S., Murugaiyan, G., Fanek, Z., et al. (2015). Targeting miR-155 restores abnormal microglia and attenuates disease in SOD1 mice. *Ann. Neurol.* 77, 75–99. doi: 10.1002/ana.24304

Caballero-Garrido, E., Pena-Philippides, J.C., Lordkipanidze, T., Bragin, D., Yang, Y., Erhardt, E.B., et al. (2015). In vivo inhibition of miR-155 promotes recovery after experimental mouse stroke. *J. Neurosci.* 35, 12446–12464. doi: 10.1523/JNEUROSCI.1641-15.2015

Cai, Q., Wang, T., Yang, W.-J., and Fen, X. (2016). Protective mechanisms of microRNA-27a against oxygen-glucose deprivation-induced injuries in hippocampal neurons. *Neural Regen. Res.* 11:1285. doi: 10.4103/1673-5374.189194

Cao, X., Yeo, G., Muotri, A. R., Kuwabara, T., and Gage, F. H. (2006). Noncoding RNAs in the mammalian central nervous system. *Annu. Rev. Neurosci.* 29, 77–103. doi: 10.1146/annurev.neuro.29.051605.112839

Cardoso, A. L., Guedes, J. R., Pereira de Almeida, L., and Pedroso de Lima, M. C. (2012). miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. *Immunology* 135, 73–88. doi: 10.1111/j.1365-2567.2011.03514.x

Chan, S. Y., and Loscalzo, J. (2010). MicroRNA-210: a unique and pleiotropic hypoxamir. *Cell Cycle* 9, 1072–1083. doi: 10.4161/cc.9.6.11006

Chan, Y. C., Banerjee, J., Choi, S. Y., and Sen, C. K. (2012). miR-210: the master hypoxamir. *Microcirculation* 19, 215–223. doi: 10.1111/j.1549-8719.2011.00154.x

Chen, F., Du, Y., Esposito, E., Liu, Y., Guo, S., Wang, X., et al. (2015). Effects of focal cerebral ischemia on exosomal versus serum miR126. *Transl. Stroke Res.* 6, 478–484. doi: 10.1007/s12975-015-0429-3

Chen, H., and Yang, T. T. (2015). Expression and significance of serum miRNA-21 control HIF-1a in newborn with asphyxia. *Chin. J. Child Health Care* 23, 32–34.

Chen, J., Liu, Z., and Yang, Y. (2014). In vitro screening of LPS-induced miRNAs in leukocytes derived from cord blood and their possible roles in regulating TLR signals. *Pediatr. Res.* 75, 595–602. doi: 10.1038/pr.2014.18

Chen, Q., Xu, J., Li, L., Li, H., Mao, S., Zhang, F., et al. (2014). MicroRNA-23a/b and microRNA-27a/b suppress Apaf-1 protein and alleviate hypoxia-induced neuronal apoptosis. *Cell Death Dis.* 5:e1132. doi: 10.1038/cddis.2014.92

Chen, T. S., Lai, R. C., Lee, M. M., Choo, A. B. H., Lee, C. N., and Lim, S. K. (2009). Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res.* 38, 215–224. doi: 10.1093/nar/gkp857

Chen, W., and Qin, C. (2015). General hallmarks of microRNAs in brain evolution and development. *RNA Biol.* 12, 701–708. doi: 10.1080/15476286.2015.1048954

Chen, Y., Song, Y., Huang, J., Qu, M., Zhang, Y., Geng, J., et al. (2017). Increased circulating exosomal miRNA-223 is associated with acute ischemic stroke. *Front. Neurol.* 8:57. doi: 10.3389/fneur.2017.00057

Cheng, L., Sharples, R. A., Scicluna, B. J., and Hill, A. F. (2014). Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. *J. Extracell. Vesicles* 3:10.3402/jev.v3.23743. doi: 10.3402/jev.v3.23743

Cheng, L.-C., Pastrana, E., Tavazoie, M., and Doetsch, F. (2009). miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat. Neurosci.* 12:399. doi: 10.1038/nn.2294

Cherubini, E., Gustincich, S., and Robinson, H. (2006). The mammalian transcriptome and the cellular complexity of the brain. *J. Physiol.* 575(Pt 2), 319–320. doi: 10.1113/jphysiol.2006.118364

Chiva-Blanch, G., Suades, R., Crespo, J., Pena, E., Padro, T., Jimenez-Xarrie, E., et al. (2016). Microparticle shedding from neural progenitor cells and vascular compartment cells is increased in ischemic stroke. *PLoS One* 11:e0148176. doi: 10.1371/journal.pone.0148176

Chow, L. C., Soliman, A., Zandian, M., Danielpour, M., and Krueger, R. C. Jr (2005). Accumulation of transforming growth factor-β2 and nitrated chondroitin sulfate proteoglycans in cerebrospinal fluid correlates with poor neurologic outcome in preterm hydrocephalus. *Neonatology* 88, 1–11. doi: 10.1159/000083945

Clovis, Y. M., Enard, W., Marinaro, F., Huttner, W. B., and De Pietri Tonelli, D. (2012). Convergent repression of Foxp2 3′UTR by miR-9 and miR-132 in embryonic mouse neocortex: implications for radial migration of neurons. *Development* 139, 3332–3342. doi: 10.1242/dev.078063

Concepcion, C. P., Bonetti, C., and Ventura, A. (2012). The microRNA-17-92 family of microRNA clusters in development and disease. *Cancer J.* 18, 262–267. doi: 10.1097/PPO.0b013e318258b60a

Contreras-Naranjo, J. C., Wu, H. J., and Ugaz, V. M. (2017). Microfluidics for exosome isolation and analysis: enabling liquid biopsy for personalized medicine. *Lab. Chip* 17, 3558–3577. doi: 10.1039/c7lc00592j

Coolen, M., Katz, S., and Bally-Cuif, L. (2013). miR-9: a versatile regulator of neurogenesis. *Front. Cell Neurosci.* 7:220. doi: 10.3389/fncel.2013.00220

Dajas-Bailador, F., Bonev, B., Garcez, P., Stanley, P., Guillemot, F., and Papalopulu, N. (2012). microRNA-9 regulates axon extension and branching by targeting Map1b in mouse cortical neurons. *Nat. Neurosci.* doi: 10.1038/nn.3082 [Epub ahead of print].

Dammann, O., and Leviton, A. (1997). Maternal intrauterine infection, cytokines, and brain damage in the preterm newborn. *Pediatr. Res.* 42, 1–8. doi: 10.1203/00006450-199707000-00001

Davis, G. M., Haas, M. A., and Pocock, R. (2015). MicroRNAs: not "fine-tuners" but key regulators of neuronal development and function. *Front. Neurol.* 6:245. doi: 10.3389/fneur.2015.00245

Davis, T. H., Cuellar, T. L., Koch, S. M., Barker, A. J., Harfe, B. D., McManus, M. T., et al. (2008). Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus. *J. Neurosci.* 28, 4322–4330. doi: 10.1523/JNEUROSCI.4815-07.2008

De Pietri Tonelli, D., Pulvers, J. N., Haffner, C., Murchison, E. P., Hannon, G. J., and Huttner, W. B. (2008). miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. *Development* 135, 3911–3921. doi: 10.1242/dev.025080

Dean, J. M., McClendon, E., Hansen, K., Azimi-Zonooz, A., Chen, K., Riddle, A., et al. (2013). Prenatal cerebral ischemia disrupts MRI-defined cortical microstructure through disturbances in neuronal arborization. *Sci. Transl. Med.* 5:168ra167. doi: 10.1126/scitranslmed.3004669

Dean, J. M., Van De Looij, Y., Sizonenko, S. V., Lodygensky, G. A., Lazeyras, F., Bolouri, H., et al. (2011). Delayed cortical impairment following lipopolysaccharide exposure in preterm fetal sheep. *Ann. Neurol.* 70, 846–856. doi: 10.1002/ana.22480

Delaloy, C., Liu, L., Lee, J. A., Su, H., Shen, F., Yang, G. Y., et al. (2010). MicroRNA-9 coordinates proliferation and migration of human embryonic stem cell-derived neural progenitors. *Cell Stem Cell* 6, 323–335. doi: 10.1016/j.stem.2010.02.015

Dharap, A., Bowen, K., Place, R., Li, L. C., and Vemuganti, R. (2009). Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome. *J. Cereb. Blood Flow Metab.* 29, 675–687. doi: 10.1038/jcbfm.2008.157

Dhillon, S. K., Gunn, A. J., Jung, Y., Mathai, S., Bennet, L., and Fraser, M. (2015). Lipopolysaccharide-induced preconditioning attenuates apoptosis and differentially regulates TLR4 and TLR7 gene expression after ischemia in the preterm ovine fetal brain. *Dev. Neurosci.* 37, 497–514. doi: 10.1159/000433422

Dillenburg, A., Ireland, G., Holloway, R. K., Davies, C. L., Evans, F. L., Swire, M., et al. (2018). Activin receptors regulate the oligodendrocyte lineage in health and disease. *Acta Neuropathol.* 135, 887–906. doi: 10.1007/s00401-018-1813-3

Doeppner, T. R., Doehring, M., Bretschneider, E., Zechariah, A., Kaltwasser, B., Müller, B., et al. (2013). MicroRNA-124 protects against focal cerebral ischemia via mechanisms involving Usp14-dependent REST degradation. *Acta Neuropathol.* 126, 251–265. doi: 10.1007/s00401-013-1142-5

Doeppner, T. R., Herz, J., Gorgens, A., Schlechter, J., Ludwig, A. K., Radtke, S., et al. (2015). Extracellular vesicles improve post-stroke neuroregeneration and prevent postischemic immunosuppression. *Stem Cells Transl. Med.* 4, 1131–1143. doi: 10.5966/sctm.2015-0078

Douglas-Escobar, M., and Weiss, M. D. (2012). Biomarkers of brain injury in the premature infant. *Front. Neurol.* 3:185. doi: 10.3389/fneur.2012.00185

Drommelschmidt, K., Serdar, M., Bendix, I., Herz, J., Bertling, F., Prager, S., et al. (2017). Mesenchymal stem cell-derived extracellular vesicles ameliorate inflammation-induced preterm brain injury. *Brain Behav. Immun.* 60, 220–232. doi: 10.1016/j.bbi.2016.11.011

Duan, Q., Mao, X., Xiao, Y., Liu, Z., Wang, Y., Zhou, H., et al. (2016). Super enhancers at the miR-146a and miR-155 genes contribute to self-regulation of inflammation. *Biochim. Biophys. Acta* 1859, 564–571. doi: 10.1016/j.bbagrm.2016.02.004

Dugas, J. C., Cuellar, T. L., Scholze, A., Ason, B., Ibrahim, A., Emery, B., et al. (2010). Dicer1 and miR-219 Are required for normal oligodendrocyte differentiation and myelination. *Neuron* 65, 597–611. doi: 10.1016/j.neuron.2010.01.027

Dzietko, M., Derugin, N., Wendland, M., Vexler, Z., and Ferriero, D. (2013). Delayed VEGF treatment enhances angiogenesis and recovery after neonatal focal rodent stroke. *Transl. Stroke Res.* 4, 189–200. doi: 10.1007/s12975-012-0221-6

Eacker, S. M., Dawson, T. M., and Dawson, V. L. (2013). The interplay of microRNA and neuronal activity in health and disease. *Front. Cell Neurosci.* 7:136. doi: 10.3389/fncel.2013.00136

Ferriero, D. M. (2016). The vulnerable newborn brain: imaging patterns of acquired perinatal injury. *Neonatology* 109, 345–351. doi: 10.1159/000444896

Fitzpatrick, J. M., Anderson, R. C., and McDermott, K. W. (2015). MicroRNA: key regulators of oligodendrocyte development and pathobiology. *Int. J. Biochem. Cell Biol.* 65, 134–138. doi: 10.1016/j.biocel.2015.05.021

Fleiss, B., and Gressens, P. (2012). Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? *Lancet Neurol.* 11, 556–566. doi: 10.1016/S1474-4422(12)70058-3

Florio, P., Luisi, S., Bruschettini, M., Grutzfeld, D., Dobrzanska, A., Bruschettini, P., et al. (2004). Cerebrospinal fluid activin a measurement in asphyxiated full-term newborns predicts hypoxic ischemic encephalopathy. *Clin. Chem.* 50, 2386–2389. doi: 10.1373/clinchem.2004.035774

Florio, P., Luisi, S., Moataza, B., Torricelli, M., Iman, I., Hala, M., et al. (2007). High urinary concentrations of activin A in asphyxiated full-term newborns with moderate or severe hypoxic ischemic encephalopathy. *Clin. Chem.* 53, 520–522. doi: 10.1373/clinchem.2005.062604

Fu, Q., Du, Y., Yang, C., Zhang, D., Zhang, N., Liu, X., et al. (2016). An oncogenic role of miR-592 in tumorigenesis of human colorectal cancer by targeting Forkhead Box O3A (FoxO3A). *Expert Opin. Ther. Targets* 20, 771–782. doi: 10.1080/14728222.2016.1181753

Galloway, D. A., and Moore, C. S. (2016). miRNAs as emerging regulators of oligodendrocyte development and differentiation. *Front. Cell Dev. Biol.* 4:59. doi: 10.3389/fcell.2016.00059

Garberg, H. T., Huun, M. U., Baumbusch, L. O., Asegg-Atneosen, M., Solberg, R., and Saugstad, O. D. (2016). Temporal profile of circulating microRNAs after global hypoxia-ischemia in newborn piglets. *Neonatology* 111, 133–139. doi: 10.1159/000449032

Gaudet, A. D., Fonken, L. K., Watkins, L. R., Nelson, R. J., and Popovich, P. G. (2017). MicroRNAs: roles in regulating neuroinflammation. *Neuroscientist* 24, 221–245. doi: 10.1177/1073858417721150

Gaudet, A. D., Fonken, L. K., Watkins, L. R., Nelson, R. J., and Popovich, P. G. (2018). MicroRNAs: roles in regulating neuroinflammation. *Neuroscientist* 24, 221–245. doi: 10.1177/1073858417721150

Gerdoni, E., Gallo, B., Casazza, S., Musio, S., Bonanni, I., Pedemonte, E., et al. (2007). Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. *Ann. Neurol.* 61, 219–227. doi: 10.1002/ana.21076

Giaume, C., Koulakoff, A., Roux, L., Holcman, D., and Rouach, N. (2010). Astroglial networks: a step further in neuroglial and gliovascular interactions. *Nat. Rev. Neurosci.* 11, 87–99. doi: 10.1038/nrn2757

Goetzl, L., Merabova, N., Darbinian, N., Martirosyan, D., Poletto, E., Fugarolas, K., et al. (2018). Diagnostic potential of neural exosome cargo as biomarkers for acute brain injury. *Ann. Clin. Transl. Neurol.* 5, 4–10. doi: 10.1002/acn3.499

Gotz, M., Nakafuku, M., and Petrik, D. (2016). Neurogenesis in the developing and adult brain-similarities and key differences. *Cold Spring Harb. Perspect. Biol.* 8:a018853. doi: 10.1101/cshperspect.a018853

Griesmaier, E., Schlager, G., Wegleiter, K., Hermann, M., Urbanek, M., Simbruner, G., et al. (2010). Role of p75NTR in NMDAR-mediated excitotoxic brain injury in neonatal mice. *Brain Res.* 1355, 31–40. doi: 10.1016/j.brainres.2010.07.095

Griffiths-Jones, S. (2006). miRBase: the microRNA sequence database. *Methods Mol. Biol.* 342, 129–138. doi: 10.1385/1-59745-123-1:129

Guo, S. C., Tao, S. C., and Dawn, H. (2018). Microfluidics-based on-a-chip systems for isolating and analysing extracellular vesicles. *J. Extracell. Vesicles* 7:1508271. doi: 10.1080/20013078.2018.1508271

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Hass, R., and Otte, A. (2012). Mesenchymal stem cells as all-round supporters in a normal and neoplastic microenvironment. *Cell Commun. Signal.* 10:26. doi: 10.1186/1478-811X-10-26

He, Y., Li, H. B., Li, X., Zhou, Y., Xia, X. B., and Song, W. T. (2018). MiR-124 promotes the growth of retinal ganglion cells derived from muller cells. *Cell Physiol. Biochem.* 45, 973–983. doi: 10.1159/000487292

Hisey, C. L., Dorayappan, K. D. P., Cohn, D. E., Selvendiran, K., and Hansford, D. J. (2018). Microfluidic affinity separation chip for selective capture and release of label-free ovarian cancer exosomes. *Lab. Chip* 18, 3144–3153. doi: 10.1039/c8lc00834e

Huang, X., Le, Q.T., and Giaccia, A.J. (2010). MiR-210–micromanager of the hypoxia pathway. *Trends Mol. Med.* 16, 230–237. doi: 10.1016/j.molmed.2010.03.004

Ilekis, J. V., Tsilou, E., Fisher, S., Abrahams, V. M., Soares, M. J., Cross, J. C., et al. (2016). Placental origins of adverse pregnancy outcomes: potential molecular targets: an Executive Workshop Summary of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. *Am. J. Obstet. Gynecol.* 215(Suppl. 1), S1–S46. doi: 10.1016/j.ajog.2016.03.001

Irmady, K., Jackman, K. A., Padow, V. A., Shahani, N., Martin, L. A., Cerchietti, L., et al. (2014). Mir-592 regulates the induction and cell death-promoting activity of p75NTR in neuronal ischemic injury. *J. Neurosci.* 34, 3419–3428. doi: 10.1523/JNEUROSCI.1982-13.2014

Iyer, A., Zurolo, E., Prabowo, A., Fluiter, K., Spliet, W. G., van Rijen, P. C., et al. (2012). MicroRNA-146a: a key regulator of astrocyte-mediated inflammatory response. *PLoS One* 7:e44789. doi: 10.1371/journal.pone.0044789

Jellema, R. K., Wolfs, T. G., Passos, V. L., Zwanenburg, A., Ophelders, D. R., Kuypers, E., et al. (2013). Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia. *PLoS One* 8:e73031. doi: 10.1371/journal.pone.0073031

Jeyaseelan, K., Lim, K. Y., and Armugam, A. (2008). MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke* 39, 959–966. doi: 10.1161/STROKEAHA.107.500736

Ji, Q., Ji, Y., Peng, J., Zhou, X., Chen, X., Zhao, H., et al. (2016). Increased Brain-Specific MiR-9 and MiR-124 in the serum exosomes of acute ischemic stroke patients. *PLoS One* 11:e0163645. doi: 10.1371/journal.pone.0163645

Jones, L. L., Sajed, D., and Tuszynski, M. H. (2003). Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: a balance of permissiveness and inhibition. *J. Neurosci.* 23, 9276–9288. doi: 10.1523/JNEUROSCI.23-28-09276.2003

Jones, L. L., Yamaguchi, Y., Stallcup, W. B., and Tuszynski, M. H. (2002). NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. *J. Neurosci.* 22, 2792–2803. doi: 10.1523/JNEUROSCI.22-07-02792.2002

Kannan, S., Saadani-Makki, F., Balakrishnan, B., Dai, H., Chakraborty, P. K., Janisse, J., et al. (2011). Decreased cortical serotonin in neonatal rabbits exposed to endotoxin in utero. *J. Cereb. Blood Flow Metab.* 31, 738–749. doi: 10.1038/jcbfm.2010.156

Katakowski, M., Buller, B., Zheng, X., Lu, Y., Rogers, T., Osobamiro, O., et al. (2013). Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett.* 335, 201–204. doi: 10.1016/j.canlet.2013.02.019

Katsumoto, A., Lu, H., Miranda, A. S., and Ransohoff, R. M. (2014). Ontogeny and functions of central nervous system macrophages. *J. Immunol.* 193, 2615–2621. doi: 10.4049/jimmunol.1400716

Khanna, S., Rink, C., Ghoorkhanian, R., Gnyawali, S., Heigel, M., Wijesinghe, D. S., et al. (2013). Loss of miR-29b following acute ischemic stroke contributes to neural cell death and infarct size. *J. Cereb. Blood Flow Metab.* 33, 1197–1206. doi: 10.1038/jcbfm.2013.68

Kim, H. Y., Kumar, H., Jo, M. J., Kim, J., Yoon, J. K., Lee, J. R., et al. (2018). Therapeutic efficacy-potentiated and diseased organ-targeting nanovesicles derived from mesenchymal stem cells for spinal cord injury treatment. *Nano Lett.* 18, 4965–4975. doi: 10.1021/acs.nanolett.8b01816

Kinney, H.C., Volpe, J.J. (2018a). "Encephalopathy of prematurity: neuropathology," in *Volpe's Neurology of the Newborn*, 6th Edn, eds J. Joseph,

M. D. Volpe, T. Basil, S. Darras Linda, J. Adré, J, Jeffrey et al (Amsterdam: Elsevier), 389–404.

Kinney, H.C., and Volpe, J.J. (2018b). "Hypoxic-ischemic injury in the term infant: neuropathology," in *Volpe's Neurology of the Newborn*, 6th Edn, eds J.J. Volpe, T.E. Inder, B.T. Darras, L.S. de Vries, A.J. du Plessis, J.J. Neil et al (Amsterdam: Elsevier), 484–499. doi: 10.1016/B978-0-323-42876-7.00018-1

Koh, S.-H., Kim, K. S., Choi, M. R., Jung, K. H., Park, K. S., Chai, Y. G., et al. (2008). Implantation of human umbilical cord-derived mesenchymal stem cells as a neuroprotective therapy for ischemic stroke in rats. *Brain Res.* 1229, 233–248. doi: 10.1016/j.brainres.2008.06.087

Koh, W., Sheng, C. T., Tan, B., Lee, Q. Y., Kuznetsov, V., Kiang, L. S., et al. (2010). Analysis of deep sequencing microRNA expression profile from human embryonic stem cells derived mesenchymal stem cells reveals possible role of let-7 microRNA family in downstream targeting of hepatic nuclear factor 4 alpha. *BMC Genomics* 11:S6. doi: 10.1186/1471-2164-11-S1-S6

Koniusz, S., Andrzejewska, A., Muraca, M., Srivastava, A. K., Janowski, M., and Lukomska, B. (2016). Extracellular vesicles in physiology, pathology, and therapy of the immune and central nervous system, with focus on extracellular vesicles derived from mesenchymal stem cells as therapeutic tools. *Front. Cell Neurosci.* 10:109. doi: 10.3389/fncel.2016.00109

Kozomara, A., and Griffiths-Jones, S. (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42(Database issue), D68–D73. doi: 10.1093/nar/gkt1181

Krichevsky, A. M., Sonntag, K. C., Isacson, O., and Kosik, K. S. (2006). Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells* 24, 857–864. doi: 10.1634/stemcells.2005-0441

Kulshreshtha, R., Ferracin, M., Wojcik, S. E., Garzon, R., Alder, H., Agosto-Perez, F. J., et al. (2007). A microRNA signature of hypoxia. *Mol. Cell Biol.* 27, 1859–1867. doi: 10.1128/MCB.01395-06

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., and Tuschl, T. (2003). New microRNAs from mouse and human. *RNA* 9, 175–179. doi: 10.1261/rna.2146903

Lau, P., Verrier, J. D., Nielsen, J. A., Johnson, K. R., Notterpek, L., and Hudson, L. D. (2008). Identification of dynamically regulated microRNA and mRNA networks in developing oligodendrocytes. *J. Neurosci.* 28, 11720–11730. doi: 10.1523/JNEUROSCI.1932-08.2008

Lederhuber, H., Baer, K., Altiok, I., Sadeghi, K., Herkner, K. R., and Kasper, D. C. (2011). MicroRNA-146: tiny player in neonatal innate immunity? *Neonatology* 99, 51–56. doi: 10.1159/000301938

Lee, J.-K., Park, S.-R., Jung, B.-K., Jeon, Y.-K., Lee, Y.-S., Kim, M.-K., et al. (2013). Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS One* 8:e84256. doi: 10.1371/journal.pone.0084256

Lehmann, S. M., Kruger, C., Park, B., Derkow, K., Rosenberger, K., Baumgart, J., et al. (2012). An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. *Nat. Neurosci.* 15, 827–835. doi: 10.1038/nn.3113

Letzen, B. S., Liu, C., Thakor, N. V., Gearhart, J. D., All, A. H., and Kerr, C. L. (2010). MicroRNA expression profiling of oligodendrocyte differentiation from human embryonic stem cells. *PLoS One* 5:e10480. doi: 10.1371/journal.pone.0010480

Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003). Prediction of mammalian microRNA targets. *Cell* 115, 787–798. doi: 10.1016/S0092-8674(03)01018-3

Li, B., Concepcion, K., Meng, X., and Zhang, L. (2017). Brain-immune interactions in perinatal hypoxic-ischemic brain injury. *Prog. Neurobiol.* 159, 50–68. doi: 10.1016/j.pneurobio.2017.10.006

Li, K., Rodosthenous, R. S., Kashanchi, F., Gingeras, T., Gould, S. J., Kuo, L. S., et al. (2018). Advances, challenges, and opportunities in extracellular RNA biology: insights from the NIH exRNA Strategic Workshop. *JCI Insight* 3:98942. doi: 10.1172/jci.insight.98942

Li, X.-Q., Lv, H.-W., Wang, Z.-L., Tan, W.-F., Fang, B., and Ma, H. (2015). MiR-27a ameliorates inflammatory damage to the blood-spinal cord barrier after spinal cord ischemia: reperfusion injury in rats by downregulating TICAM-2 of the TLR 4 signaling pathway. *J. Neuroinflam.* 12:25. doi: 10.1186/s12974-015-0246-3

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., et al. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769–773. doi: 10.1038/nature03315

Liu, A., Li, J., Marin-Husstege, M., Kageyama, R., Fan, Y., Gelinas, C., et al. (2006). A molecular insight of Hes5-dependent inhibition of myelin gene expression: old partners and new players. *EMBO J.* 25, 4833–4842. doi: 10.1038/sj.emboj.7601352

Liu, D. Z., Tian, Y., Ander, B. P., Xu, H., Stamova, B. S., Zhan, X., et al. (2010). Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. *J. Cereb. Blood Flow Metab.* 30, 92–101. doi: 10.1038/jcbfm.2009.186

Liu, J., Zhang, K. S., Hu, B., Li, S. G., Li, Q., Luo, Y. P., et al. (2018). Systematic analysis of RNA regulatory network in rat brain after ischemic stroke. *Biomed. Res. Int.* 2018:8354350. doi: 10.1155/2018/8354350

Liu, M., Zhi, Q., Wang, W., Zhang, Q., Fang, T., and Ma, Q. (2015). Up-regulation of miR-592 correlates with tumor progression and poor prognosis in patients with colorectal cancer. *Biomed. Pharmacother.* 69, 214–220. doi: 10.1016/j.biopha.2014.12.001

Liu, X. S., Chopp, M., Pan, W. L., Wang, X. L., Fan, B. Y., Zhang, Y., et al. (2017). MicroRNA-146a promotes oligodendrogenesis in stroke. *Mol. Neurobiol.* 54, 227–237. doi: 10.1007/s12035-015-9655-7

Liu, X. S., Chopp, M., Zhang, R. L., Tao, T., Wang, X. L., Kassis, H., et al. (2011). MicroRNA profiling in subventricular zone after stroke: MiR-124a regulates proliferation of neural progenitor cells through Notch signaling pathway. *PLoS One* 6:e23461. doi: 10.1371/journal.pone.0023461

Liu, Z., Wu, R., Li, G., Sun, P., Xu, Q., and Liu, Z. (2015). MiR-592 inhibited cell proliferation of human colorectal cancer cells by suppressing of CCND3 expression. *Int. J. Clin. Exp. Med.* 8:3490.

Long, Q., Upadhya, D., Hattiangady, B., Kim, D. K., An, S. Y., Shuai, B., et al. (2017). Intranasal MSC-derived A1-exosomes ease inflammation, and prevent abnormal neurogenesis and memory dysfunction after status epilepticus. *Proc. Natl. Acad. Sci. U.S.A.* 114, E3536–E3545. doi: 10.1073/pnas.1703920114

Looney, A. M., Ahearne, C. E., Hallberg, B., Boylan, G. B., and Murray, D. M. (2017). Downstream mRNA target analysis in neonatal hypoxic-ischaemic encephalopathy identifies novel marker of severe injury: a proof of concept paper. *Mol. Neurobiol.* 54, 8420–8428. doi: 10.1007/s12035-016-0330-4

Looney, A. M., Walsh, B. H., Moloney, G., Grenham, S., Fagan, A., O'Keeffe, G. W., et al. (2015). Downregulation of umbilical cord blood levels of miR-374a in neonatal hypoxic ischemic encephalopathy. *J. Pediatr.* 167, 269–273.e2. doi: 10.1016/j.jpeds.2015.04.060

Luarte, A., Batiz, L. F., Wyneken, U., and Lafourcade, C. (2016). Potential therapies by stem cell-derived exosomes in cns diseases: focusing on the neurogenic niche. *Stem Cells Int.* 2016:5736059. doi: 10.1155/2016/5736059

Ludwig, A. K., and Giebel, B. (2012). Exosomes: small vesicles participating in intercellular communication. *Int. J. Biochem. Cell Biol.* 44, 11–15. doi: 10.1016/j.biocel.2011.10.005

Lv, Y.-N., Ou-yang, A.-J., and Fu, L.-S. (2017). MicroRNA-27a negatively modulates the inflammatory response in lipopolysaccharide-stimulated microglia by targeting TLR4 and IRAK4. *Cell. Mol. Neurobiol.* 37, 195–210. doi: 10.1007/s10571-016-0361-4

Ma, Q., Dasgupta, C., Li, Y., Bajwa, N. M., Xiong, F., Harding, B., et al. (2016). Inhibition of microRNA-210 provides neuroprotection in hypoxic-ischemic brain injury in neonatal rats. *Neurobiol. Dis.* 89, 202–212. doi: 10.1016/j.nbd.2016.02.011

Ma, Q., Dasgupta, C., Li, Y., Huang, L., and Zhang, L. (2017). MicroRNA-210 suppresses junction proteins and disrupts blood-brain barrier integrity in neonatal rat hypoxic-ischemic brain injury. *Int. J. Mol. Sci.* 18:E1356. doi: 10.3390/ijms18071356

Maiorano, N. A., and Mallamaci, A. (2009). Promotion of embryonic cortico-cerebral neuronogenesis by miR-124. *Neural Dev.* 4:40. doi: 10.1186/1749-8104-4-40

Makeyev, E. V., Zhang, J., Carrasco, M. A., and Maniatis, T. (2007). The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol. Cell* 27, 435–448. doi: 10.1016/j.molcel.2007.07.015

Malaeb, S., and Dammann, O. (2009). Fetal inflammatory response and brain injury in the preterm newborn. *J. Child Neurol.* 24, 1119–1126. doi: 10.1177/0883073809338066

Mann, M., Mehta, A., Zhao, J. L., Lee, K., Marinov, G. K., Garcia-Flores, Y., et al. (2017). An NF-kappaB-microRNA regulatory network tunes macrophage

inflammatory responses. *Nat. Commun.* 8:851. doi: 10.1038/s41467-017-00972-z

Mastoridis, S., Bertolino, G. M., Whitehouse, G., Dazzi, F., Sanchez-Fueyo, A., and Martinez-Llordella, M. (2018). Multiparametric analysis of circulating exosomes and other small extracellular vesicles by advanced imaging flow cytometry. *Front. Immunol.* 9:1583. doi: 10.3389/fimmu.2018.01583

McKeon, R. J., Jurynec, M. J., and Buck, C. R. (1999). The chondroitin sulfate proteoglycans neurocan and phosphacan are expressed by reactive astrocytes in the chronic CNS glial scar. *J. Neurosci.* 19, 10778–10788. doi: 10.1523/JNEUROSCI.19-24-10778.1999

Meng, Z. Y., Kang, H. L., Duan, W., Zheng, J., Li, Q. N., and Zhou, Z. J. (2018). MicroRNA-210 promotes accumulation of neural precursor cells around ischemic foci after cerebral ischemia by regulating the SOCS1-STAT3-VEGF-C Pathway. *J. Am. Heart Assoc.* 7:e005052. doi: 10.1161/JAHA.116.005052

Michell-Robinson, M. A., Touil, H., Healy, L. M., Owen, D. R., Durafourt, B. A., Bar-Or, A., et al. (2015). Roles of microglia in brain development, tissue maintenance and repair. *Brain* 138(Pt 5), 1138–1159. doi: 10.1093/brain/awv066

Miska, E. A., Alvarez-Saavedra, E., Townsend, M., Yoshii, A., Sestan, N., Rakic, P., et al. (2004). Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol.* 5:R68. doi: 10.1186/gb-2004-5-9-r68

Mokarizadeh, A., Delirezh, N., Morshedi, A., Mosayebi, G., Farshid, A.-A., and Mardani, K. (2012). Microvesicles derived from mesenchymal stem cells: potent organelles for induction of tolerogenic signaling. *Immunol. Lett.* 147, 47–54. doi: 10.1016/j.imlet.2012.06.001

Mondello, S., Thelin, E. P., Shaw, G., Salzet, M., Visalli, C., Cizkova, D., et al. (2018). Extracellular vesicles: pathogenetic, diagnostic and therapeutic value in traumatic brain injury. *Expert Rev. Proteomics* 15, 451–461. doi: 10.1080/14789450.2018.1464914

Monfils, M. H., Driscoll, I., Kamitakahara, H., Wilson, B., Flynn, C., Teskey, G. C., et al. (2006). FGF-2-induced cell proliferation stimulates anatomical, neurophysiological and functional recovery from neonatal motor cortex injury. *Eur. J. Neurosci.* 24, 739–749. doi: 10.1111/j.1460-9568.2006.04939.x

Moon, J. M., Xu, L., and Giffard, R. G. (2013). Inhibition of microRNA-181 reduces forebrain ischemia-induced neuronal loss. *J. Cereb. Blood Flow Metab.* 33, 1976–1982. doi: 10.1038/jcbfm.2013.157

Morris, D. C., Chopp, M., Zhang, L., Lu, M., and Zhang, Z. G. (2010). Thymosin beta4 improves functional neurological outcome in a rat model of embolic stroke. *Neuroscience* 169, 674–682. doi: 10.1016/j.neuroscience.2010.05.017

Mueller, M., Zhou, J., Yang, L., Gao, Y., Wu, F., Schoeberlein, A., et al. (2014). PreImplantation factor promotes neuroprotection by targeting microRNA let-7. *Proc. Natl. Acad. Sci. U.S.A.* 111, 13882–13887. doi: 10.1073/pnas.1411674111

Nahid, M. A., Satoh, M., and Chan, E. K. (2011). MicroRNA in TLR signaling and endotoxin tolerance. *Cell Mol. Immunol.* 8, 388–403. doi: 10.1038/cmi.2011.26

Nallamshetty, S., Chan, S.Y., and Loscalzo, J. (2013). Hypoxia: a master regulator of microRNA biogenesis and activity. *Free Radic. Biol. Med.* 64, 20–30. doi: 10.1016/j.freeradbiomed.2013.05.022

Narayan, A., Bommakanti, A., and Patel, A. A. (2015). High-throughput RNA profiling via up-front sample parallelization. *Nat. Methods* 12, 343–346. doi: 10.1038/nmeth.3311

Nayak, D., Roth, T. L., and McGavern, D. B. (2014). Microglia development and function. *Annu. Rev. Immunol.* 32, 367–402. doi: 10.1146/annurev-immunol-032713-120240

O'Connell, R. M., Taganov, K. D., Boldin, M. P., Cheng, G., and Baltimore, D. (2007). MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1604–1609. doi: 10.1073/pnas.0610731104

Ohab, J. J., Fleming, S., Blesch, A., and Carmichael, S. T. (2006). A neurovascular niche for neurogenesis after stroke. *J. Neurosci.* 26, 13007–13016. doi: 10.1523/JNEUROSCI.4323-06.2006

Omran, A., Peng, J., Zhang, C., Xiang, Q. L., Xue, J., Gan, N., et al. (2012). Interleukin-1beta and microRNA-146a in an immature rat model and children with mesial temporal lobe epilepsy. *Epilepsia* 53, 1215–1224. doi: 10.1111/j.1528-1167.2012.03540.x

O'Neill, L. A., Sheedy, F. J., and McCoy, C. E. (2011). MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nat. Rev. Immunol.* 11, 163–175. doi: 10.1038/nri2957

Ono, M., Kosaka, N., Tominaga, N., Yoshioka, Y., Takeshita, F., Takahashi, R.-U., et al. (2014). Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci. Signal.* 7:ra63 doi: 10.1126/scisignal.2005231

Ophelders, D. R., Wolfs, T. G., Jellema, R. K., Zwanenburg, A., Andriessen, P., Delhaas, T., et al. (2016). Mesenchymal stromal cell-derived extracellular vesicles protect the fetal brain after hypoxia-ischemia. *Stem Cells Transl. Med.* 5, 754–763. doi: 10.5966/sctm.2015-0197

Otaegi, G., Pollock, A., Hong, J., and Sun, T. (2011). MicroRNA miR-9 modifies motor neuron columns by a tuning regulation of FoxP1 levels in developing spinal cords. *J. Neurosci.* 31, 809–818. doi: 10.1523/JNEUROSCI.4330-10.2011

Ouyang, Y. B., Xu, L., Yue, S., Liu, S., and Giffard, R. G. (2014). Neuroprotection by astrocytes in brain ischemia: importance of microRNAs. *Neurosci. Lett.* 565, 53–58. doi: 10.1016/j.neulet.2013.11.015

Pandit, A. S., Ball, G., Edwards, A. D., and Counsell, S. J. (2013). Diffusion magnetic resonance imaging in preterm brain injury. *Neuroradiology* 55, 65–95. doi: 10.1007/s00234-013-1242-x

Park, K. K., Liu, K., Hu, Y., Smith, P. D., Wang, C., Cai, B., et al. (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science* 322, 963–966. doi: 10.1126/science.1161566

Patz, S., Trattnig, C., Grunbacher, G., Ebner, B., Gully, C., Novak, A., et al. (2013). More than cell dust: microparticles isolated from cerebrospinal fluid of brain injured patients are messengers carrying mRNAs, miRNAs, and proteins. *J. Neurotrauma* 30, 1232–1242. doi: 10.1089/neu.2012.2596

Pena, J. T., Sohn-Lee, C., Rouhanifard, S. H., Ludwig, J., Hafner, M., Mihailovic, A., et al. (2009). miRNA in situ hybridization in formaldehyde and EDC-fixed tissues. *Nat. Methods* 6, 139–141. doi: 10.1038/nmeth.1294

Pena-Philippides, J.C., Caballero-Garrido, E., Lordkipanidze, T., and Roitbak, T. (2016). In vivo inhibition of miR-155 significantly alters post-stroke inflammatory response. *J. Neuroinflam.* 13:287. doi: 10.1186/s12974-016-0753-x

Petri, R., Malmevik, J., Fasching, L., Akerblom, M., and Jakobsson, J. (2014). miRNAs in brain development. *Exp. Cell Res.* 321, 84–89. doi: 10.1016/j.yexcr.2013.09.022

Podolska, A., Kaczkowski, B., Kamp Busk, P., Sokilde, R., Litman, T., Fredholm, M., et al. (2011). MicroRNA expression profiling of the porcine developing brain. *PLoS One* 6:e14494. doi: 10.1371/journal.pone.0014494

Ponnusamy, V., Kapellou, O., Yip, E., Evanson, J., Wong, L. F., Michael-Titus, A., et al. (2016). A study of microRNAs from dried blood spots in newborns after perinatal asphyxia: a simple and feasible biosampling method. *Pediatr. Res.* 79, 799–805. doi: 10.1038/pr.2015.276

Ponomarev, E. D., Veremeyko, T., Barteneva, N., Krichevsky, A. M., and Weiner, H. L. (2011). MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. *Nat. Med.* 17, 64–70. doi: 10.1038/nm.2266

Ponomarev, E. D., Veremeyko, T., and Weiner, H. L. (2013). MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and macrophages in normal and diseased CNS. *Glia* 61, 91–103. doi: 10.1002/glia.22363

Potzner, M. R., Griffel, C., Lutjen-Drecoll, E., Bosl, M. R., Wegner, M., and Sock, E. (2007). Prolonged Sox4 expression in oligodendrocytes interferes with normal myelination in the central nervous system. *Mol. Cell Biol.* 27, 5316–5326. doi: 10.1128/MCB.00339-07

Qiu, J., Zhou, X. Y., Zhou, X. G., Cheng, R., Liu, H. Y., and Li, Y. (2013a). Neuroprotective effects of microRNA-210 against oxygen-glucose deprivation through inhibition of apoptosis in PC12 cells. *Mol. Med. Rep.* 7, 1955–1959. doi: 10.3892/mmr.2013.1431

Qiu, J., Zhou, X. Y., Zhou, X. G., Cheng, R., Liu, H. Y., and Li, Y. (2013b). Neuroprotective effects of microRNA-210 on hypoxic-ischemic encephalopathy. *Biomed. Res. Int.* 2013:350419. doi: 10.1155/2013/350419

Quinn, S. R., and O'Neill, L. A. (2011). A trio of microRNAs that control Toll-like receptor signalling. *Int. Immunol.* 23, 421–425. doi: 10.1093/intimm/dxr034

Radhakrishnan, B., and Alwin Prem Anand, A. (2016). Role of miRNA-9 in Brain Development. *J. Exp. Neurosci.* 10, 101–120. doi: 10.4137/JEN.S32843

Rao, P., Benito, E., and Fischer, A. (2013). MicroRNAs as biomarkers for CNS disease. *Front. Mol. Neurosci.* 6:39. doi: 10.3389/fnmol.2013.00039

Rao, V. T., Ludwin, S. K., Fuh, S. C., Sawaya, R., Moore, C. S., Ho, M. K., et al. (2016). MicroRNA expression patterns in human astrocytes in relation

to anatomical location and age. *J. Neuropathol. Exp. Neurol.* 75, 156–166. doi: 10.1093/jnen/nlv016

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383. doi: 10.1083/jcb.201211138

Reemst, K., Noctor, S. C., Lucassen, P. J., and Hol, E. M. (2016). The indispensable roles of microglia and astrocytes during brain development. *Front. Hum. Neurosci.* 10:566. doi: 10.3389/fnhum.2016.00566

Repetto, E., Briata, P., Kuziner, N., Harfe, B. D., McManus, M. T., Gherzi, R., et al. (2012). Let-7b/c enhance the stability of a tissue-specific mRNA during mammalian organogenesis as part of a feedback loop involving KSRP. *PLoS Genet.* 8:e1002823. doi: 10.1371/journal.pgen.1002823

Riddle, A., Maire, J., Gong, X., Chen, K. X., Kroenke, C. D., Hohimer, A. R., et al. (2012). Differential susceptibility to axonopathy in necrotic and non-necrotic perinatal white matter injury. *Stroke* 43, 178–184. doi: 10.1161/STROKEAHA.111.632265

Robinson, S., Li, Q., DeChant, A., and Cohen, M. L. (2006). Neonatal loss of γ–aminobutyric acid pathway expression after human perinatal brain injury. *J. Neurosurg.* 104, 396–408. doi: 10.3171/ped.2006.104.6.396

Roitbak, T. (2018). Silencing a Multifunctional microrna is beneficial for stroke recovery. *Front. Mol. Neurosci.* 11:58. doi: 10.3389/fnmol.2018.00058

Salomon, C., Nuzhat, Z., Dixon, C. L., and Menon, R. (2018). Placental exosomes during gestation: liquid biopsies carrying signals for the regulation of human parturition. *Curr. Pharm. Des.* 24, 974–982. doi: 10.2174/1381612824666180125164429

Santra, M., Zhang, Z. G., Yang, J., Santra, S., Santra, S., Chopp, M., et al. (2014). Thymosin beta4 up-regulation of microRNA-146a promotes oligodendrocyte differentiation and suppression of the Toll-like proinflammatory pathway. *J. Biol. Chem.* 289, 19508–19518. doi: 10.1074/jbc.M113.529966

Sanuki, R., Onishi, A., Koike, C., Muramatsu, R., Watanabe, S., Muranishi, Y., et al. (2011). miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression. *Nat. Neurosci.* 14, 1125–1134. doi: 10.1038/nn.2897

Schwab, J. M., Postler, E., Nguyen, T. D., Mittelbronn, M., Meyermann, R., and Schluesener, H. J. (2000). Connective tissue growth factor is expressed by a subset of reactive astrocytes in human cerebral infarction. *Neuropathol. Appl. Neurobiol.* 26, 434–440. doi: 10.1046/j.1365-2990.2000.00271.x

Selvamani, A., Sathyan, P., Miranda, R. C., and Sohrabji, F. (2012). An antagomir to microRNA Let7f promotes neuroprotection in an ischemic stroke model. *PLoS One* 7:e32662. doi: 10.1371/journal.pone.0032662

Sempere, L. F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E., and Ambros, V. (2004). Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.* 5:R13. doi: 10.1186/gb-2004-5-3-r13

Shenoy, A., Danial, M., and Blelloch, R. H. (2015). Let-7 and miR-125 cooperate to prime progenitors for astrogliogenesis. *EMBO J.* 34, 1180–1194. doi: 10.15252/embj.201489504

Shi, Y., Zhao, X., Hsieh, J., Wichterle, H., Impey, S., Banerjee, S., et al. (2010). MicroRNA regulation of neural stem cells and neurogenesis. *J. Neurosci.* 30, 14931–14936. doi: 10.1523/JNEUROSCI.4280-10.2010

Shin, D., Shin, J. Y., McManus, M. T., Ptacek, L. J., and Fu, Y. H. (2009). Dicer ablation in oligodendrocytes provokes neuronal impairment in mice. *Ann. Neurol.* 66, 843–857. doi: 10.1002/ana.21927

Shindo, A., Maki, T., Itoh, K., Miyamoto, N., Egawa, N., Liang, A. C., et al. (2016). "Crosstalk between cerebral endothelium and oligodendrocyte after stroke," in *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, eds J. Chen, J. Zhang, and X. Hu (Cham: Springer), 151–170.

Silber, J., Lim, D. A., Petritsch, C., Persson, A. I., Maunakea, A. K., Yu, M., et al. (2008). miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med.* 6:14. doi: 10.1186/1741-7015-6-14

Smirnova, L., Grafe, A., Seiler, A., Schumacher, S., Nitsch, R., and Wulczyn, F. G. (2005). Regulation of miRNA expression during neural cell specification. *Eur. J. Neurosci.* 21, 1469–1477. doi: 10.1111/j.1460-9568.2005.03978.x

Smith, B., Treadwell, J., Zhang, D., Ly, D., McKinnell, I., Walker, P. R., et al. (2010). Large-scale expression analysis reveals distinct microRNA profiles at different stages of human neurodevelopment. *PLoS One* 5:e11109. doi: 10.1371/journal.pone.0011109

Steinberg, G. K., Kondziolka, D., Wechsler, L. R., Lunsford, L. D., Coburn, M. L., Billigen, J. B., et al. (2016). Clinical outcomes of transplanted modified bone marrow–derived mesenchymal stem cells in stroke: a phase 1/2a study. *Stroke* 47, 1817–1824. doi: 10.1161/STROKEAHA.116.012995

Stolt, C. C., Schlierf, A., Lommes, P., Hillgartner, S., Werner, T., Kosian, T., et al. (2006). SoxD proteins influence multiple stages of oligodendrocyte development and modulate SoxE protein function. *Dev. Cell* 11, 697–709. doi: 10.1016/j.devcel.2006.08.011

Su, W., Aloi, M. S., and Garden, G. A. (2016). MicroRNAs mediating CNS inflammation: small regulators with powerful potential. *Brain Behav. Immun.* 52, 1–8. doi: 10.1016/j.bbi.2015.07.003

Suh, M. R., Lee, Y., Kim, J. Y., Kim, S. K., Moon, S. H., Lee, J. Y., et al. (2004). Human embryonic stem cells express a unique set of microRNAs. *Dev. Biol.* 270, 488–498. doi: 10.1016/j.ydbio.2004.02.019

Sun, L.-Q., Guo, G.-L., Zhang, S., and Yang, L.-L. (2018). Effects of MicroRNA-592-5p on hippocampal neuron injury following hypoxic-ischemic brain damage in neonatal mice-involvement of PGD2/DP and PTGDR. *Cell. Physiol. Biochem.* 45, 458–473. doi: 10.1159/000486923

Sun, Y., Luo, Z. M., Guo, X. M., Su, D. F., and Liu, X. (2015). An updated role of microRNA-124 in central nervous system disorders: a review. *Front. Cell Neurosci.* 9:193. doi: 10.3389/fncel.2015.00193

Taganov, K. D., Boldin, M. P., Chang, K. J., and Baltimore, D. (2006). NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12481–12486. doi: 10.1073/pnas.0605298103

Tan, K. S., Armugam, A., Sepramaniam, S., Lim, K. Y., Setyowati, K. D., Wang, C. W., et al. (2009). Expression profile of MicroRNAs in young stroke patients. *PLoS One* 4:e7689. doi: 10.1371/journal.pone.0007689

Taverna, E., Gotz, M., and Huttner, W. B. (2014). The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. *Annu. Rev. Cell Dev. Biol.* 30, 465–502. doi: 10.1146/annurev-cellbio-101011-155801

Tay, T. L., Savage, J. C., Hui, C. W., Bisht, K., and Tremblay, M. E. (2017). Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. *J. Physiol.* 595, 1929–1945. doi: 10.1113/JP272134

Taylor, D. D., and Gercel-Taylor, C. (2014). Exosome platform for diagnosis and monitoring of traumatic brain injury. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20130503. doi: 10.1098/rstb.2013.0503

Teng, H., Zhang, Z. G., Wang, L., Zhang, R. L., Zhang, L., Morris, D., et al. (2008). Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. *J. Cereb. Blood Flow Metab.* 28, 764–771. doi: 10.1038/sj.jcbfm.9600573

Thion, M. S., Low, D., Silvin, A., Chen, J., Grisel, P., Schulte-Schrepping, J., et al. (2018). Microbiome influences prenatal and adult microglia in a sex-specific manner. *Cell* 172, 500–516.e16. doi: 10.1016/j.cell.2017.11.042

Uccelli, A., Laroni, A., and Freedman, M. S. (2011). Mesenchymal stem cells for the treatment of multiple sclerosis and other neurological diseases. *Lancet Neurol.* 10, 649–656. doi: 10.1016/S1474-4422(11)70121-1

Vaccarino, F. M., Schwartz, M. L., Raballo, R., Rhee, J., and Lyn-Cook, R. (1999). Fibroblast growth factor signaling regulates growth and morphogenesis at multiple steps during brain development. *Curr. Top. Dev. Biol.* 46, 179–200. doi: 10.1016/S0070-2153(08)60329-4

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., and Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., et al. (2016). Impaired oligodendrocyte maturation in preterm infants: potential therapeutic targets. *Prog. Neurobiol.* 136, 28–49. doi: 10.1016/j.pneurobio.2015.11.002

van Velthoven, C. T., Kavelaars, A., van Bel, F., and Heijnen, C. J. (2010). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain Behav. Immun.* 24, 387–393. doi: 10.1016/j.bbi.2009.10.017

Vasudevan, S. (2012). Posttranscriptional upregulation by microRNAs. *Wiley Interdiscip. Rev. RNA* 3, 311–330. doi: 10.1002/wrna.121

Ventura, A., Young, A. G., Winslow, M. M., Lintault, L., Meissner, A., Erkeland, S. J., et al. (2008). Targeted deletion reveals essential and overlapping functions

of the miR-17 through 92 family of miRNA clusters. *Cell* 132, 875–886. doi: 10.1016/j.cell.2008.02.019

Visvanathan, J., Lee, S., Lee, B., Lee, J. W., and Lee, S. K. (2007). The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes Dev.* 21, 744–749. doi: 10.1101/gad.1519107

Voloboueva, L. A., Sun, X., Xu, L., Ouyang, Y. B., and Giffard, R. G. (2017). Distinct Effects of miR-210 reduction on neurogenesis: increased neuronal survival of inflammation but reduced proliferation associated with mitochondrial enhancement. *J. Neurosci.* 37, 3072–3084. doi: 10.1523/JNEUROSCI.1777-16.2017

Volosin, M., Song, W., Almeida, R. D., Kaplan, D. R., Hempstead, B. L., and Friedman, W. J. (2006). Interaction of survival and death signaling in basal forebrain neurons: roles of neurotrophins and proneurotrophins. *J. Neurosci.* 26, 7756–7766. doi: 10.1523/JNEUROSCI.1560-06.2006

Volpe, J. J., Kinney, H. C., Jensen, F. E., and Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *Int. J. Dev. Neurosci.* 29, 423–440. doi: 10.1016/j.ijdevneu.2011.02.012

Wang, J., Wang, H., Liu, A., Fang, C., Hao, J., and Wang, Z. (2015). Lactate dehydrogenase A negatively regulated by miRNAs promotes aerobic glycolysis and is increased in colorectal cancer. *Oncotarget* 6, 19456–19468. doi: 10.18632/oncotarget.3318

Wang, L., Ke, J., Li, Y., Ma, Q., Dasgupta, C., Huang, X., et al. (2017). Inhibition of miRNA-210 reverses nicotine-induced brain hypoxic-ischemic injury in neonatal rats. *Int. J. Biol. Sci.* 13, 76–84. doi: 10.7150/ijbs.17278

Wang, Y., and Yang, G. Y. (2013). MicroRNAs in Cerebral Ischemia. *Stroke Treat.* 2013:276540. doi: 10.1155/2013/276540

Weaver-Mikaere, L., Gunn, A. J., Mitchell, M. D., Bennet, L., and Fraser, M. (2013). LPS and TNF alpha modulate AMPA/NMDA receptor subunit expression and induce PGE2 and glutamate release in preterm fetal ovine mixed glial cultures. *J. Neuroinflam.* 10:916. doi: 10.1186/1742-2094-10-153

Wei, J., Blenkiron, C., Tsai, P., James, J. L., Chen, Q., Stone, P. R., et al. (2017). Placental trophoblast debris mediated feto-maternal signalling via small RNA delivery: implications for preeclampsia. *Sci. Rep.* 7:14681. doi: 10.1038/s41598-017-14180-8

Werner, J. K., and Stevens, R. D. (2015). Traumatic brain injury: recent advances in plasticity and regeneration. *Curr. Opin. Neurol.* 28, 565–573. doi: 10.1097/WCO.0000000000000265

Whitehead, C. L., Teh, W. T., Walker, S. P., Leung, C., Larmour, L., and Tong, S. (2013). Circulating MicroRNAs in maternal blood as potential biomarkers for fetal hypoxia in-utero. *PLoS One* 8:e78487. doi: 10.1371/journal.pone.0078487

Willis, G. R., Kourembanas, S., and Mitsialis, S. A. (2017). Toward exosome-based therapeutics: isolation, heterogeneity, and fit-for-purpose potency. *Front. Cardiovasc. Med.* 4:63. doi: 10.3389/fcvm.2017.00063

Wu, M. L., Zhu, C. C., Qi, Y. Y., Shi, Y. X., Xu, H. N., and Yang, J. R. (2018). [Isolation, Identification and Degradation Characteristics of a 17beta-estradiol Degrading Strain Fusarium sp. KY123915]. *Huan Jing Ke Xue* 39, 4802–4808. doi: 10.13227/j.hjkx.201711077

Xia, H., Cheung, W. K., Ng, S. S., Jiang, X., Jiang, S., Sze, J., et al. (2012). Loss of brain-enriched miR-124 microRNA enhances stem-like traits and invasiveness of glioma cells. *J. Biol. Chem.* 287, 9962–9971. doi: 10.1074/jbc.M111.332627

Xin, H., Li, Y., Buller, B., Katakowski, M., Zhang, Y., Wang, X., et al. (2012). Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells* 30, 1556–1564. doi: 10.1002/stem.1129

Xin, H., Li, Y., and Chopp, M. (2014). Exosomes/miRNAs as mediating cell-based therapy of stroke. *Front. Cell. Neurosci.* 8:377. doi: 10.3389/fncel.2014.00377

Xin, H., Li, Y., Liu, Z., Wang, X., Shang, X., Cui, Y., et al. (2013). MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem Cells* 31, 2737–2746. doi: 10.1002/stem.1409

Xiong, Y., Mahmood, A., and Chopp, M. (2017). Emerging potential of exosomes for treatment of traumatic brain injury. *Neural Regen. Res.* 12, 19–22. doi: 10.4103/1673-5374.198966

Xiong, Y., Mahmood, A., Meng, Y., Zhang, Y., Zhang, Z. G., Morris, D. C., et al. (2012). Neuroprotective and neurorestorative effects of thymosin beta4 treatment following experimental traumatic brain injury. *Ann. N. Y. Acad. Sci.* 1270, 51–58. doi: 10.1111/j.1749-6632.2012.06683.x

Yang, J., Zhang, X., Chen, X., Wang, L., and Yang, G. (2017). Exosome mediated delivery of miR-124 promotes neurogenesis after ischemia. *Mol. Ther. Nucleic Acids* 7, 278–287. doi: 10.1016/j.omtn.2017.04.010

Yeh, Y. M., Chuang, C. M., Chao, K. C., and Wang, L. H. (2013). MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1alpha. *Int. J. Cancer* 133, 867–878. doi: 10.1002/ijc.28086

Yeo, R. W., Lai, R. C., Zhang, B., Tan, S. S., Yin, Y., Teh, B. J., et al. (2013). Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv. Drug Deliv. Rev.* 65, 336–341. doi: 10.1016/j.addr.2012.07.001

Yin, K.-J., Hamblin, M., and Eugene Chen, Y. (2015). Angiogenesis-regulating microRNAs and ischemic stroke. *Curr. Vasc. Pharmacol.* 13, 352–365. doi: 10.2174/1570161113119990016

Yin, K.-J., Olsen, K., Hamblin, M., Zhang, J., Schwendeman, S. P., and Chen, Y. E. (2012). Vascular endothelial cell-specific microRNA-15a inhibits angiogenesis in hindlimb ischemia. *J. Biol. Chem.* 287, 27055–27064. doi: 10.1074/jbc.M112.364414

Yuan, Y., Wang, J. Y., Xu, L. Y., Cai, R., Chen, Z., and Luo, B. Y. (2010). MicroRNA expression changes in the hippocampi of rats subjected to global ischemia. *J. Clin. Neurosci.* 17, 774–778. doi: 10.1016/j.jocn.2009.10.009

Zeng, L., He, X., Wang, Y., Tang, Y., Zheng, C., Cai, H., et al. (2014). MicroRNA-210 overexpression induces angiogenesis and neurogenesis in the normal adult mouse brain. *Gene Ther.* 21, 37–43. doi: 10.1038/gt.2013.55

Zhang, J., Li, Y., Chen, J., Cui, Y., Lu, M., Elias, S. B., et al. (2005). Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. *Exp. Neurol.* 195, 16–26. doi: 10.1016/j.expneurol.2005.03.018

Zhang, Y., Chopp, M., Liu, X. S., Kassis, H., Wang, X., Li, C., et al. (2015a). MicroRNAs in the axon locally mediate the effects of chondroitin sulfate proteoglycans and cGMP on axonal growth. *Dev. Neurobiol.* 75, 1402–1419. doi: 10.1002/dneu.22292

Zhang, Y., Chopp, M., Meng, Y., Katakowski, M., Xin, H., Mahmood, A., et al. (2015b). Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. *J. Neurosurg.* 122, 856–867. doi: 10.3171/2014.11.Jns14770

Zhang, Y., Ueno, Y., Liu, X. S., Buller, B., Wang, X., Chopp, M., et al. (2013). The MicroRNA-17-92 cluster enhances axonal outgrowth in embryonic cortical neurons. *J. Neurosci.* 33, 6885–6894. doi: 10.1523/JNEUROSCI.5180-12.2013

Zhao, C., Sun, G., Li, S., and Shi, Y. (2009). A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. *Nat. Struct. Mol. Biol.* 16, 365–371. doi: 10.1038/nsmb.1576

Zhao, X., He, X., Han, X., Yu, Y., Ye, F., Chen, Y., et al. (2010). MicroRNA-mediated control of oligodendrocyte differentiation. *Neuron* 65, 612–626. doi: 10.1016/j.neuron.2010.02.018

Zheng, K., Li, H., Huang, H., and Qiu, M. (2012). MicroRNAs and glial cell development. *Neuroscientist* 18, 114–118. doi: 10.1177/1073858411398322

Zheng, K., Li, H., Zhu, Y., Zhu, Q., and Qiu, M. (2010). MicroRNAs are essential for the developmental switch from neurogenesis to gliogenesis in the developing spinal cord. *J. Neurosci.* 30, 8245–8250. doi: 10.1523/JNEUROSCI.1169-10.2010

Zhou, J., and Zhang, J. (2014). Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction. *Mol. Med. Rep.* 10, 971–976. doi: 10.3892/mmr.2014.2245

Zhou, T., Huang, Y. X., Song, J. W., and Ma, Q. M. (2015). Thymosin beta4 inhibits microglia activation through microRNA 146a in neonatal rats following hypoxia injury. *Neuroreport* 26, 1032–1038. doi: 10.1097/WNR.0000000000000463

Ziats, M. N., and Rennert, O. M. (2014). Identification of differentially expressed microRNAs across the developing human brain. *Mol. Psychiatry* 19, 848–852. doi: 10.1038/mp.2013.93

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Cho, Xu, Blenkiron and Fraser. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 02 April 2019
doi: 10.3389/fphys.2019.00307

Cognitive Development Trajectories in Preterm Children With Very Low Birth Weight Longitudinally Followed Until 11 Years of Age

Sofia Ryytty Stålnacke[1], Mesfin Tessma[2], Birgitta Böhm[1†] and Eric Herlenius[1†]*

[1] Department of Women's and Children's Health, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden,
[2] Department of Learning, Informatics, Management and Ethics – LIME, Karolinska Institutet, Stockholm, Sweden

Background: There is a high prevalence of cognitive dysfunction in very low birthweight (500–1250 g) infants (VLBW). Understanding long-term risk factors associated with cognitive development in preterm children requires longitudinal characterization. Thus, follow-up evaluations, including identification of risks and resilience influences–are important to promote health and cognitive abilities of children born preterm.

Aim: To examine changes in cognitive development from birth until 11 years of age in preterm children with very low birthweight.

Methods: 24 VLBW infants, at the Karolinska University Hospital, Stockholm, were assessed with regards to cognitive functioning at three times during development at 18 months, 5 and 11 years of age using standardized tests. Longitudinal data were analyzed using Generalized Estimating Equation (GEE) univariate and multivariate models.

Results: The follow-up rate was 100%. Level of cognitive functioning at 18 months and at 11 years was similar. Females had higher cognitive scores than males at all three timepoints. We found that intraventricular hemorrhage (IVH) and prolonged invasive ventilatory support (>7 days) had a negative effect on cognitive functioning. Higher levels of parental education had a favorable influence on cognitive functioning over time.

Conclusion: Level of cognitive development at 18 months was highly predictive of level of cognitive function at 11 years of age and differences in assessment scores between male and female VLBW infants persisted. Additional longitudinal studies, performed before school entry and across childhood, are needed to further elucidate the cognitive trajectories of preterm children.

Keywords: preterm (birth), development, cognitive stability, medical complications, academic achievements

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Adam John Watkins,
University of Nottingham,
United Kingdom
Alistair Jan Gunn,
The University of Auckland,
New Zealand

****Correspondence:***
Eric Herlenius
eric.herlenius@ki.se

† Shared last authorship

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *15 October 2018*
Accepted: *07 March 2019*
Published: *02 April 2019*

Citation:
Stålnacke SR, Tessma M, Böhm B and Herlenius E (2019) Cognitive Development Trajectories in Preterm Children With Very Low Birth Weight Longitudinally Followed Until 11 Years of Age. Front. Physiol. 10:307. doi: 10.3389/fphys.2019.00307

INTRODUCTION

Pre-term birth is associated with dysfunctional development of vital organs and increased risk of cognitive impairment later in life. Some problems appear during the first weeks of life and can be successfully treated, whilst others have a permanent influence on the development. Brain injury such as intraventricular hemorrhage (IVH) and periventricular leukomalacia (PVL) are associated with a high risk of neurodevelopmental disability (Volpe, 1980; Volpe et al., 2011). Preventing

brain injury by supporting the respiratory control systems in the preterm infant is crucial. Apnea of prematurity can prolong the need for invasive ventilatory support and bronchopulmonary dysplasia, which are both associated with neurodevelopmental impairment (Janvier et al., 2004; Hofstetter et al., 2008; Doyle and Anderson, 2009). The degree of prematurity and the presence of comorbidities of more than one harmful factor influence the severity of developmental deficiencies in cognitive functioning, as well as in academic achievements (Schmidt et al., 2015). Inflammation has emerged as a critical contributor to both normal development and injury outcome in the immature brain (Hagberg et al., 2015). Neonatal factors found to predict a lower adulthood IQ include: respiratory distress syndrome, IVH, mechanical ventilation, mobility problems, parenteral nutrition, low to middle socioeconomic status of parents, and poor parent-infant relationship (Breeman et al., 2017). Furthermore, a range of perinatal vulnerability factors have been associated with male sex supporting the concept that male sex is an important biological risk factor in extremely preterm infants. Future prospects for preterm children are of utmost interest for parents, pediatric medicine, schools, and society (Moore et al., 2012; Aylward, 2014).

The results of cognitive assessments in children who were born very (week 28–32) or extremely (<week 28) preterm, range from severe and mild levels of intellectual disability to cognitive levels above average. The prevalence for severe cognitive delay is higher in populations of very (Murray et al., 2014) and extremely premature children (Johnson et al., 2009). Though the majority of preterm children perform within normal range of general cognitive functioning, as a group they perform 0.5–1 SD below that of full term children (Bohm et al., 2002; Rose et al., 2011; Luttikhuizen dos Santos et al., 2013; Linsell et al., 2018). Specific cognitive functions such as attention, working memory, and processing speed are also often delayed (Rose et al., 2012; Murray et al., 2014).

Early developmental assessment of cognition from 18 to 24 months post term age (corrected for prematurity) tend to be stable in preterm children with average cognitive development, but future cognitive functioning seems harder to predict when children are performing 1 to 2 SD below expectation, especially in VLBW infants (Roberts et al., 2010; Luttikhuizen dos Santos et al., 2013; Wong et al., 2016).

There is strong evidence that parental education acts as a predictor for cognitive development in preterm children (Bohm et al., 2002; Breeman et al., 2017). In addition, parental level of education, employment and income have additionally shown independent, and additive effects on cognitive gain across preschool years (Manley et al., 2015; Beauregard et al., 2018). Cognitive outcome after preterm birth is heterogeneous, and group level analyses may disguise individual variability in development.

Thus, long-term studies that address individual patterns and explore trajectories in cognitive development are emerging (Stalnacke et al., 2015; Wong et al., 2016; Mangin et al., 2017). A significant number of children born very preterm or VLBW experience difficulties in school. To be able to reduce the long-term risks associated with VLBW birth, an improved understanding of the mechanisms and risk factors placing these children at risk of cognitive delay and dysfunction is necessary. Identifying factors affecting the predictive accuracy of early neurodevelopmental assessments and individual trajectories of overall, as well as specific, cognitive function is important in order to enable earlier support and intervention.

Aim

The aim of the present study was to investigate trajectories of cognitive functioning at the age of 18 months, 5 and 11 years in a Swedish cohort of preterm children with very low birth-weight (500–1250 g). The concordance over time in different aspects of cognition were studied as well as the differences within the cohort predicted by sex, preterm birth factors, medical risk factors, and parental level of education.

MATERIALS AND METHODS

The Swedish cohort is part of an international multisite follow-up study, the Caffeine for Apnea of Prematurity trial (CAP), a randomized and placebo-controlled study of the safety and efficacy of neonatal Caffeine citrate (Methylxanthines), for management and/or prevention of apnea in premature children with a birth-weight of 500–1250 g (Schmidt, 2005; Schmidt et al., 2011). Information about the random assignment is confidential to members of the double-blind CAP trial. Therefore, effects of drug treatment are not evaluated in this study.

Participants

The present Swedish cohort consists of 24 VLBW infants, born at the Karolinska University Hospital between 2001–2004. They were enrolled in the study during the first week after birth and received caffeine therapy or placebo, until it was no longer needed during the neonatal period (Schmidt et al., 2006). The characteristics of the preterm infants and parental education is presented in **Table 1**.

Ethics Statement

The study was performed in accordance with European Community guidelines. The regional ethics committees at the Karolinska Institutet and Stockholm County approved the study (2012/1401). Informed written consent was obtained from the parents. Feedback to parents was communicated after assessments.

Procedure

Follow-up in terms of medical, motor, and cognitive assessment was performed three times. The cognitive assessment was performed by clinical psychologists at 18–24 months and at the 5th and 11th years of age. The assessment at 18 months, at 5 years as well as at 11 years were corrected for preterm birth. Child and parent ratings of behavior was collected at 11 years: Presentation of motor assessments and behavior ratings have been planned for in the near future.

TABLE 1 | Characteristics of preterm infants and parents' educational level, Swedish CAP cohort.

Variables	Descriptive statistics
Infants Characteristics, *n* = 24	
Birth weight, mean (SD), g	981 (167)
Gestational age, mean (SD), week	27 (1.1)
Female, no. (%)	10 (42)
Very preterm (28–29 weeks), no. (%)	6 (25)
Extremely preterm (<28 weeks), no. (%)	18 (75)
SGA, no. (%)	8 (33)
Singleton birth, no. (%)	20 (83)
Respiratory support <8 days no. (%)	7 (25)
Invasive ventilatory support <6 days no. (%)	13 (54)
Invasive ventilatory support >7 days no. (%)	4 (17)
Medical complications	
CLD, no. (%)	4 (17)
BPD, no. (%)	7 (29)
IVH, no. (%)	5 (21)
ROP, grade >3, treated, no. (%)	4 (17)
ROP, grade 1–2, no. (%)	3 (13)
Sepsis, no. (%)	16 (67)
Parental Education Level, at 11 years (*n* = 48)	
Less than elementary school level yes, no. (%)	2 (4)
High school, yes, no. (%)	21 (45)
Diploma, yes, no. (%)	11(23)
Bachelor/masters, yes, no. (%)	11(23)
PhD, yes, no. (%)	2 (4)

Respiratory support (Invasive and non-invasive ventilatory support combined) <8 days. CLD = chronic lung disease, BPD = bronchopulmonary dysplasia, IVH = intraventricular hemorrhage, ROP = retinopathy of prematurity.

Tests and Measures

General cognitive development/functioning was estimated at the three assessments points, with the second edition of the Bayley Scales of Infant Development, (BSID-II), mental development index (MDI), WPPSI-III full scale index (FSIQ), and WISC-IV full scale index (FSIQ), respectively. A validated WISC-IV-short form was used (Crawford et al., 2010) in exchange for WASI-II and DLS Swedish Reading and Spelling tests replaced the corresponding sub-tests from WRAT-4. Standard scores on cognitive indexes have a mean of 100 and standard deviation (SD) of 15, with higher scores indicating a higher level of cognitive development/functioning (Bayley, 1993; David, 2006, 2007). Tests and measures evaluated in the study are presented in **Table 2**.

Statistical Methods

Descriptive statistics are presented either as means, SD, and medians (ranges) for continuous data, or as frequencies or percentages for categorical variables.

For continuous variables the paired *t* test was used to examine within subject changes between two assessment time points and the independent *t* test was applied to examine difference between males and females. The Mann-Whitney test was employed when there was a violation of the assumptions of normality and equal variance.

Based on the three indexes, BSID-II (MDI), WPPSI-III (FSIQ), and WISC-IV (FSIQ) a standardized cognitive scale was created (-2 to $+2$). The Friedman ANOVA was performed to compare the three scales considering the ordinal property of the outcome variable. *Post hoc* analysis with Wilcoxon signed-rank tests was conducted with a Bonferroni correction applied since the overall Friedman ANOVA was significant.

We employed Generalized Estimating Equation (GEE) to analyze the change over time and to examine the fixed effects of male/female sex. A time x sex interaction term was introduced in the model to examine heterogeneity effect. GEE was also performed to control for potential confounders. Explanatory variables were selected based on clinical relevance, earlier research findings and univariate GEE models. Upon completion of the univariate analyses, we selected variables for the multivariate analyses including factors judged to be potential confounders. We have also employed Linear mixed model (LMM) to examine the relationship between the response

TABLE 2 | Cognitive test during follow up.

Age	Test methods	Measures
18 month	BSID-II, Bayley scales of infant development – second edition	Mental development index (MDI)
5 year	WPPSI-III, Wechsler's Preschool and Primary Scale of Intelligence - Third edition	Full scale IQ (FSIQ): verbal function (VI) visual function (PI) and processing speed (SI)
5 and 11 year	Beery -Buktenica Developmental Test of Visual-Motor Integration (VMI) – sixth edition	Visuomotor integration (VMI), visual perception (VP) and fine motor coordination (FMC)
11 year	WISC-IV, Wechsler's Intelligence Scale for Children – fourth edition, short form	Full scale IQ (FSIQ): 7 subtests. Verbal function index (VI): subtests similarities and vocabulary. Performance index (PI): subtests block design and matrix reasoning. Index speed of process (SI): subtests coding and symbol search. Working memory: subtest digit span
	RCFT: rey complex figure test and recognition trial	Copy score and Recall score
	TEA-Ch; test of everyday attention for children	Selective (subtest sky search), sustained (subtest score), divided (subtest sky search dt) and shifting (subtest creature)
	WRAT-4: the wide range achievement test	Mathematics (numerical operations)
	DLS for school year 4–6; diagnostic material for reading and writing. Swedish tests	Spelling 36 words and Reading 47 words

IQ score as numerical continuous outcome variable and clinical and demographic explanatory variables. Both GEE and LMM simultaneously examine the relationship between each predictor and the outcome variable and the relationship between changes in the predictors and changes in the dependent variable. The dependent variable cognitive level was coded as −2, −1, 0, 1, and 2 for each timepoint, based on the standardized cognitive level which represent severe delay (≤ = 70), moderate delay (71–85), normal (86–114), high (115–130), and superior (≥131) cognitive levels, respectively. Categorical explanatory variables were coded depending on their level. If only two levels existed, the reference category was the category with the higher code number: The variable sex was coded as female = 1 and male = 2, thus male was the reference category. The categorical variable "educational level" constituted six levels, which were coded as 1 = less than elementary, 2 = elementary, 3 = high school, 4 = diploma, 5 = university degree (Bachelor or Masters), and 6 = Ph.D. holder. Less than elementary was the reference category. IVH was coded as 0 = no IVH, 1 = Grade 1, 2 = Grade 2; grade 2 was the reference category. Time was coded as 1 = 18 months, 2 = 5 years, and 3 = 11 years (the reference category). Level of respiratory support was coded as: 0 = Invasive (endotracheal tube *in situ*) and non-invasive (continuous positive airway pressure- CPAP) ventilatory support combined <8 days, 1 = mild (combined ventilatory support >8 days but invasive ventilatory support ≤6 days) and 2 = prolonged (invasive ventilatory support >6 days). The results of GEE are presented as estimate (regression coefficient), standard error (SE), Wald Chi-square value and *p*-value. Results of LMM are included in **Supplementary Table 1**. SPSS version 25.0 (IBM, NY, United States) was used for all data analyses and Statistica 13 for case profile graphical presentation. The level of significance was specified as $p = 0.05$.

RESULTS

All 24 children in the Swedish cohort participated in all three assessments, thus the follow up was 100%. **Table 1** shows the characteristics of preterm birth, medical and social background factors for the complete group as well as separated for by sex. At the 5-year and 11-year assessment, no child was deaf, blind or had cerebral palsy. Preterm medical risk variables that were considered to have too few participants to be included in further analyses are not shown in the tables. The level of the cognitive index score at the three assessment points for each gender are summarized in **Figure 1**. **Table 2** shows all tests, including abbreviations, cognitive, and academic measures for the Swedish CAP cohort. The test results are summarized in **Table 3**. The mean test score of the study subjects is presented by sex, maternal education level, paternal education level, and IVH (**Figures 2A–D**, respectively). The independent sample *t*-test was employed to compare means for between group differences and the paired *t*-test for within-subject change (**Table 4**).

Cognitive Level Through Development

Friedman test revealed a statistically significant difference in the standardized cognitive index over time, $\chi^2 = 18.7$, df = 2, $p < 0.001$. *Post hoc* analysis with Wilcoxon signed-rank tests was conducted with a Bonferroni correction applied, resulting in a significance level set at $p = 0.017$. We observed a statistically significant difference between Bayley-II MDI (at 18 months) and WPPSI-III FSIQ (at 5 years) ($p = 0.001$) and between WPPSI-III FSIQ (at 5 years) and WISC-IV FSIQ (at 11 years) ($p = 0.002$). However, we did not observe significant differences between BSID-II MDI and WISC-IV FSIQ ($Z = -1.67$, $p = 0.096$). WISC-FI was significantly related to WISC-SI, Mathematics SS, sex, and IVH (**Supplementary Table 1**). LMM also revealed that time was a statistically significant predictor of cognitive score. We observed a statistically significant difference between WPPSI-III FSIQ (at 5 years) and WISC-IV FSIQ at 11 years ($p = 0.02$). However, we did not observe a significant difference between BSID-II MDI and WISC-IV FSIQ ($p = 0.79$).

The univariate and multivariable General Estimating Equation (GEE) analysis were carried out and are presented in **Tables 5**, **6** respectively. Sex was observed to be a predictor of standardized

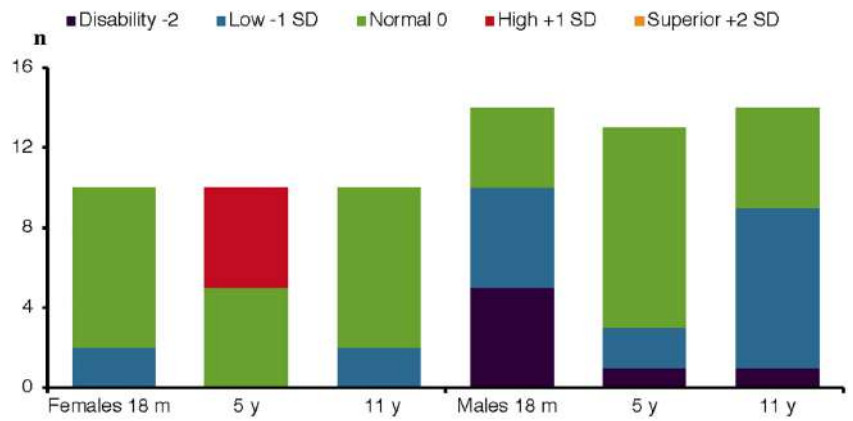


FIGURE 1 | Cognitive level for females and males at three assessment points. Cognitive index scores standardized in levels. Index score <70 = disability/severe delay. 71–85 = low/moderate delay. 86–115 = normal/average. 116–130 = high. <131 = superior. Assessment at 18 months, 5 and 11 years of age, 10 female and 14 male preterm VLBW children.

TABLE 3 | Test results for the Swedish cohort.

Tests	Swedish data: *N* = 24 mean (SD)	Female *N* = 10 mean (SD)	Male *N* = 14 mean (SD)	Total *N* = 24 min.– max. Results
BSID-II MDI **18 months**	85.7 (17.0)	97.8 (15.1)	77.1 (12.7)	55 – 114
WPPSI-III FSIQ **5 year** (full scale)	102.0 (16.6)	110.4 (14.6)	95.9 (15.7)	57 – 128
WPPSI-III VI (verbal)	98.1 (17.8)	107.0 (15.9)	91.8 (16.8)	47 – 137
WPPSI-III PI (visual)	97.3 (12.9)	100.8 (11.8)	94.7 (13.4)	70 – 120
WPPSI-III SI (speed)	79.0 (12.8)	87.9 (12.6)	72.6 (8.7)	46 – 114
WISC-IV FSIQ **11 year**	86.9 (13.4)	95.1 (12.8)	81.0 (10.8)	56 – 113
WISC-IV VI (verbal)	89.0 (15.0)	98.9 (14.8)	86.2 (11.3)	70 – 122
WISC-IV PI (visual)	95.5 (12.0)	101.2 (12.9)	91.4 (9.8)	77 – 121
WISC-IV SI (speed)	89.4 (14.1)	95.6 (12.4)	84.9 (13.8)	55 – 116
Beery VMI **5 year** (copying)	96.3 (16.8)	102.9 (21.7)	91.6 (10.8)	70 – 132
VMI VP (perception)	105.9 (23.6)	111.1 (23.7)	102.1 (23.7)	45 – 132
VMI FMC (fine-motor control)	92.4 (21.0)	101.3 (21.7)	86.1 (18.8)	45 – 130
Beery VMI **11 year** (copying)	90.5 (15.1)	98.4 (10.4)	84.9 (15.7)	62 – 115
VMI VP (perception)	96.0 (17.3)	100.9 (16.1)	92.5 (17.7)	45 – 113
VMI FMC (fine-motor control)	87.8 (15.7)	95.1 (15.3)	82.6 (14.3)	45 – 114
WRAT-4, Mathematic	81.2 (16.4)	86.7 (16.4)	77.3 (9.7)	16 – 40
Reading 47 words; %	80.0 (21.0)	87.9 (14.1)	74.4 (23.7)	4 – 47
Spelling 36; stanine	3.3 (1.9)	4.4 (1.7)	2.5 (1.7)	3 – 32
TEA-Ch: selective att.	7.6 (3.3)	9.1 (3.3)	6.5 (2.9)	2 – 13
TEA-Ch: sustained	7.1 (2.1)	8.1 (2.2)	6.4 (1.8)	4 – 13
TEA-Ch: divided	5.3 (2.4)	5.4 (2.8)	5.2 (2.2)	1 – 10
TEA-Ch: shifting	9.3 (3.3)	10.2 (3.4)	8.6 (3)	3 – 13
WISC-IV digit span, forward	5.0 (1.0)	5.2 (1.3)	4.9 (0.8)	3 – 7
WISC-IV digit span, backward	3.6 (0.9)	3.8 (1.0)	3.4 (0.8)	2 – 6
RCFT, copy	24.3 (9.6)	28.8 (5.8)	21.1 (10.6)	3 – 36
RCFT, delayed recall	11.7 (7.9)	13.9 (5.0)	10.0 (9.4)	0 – 25.5

cognitive score. Female sex was positively associated with standardized score in all multivariable models, **Table 6**. Univariate GEE revealed that the likelihood of having a higher cognitive score was positively related to time at 5 years ($B = 1.58$, Wald Chi-square = 10.8, $p = 0.001$) with 11 years as a reference. However, when the dependent variable was the standardized Z score, GEE revealed that the likelihood of having a higher Z cognitive score was positively related to time (Wald Chi square 18.7, df = 2, $p < 0.001$). Higher Z score value was positively related to time at 5 years ($B = 2.1$, Wald Chi-square = 12.9, df = 1, $p < 0.001$) with 11 years as a reference.

General Estimating Equation showed that IVH was a predictor of standardized cognitive score. In addition, level of respiratory support was a significant predictor of standardized cognitive score in the univariate ($p = 0.03$) and multivariable GEE analysis when controlling for sex and time in the latter analysis ($p = 0.04$). SGA and BPD were not statistically significant in either univariate and multivariable GEE analyses, when controlled for sex and time, **Tables 5**, **6**.

Univariate GEE analysis revealed a positive relationship between parental education levels and the standardized score (**Table 5**). Both maternal and paternal educational levels were positively associated with the standardized score in all multivariable models when controlling for sex and time (**Supplementary Table 2**). We did not observe interactions between time and other clinical and demographic factors in all used multivariable models.

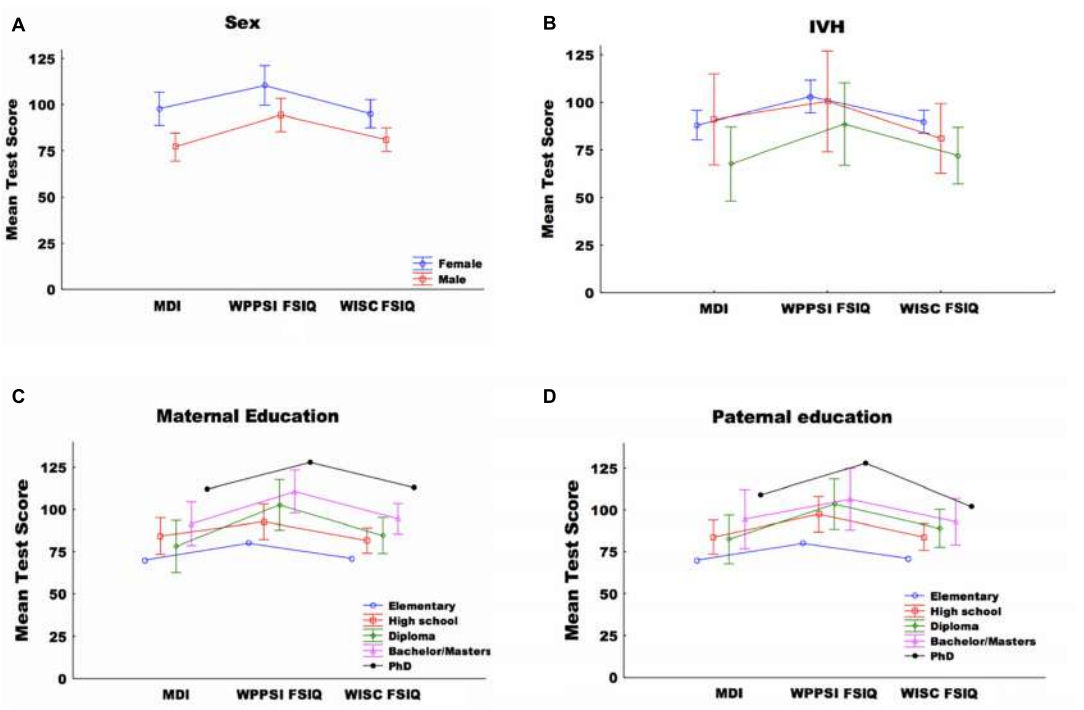


FIGURE 2 | (A) Mean test score by sex and test type. **(B)** Mean test score by IVH and test type. **(C)** Mean test score by maternal educational level and test type, **(D)** mean test score by paternal educational level and test type. Mental development index (MDI), WPPSI-III Full scale index (FSIQ), and WISC-IV Full scale index (FSIQ) 18 month, 5 and 11 years, respectively. Data is presented as mean + SEM.

TABLE 4 | Results of the paired and independent *t* tests of the Swedish cohort.

Scale	Mean difference (95% CI)	*P*-value
Paired *t* test		
2 VMI SS – VMI SS	−5.5 (−9.8, −1.3)	0.01
2 VMI VP – VMI VP	−10.1 (−17.0, −3.2)	<0.01
2 VMI Motor – VMI motor	−5.2 (−11.6, 1.1)	0.1
WISC VI – WPPSI VI	−13.1 (−17.3, −8.8)	<0.001
WISC PI – WPPSI PI	−8.8 (−12.6, −4.9)	<0.001
WISC SI – WPPSI S	10.4 (5.8, 15.6)	<0.001
Independent *t* test (female – male)		
2VMI SS	13.5 (1.6, 25.3)	0.03
2VMI VP	0.03 (−0.4, 0.5)	0.42
2VMI FMC	13.1 (0.5, 25.7)	0.04
WPPSI FSIQ	14.6 (1.6, 27.7)	0.03
WPPSI VI (verbal)	16.0 (1.1, 30.8)	0.04
WPPSI PI (visual)	6.5 (−4.9, 18.0)	0.25
WPPSI SI (speed)	17.2 (−4.9, 27.2)	<0.001
WISC FSIQ	14.1 (4.1, 24.1)	<0.01
WISC VI (verbal)	12.6 (1.7, 23.7)	0.03
WISC PI (visual)	9.8 (0.2, 19.3)	<0.05
WISC SI (speed)	10.7 (−0.7, 22.1)	0.17

Mean differences, 95% confidence interval (CI) and p-values for paired t and independent t tests.

TABLE 5 | Results of GEE univariate analysis with parameter estimates, standard errors and Wald Chi square for the cohort for the dependent variable cognitive level.

Variable	B	SE	Wald Chi-square	*P*-value
Sex = Female	2.25	0.79	8.14	0.004
Time			16.2	<0.001
Time = 18 months	−0.41	0.31	1.78	0.18
Time = 5 years	1.58	0.48	10.8	0.001
SGA = 0	1.1	0.72	2.34	0.12
BPD = 0	−0.12	0.67	0.03	0.86
IVH			8.6	0.01
IVH = 0	2.4	0.93	6.45	0.01
IVH = 1	2.9	1.00	8.43	0.004
Maternal education*				
University	5.5	0.86	40.6	<0.001
Bachelor/masters	3.4	0.83	16.5	<0.001
Diploma	1.7	0.42	16.3	<0.001
Elementary	1.9	0.72	7.2	0.007
Paternal education*				
University	5.3	0.91	34.1	<0.001
Bachelor/masters	2.9	0.92	10.1	0.001
Diploma	3.8	0.59	41.6	<0.001
High School	2.2	0.42	27.4	<0.001
Elementary	1.9	0.67	7.8	0.005
Respiratory[#] support level				
Level = 0	1.7	0.70	5.5	0.019
Level = 1	1.6	0.65	5.8	0.015

**Reference: less than elementary. [#]Reference: respiratory support = level 2 (invasive ventilatory support >6 days).*

The independent *t*-test revealed that there was statistically significant score differences in between the sexes for many items indicating higher score for girls compared to boys (**Table 4**). The paired mean differences were also statistically significant for most items (**Table 4**).

Cognitive Functioning and Academic Achievement at 11 Years of Age

Data analyses revealed broad confidence intervals for results of specific cognitive measures (TEA-Ch, RCFT, and WISC-IV digit span) at 11 years of age. WISC-IV SI was found significantly lower than WISC-IV FSIQ at 11 years ($p \leq 0.001$). Measures of academic achievement showed sex difference in Mathematic WRAT-4 ($p = 0.04$) and Spelling DLS measures ($p = 0.01$), favoring girls. We did not observe differences in reading ability.

DISCUSSION

This prospective cohort study has three assessment points of cognitive development and a follow-up rate of 100%, which adds stability to the results and bolster our conclusions. We found that, male sex and parental education had a significant impact on cognitive test results. This concurs with previous studies but the present data further underline the effect of sex and parental education in long term cognitive and academic outcomes (Bohm et al., 2002; Linsell et al., 2015, 2018; Mangin et al., 2017). IVH was identified as a strong predictor of cognitive outcome, and so was the cumulative duration of invasive ventilatory support. This is in accordance with recent studies, e.g., (Breeman et al., 2017). However, other medical complications (ROP, CLD/BPD, SGA, and sepsis) did not contribute to the explained variance. Sex differences were seen in all tests given, with females acquiring higher scores than males. Parental education was on average high in both mothers and fathers and all levels significantly influenced the cognitive outcome in the multivariate analyses.

The GEE is an appropriate statistical method to fit a marginal model for longitudinal data analysis since we have repeated measures over time. We measured 24 children at three time points with three different cognitive tests to examine their cognitive development/functioning. The repeated measurements thus provides a multivariate response of similar individuals. GEE is a common approach to longitudinal data based on population-averaged (marginal) approach. The GEE models the average response over the subpopulation sharing a common value of the predictors, as a function of the predictors (Liang and Zeger, 1986).

Cognitive z-scores from −2 to +2 are represented in this Swedish cohort of VLBW infants. GEE showed that the cognitive results were similar at 18 months and 11 years of age. We found the results to be important since predictability of early assessments varies. Robust findings based on meta-analyses and single studies imply that the predictability for later cognitive functioning in pre-school and school-aged children vary, from moderate in very preterm to poor in extremely preterm infants. In contrast, several studies of cognitive outcome between pre-school and middle-school age as well as adolescence,

TABLE 6 | Results of GEE multivariable analysis with parameter estimates, standard errors and Wald Chi square for the cohort for the dependent variable cognitive level.

Variable	B	SE	Wald Chi-square	*P*-value
Model 1 with SGA				
SGA	0.82	0.76	1.2	0.28
Sex = female	2.51	0.91	7.51	0.006
Time = 18 months	−0.70	0.42	2.81	0.09
Time = 5 years	1.88	0.49	15.0	<0.001
Model 2 with IVH				
IVH = 0	1.6	1.08	2.12	0.14
IVH = 1	3.6	1.07	11.2	0.001
Sex = female	2.76	1.00	7.6	0.006
Time = 18 months	−0.75	0.47	2.5	0.11
Time = 5 years	2.01	0.52	14.7	<0.001
Model 3* Maternal education				
University	5.1	1.26	16.4	<0.001
Bachelor/masters	3.6	0.97	13.8	<0.001
Diploma	1.9	0.68	7.81	0.005
Elementary	1.5	0.90	2.8	0.094
Sex = female	2.68	0.96	7.77	0.005
Time = 18 months	−0.77	0.45	2.86	0.09
Time = 5 years	1.51	0.84	3.21	0.07
Model 4* Paternal Education				
University	4.51	1.22	12.1	<0.001
Bachelor/Masters	1.93	0.95	4.0	0.047
Diploma	2.43	1.25	3.78	
High school	2.60	0.72	13.4	<0.001
Elementary	1.74	0.78	4.91	0.027
Sex = female	2.53	1.26	3.93	0.047
Time = 18 months	−0.78	0.48	2.71	0.10
Time = 5 years	2.04	0.54	14.3	<0.001
Model 5 Respiratory support#				
Level = 0	3.15	1.25	6.3	0.012
Level = 1	2.35	1.25	3.5	0.06

**Reference: less than elementary; #Reference: respiratory support = level 2. The multivariable models include the variables sex and time with the interaction.*

report stability in cognitive development (Stalnacke et al., 2015; Mangin et al., 2017).

Summarizing other studies of cognitive development from infancy to adolescence is challenging due to methodological deficiencies. Outcome studies often have only one or two assessment points, lack of a control group, loss to follow up, and a varied application of standardized test norms and statistical methodologies, rendering the conclusions of these studies hard to compare (Wong et al., 2016). Based on our study design and results we find it important to take into account that time points and time between cognitive assessments can affect findings in cognitive follow-up of preterm children. Of equal importance are longitudinal studies, and maintaining high retention throughout follow-up (Doyle and Anderson, 2018). Recently, the longitudinal EPI Cure study showed that cognitive test score in infancy and early childhood reflect early adult outcomes (Linsell et al., 2018).

The cognitive results at 5 years of age were markedly higher compared to cognitive levels at both 18 months and 11 years of age, which requires an account of potential methodological issues in the performance of said test. The Swedish WPPSI-III norms are based on British norms and validated in a limited sample of Swedish children (David, 2006). Thus, the high results at 5 years of age might be due to an incomplete Swedish validation of the British WPPSI-III norms. Test construction, validity and reliability issues of cognitive assessment in preterm children can have an impact on cognitive results and are also addressed in other studies (Luttikhuizen dos Santos et al., 2013; Spencer-Smith et al., 2015). The results are similar at 18 months and 11 years, both at individual and group level. Thus, the apparent increase at the 5-year assessment are likely due to test norm differences. Nevertheless, the heterogeneous nature of cognitive outcomes in individuals emphasize the importance of long-term follow-up and monitoring of infants born VLBW (Manley et al., 2015; Beauregard et al., 2018).

We know little about the developmental pace regarding different aspects of cognition, especially for preterm children, with their risk of suboptimal neurocognitive development/cognition. There is still considerable debate and uncertainty with regards to whether very preterm children grow into or out of their cognition problems (Mangin et al., 2017). Our and other long-term studies, that address individual patterns and explore trajectories in cognitive development, indicate that cognitive test scores in infancy and early childhood may reflect cognition and academic performance during early school years (Stalnacke et al., 2015; Wong et al., 2016; Mangin et al., 2017; Linsell et al., 2018). Notably, cognitive trajectories may differ. Recently, several distinct language trajectories were revealed, in very preterm and full term infants examined at 2, 5, 7, and 13 years (Nguyen et al., 2018). This and our study underline the importance of monitoring cognition in children born very preterm before school entry and across childhood.

The process of cognitive maturation is complex, multidimensional and influenced by genetic predisposition, environmental factors and experience (Fuster, 2005) and ruptures in development (Anderson, 2001; Anderson et al., 2008). Effects on brain development (Haynes et al., 2011; Volpe et al., 2011) and coherent alternated cognitive trajectories (Mangin et al., 2017) are found in preterm children (Thompson et al., 2018). Comparing the cognitive results at 5 years and 11 years of age indicated both stability and change in the cohort. Level of verbal intelligence (VI) was significantly lower at 11 years and so was the visuo-constructive measure (Beery VMI). Perceptual intelligence (PI) was found to be stable. Processing speed (SI) was higher at 11 years. The visuo-constructive measure (Beery VMI) was significantly lower at 11 years. Declining results and differences between cognitive functions may be affected by specific deficits especially in executive functions. In our study it was not possible to draw a conclusion from executive tests at 11 years since the single specific cognitive measures, TEA-Ch, RCFT, and Digit Span, had broad confidence intervals and in combination with the effect of the cohort size could lead to random results. Problems with attention, working memory and processing speed are significantly more present among

preterm children and tend to emerge during development (Rose et al., 2011, 2012; Murray et al., 2014). Deficits in attention and processing speed are identified as important abilities contributing to lower level of cognitive intelligence in preterm children (Rose et al., 2009, 2011, 2012). Cognitive functioning in preterm children is of great importance for school performance. Results in academic achievement are of special interest, as they will show any issues that may be a problem in school. Our test battery included mathematics, reading and spelling. Mathematics were significantly correlated to FSIQ at 11 years (r = 0.77) and WISC PI, WISC SI, and IVH-2 were found to significantly explain most of the variance (R^2 = 0.64) of the dependent variable Mathematics.

Sex differences were seen in all tests given, with females acquiring higher scores than males. Except for the VMI visual perception (VP) and fine motor coordination, this was also consistent between the two assessment points. Females also performed higher in academics, mathematics and spelling. Male sex is known to be a disadvantage with regards to mortality, morbidity and incidence of brain injury in preterm children (Marlow et al., 2005; Hintz et al., 2006; Skiold et al., 2014). The major type of brain injury involves cerebral white matter and the principal cellular target is the developing oligodendrocytes (Volpe et al., 2011). Neonatal white matter abnormalities are associated with cognitive impairment across childhood (Mangin et al., 2017). The view of male sex as a risk factor for cognitive development varies. Extremely premature boys have an increased risk of lower cognitive outcome (Linsell et al., 2018) and of developing severe cognitive disability (Marlow et al., 2005). However, in very preterm children the difference based on male sex decreases with age and environmental factors become more significant (Linsell et al., 2015; Mangin et al., 2017). The present results are in line with previous as well as recent studies (Doyle et al., 2015; Linsell et al., 2018; Thompson et al., 2018) and underlines the importance of considering sex in the potential cognitive developmental of preterm infants. Structural asymmetries and sexual brain dimorphism already exist at 1 month after birth in healthy term infants (Dean et al., 2018). In general, males have larger total brain volume and volumes differ by sex in regionally specific brain regions. We speculate that the different rates of maturation between the sexes renders the male brain, and hence male sex, a risk factor for long term cognitive outcome especially in VLBW preterm infants. Some of the underlying mechanisms for sex differences found, are delayed myelinization in preterm males, lower white matter volumes in males and differences in cerebral white matter microstructure compared to preterm females (Constable et al., 2008; Skiold et al., 2014).

We cannot exclude the effect of neonatal caffeine therapy from our findings. However, no adverse long-term effects of caffeine on development have been shown in the CAP studies, e.g., (Schmidt et al., 2006, 2017; Mürner-Lavanchy et al., 2018). The CAP-trial indicated a positive developmental trend in cognitive scores, between 18 months and 5 years of age, independent of treatment. Thus, our data are consistent with the results from this larger cohort (Schmidt et al., 2012). However, in the present cohort, the data and trajectories suggest that the increase was temporary and likely due to test norm differences. Nevertheless, cognitive results in our cohort indicate similar levels between 18 months and 11 years of age. Thus, on a group level, this does not support the suggestion that cognitive outcomes for preterm VLBW infants may improve throughout childhood (Ment et al., 2003). However, they are in line with, and underscore previous findings with regards to the importance of IVH, level of ventilatory support, sex as well as parental education for the long-term cognitive and academic outcomes (Doyle et al., 2015). These data emphasize the importance of early and repeated individual assessment enabling early intervention as well as adequate support during early childhood into adolescence, especially for those with cognitive delay and at risk for cognitive decline (Stalnacke et al., 2015; Mangin et al., 2017).

A strength of the present study is the 100% follow-up rate. The small group size is a limitation, which is why all medical variables and possible background variables could not be included in the model. The specific cognitive measures at 11 years of age, were found to have broad confidence intervals and thus in combination with the effect of cohort size these results could be random.

In summary analyzing measures of cognitive development across time better clarify changes and risk factors. We suggest long-term study designs, with more than two assessment points and with a substantial time between follow-up. It is possible to explore trajectories of cognitive function in a small cohort, when the cohort remains the same. Exploring individual patterns of cognition and brain development, as well as the underlying mechanisms associated with these, is necessary to increase knowledge about the maturation of preterm children. Cognitive development extends well beyond adolescence; and thus future follow-up should continue beyond 11 years. Early identification of children in need of support to promote development is an imperative necessity, especially for those with cognitive delay and at risk for cognitive decline.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

EH and BB conceptualized and designed the study. EH, SS, and BB acquired the data and revised the manuscript. All authors analyzed the data, drafted a significant portion of the manuscript or figures, and accepted the final version of the manuscript.

FUNDING

This study was supported by the Swedish Research Council (2009-3724 and 2016-0111), the Stockholm County Council (ALF projects 2012-0465 and 20140011), the Karolinska Institutet, and grants from the Swedish Brain (2015-0020), Axel Tielman (2015-00220 and 2018-00418),

Freemasons Children's House, Astrid Lindgren Children's Hospital and Swedish National Heart and Lung (2015-0558) Foundations. The funding sources of the study had no role in the study design, data collection, analysis, interpretation, or writing of the results of this study, or in the decision to submit.

ACKNOWLEDGMENTS

We acknowledge research nurse Lena Legnevall for providing technical assistance and psychologist Anette Holm and Stephanie Cullberg-Sundén for assisting with the testing procedures. We thank Dr. Louise Steinhoff and Ph.D. Wiktor Phillips for English language assistance and Prof. Peter J. Anderson for discussion and advice. We are indebted to the CAP investigators who made this study possible and importantly the children and their families who participated in this follow-up study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00307/full#supplementary-material

REFERENCES

Anderson, V. (2001). *Developmental Neuropsychology: A Clinical Approach*. Hove: Psychology Press.

Anderson, V., Jacobs, R., and Anderson, P. (2008). *Executive Functions And The Frontal Lobes: A Lifespan Perspective*. Didcot: Taylor & Francis.

Aylward, G. P. P. A. (2014). Neurodevelopmental outcomes of infants born prematurely. *J. Dev. Behav. Pediatr.* 35, 394–407. doi: 10.1097/01.DBP.0000452240.39511.d4

Bayley, N. (1993). *Bayley Scales of Infant Development*, 2nd Edn. San Antonio, TX: The Psychological Corporation.

Beauregard, J. L., Drews-Botsch, C., Sales, J. M., Flanders, W. D., and Kramer, M. R. (2018). Does socioeconomic status modify the association between preterm birth and children's early cognitive ability and kindergarten academic achievement in the United States? *Am. J. Epidemiol.* 187, 1704–1713. doi: 10.1093/aje/kwy068

Bohm, B., Katz-Salamon, M., Institute, K., Smedler, A. C., Lagercrantz, H., and Forssberg, H. (2002). Developmental risks and protective factors for influencing cognitive outcome at 5 1/2 years of age in very-low-birthweight children. *Dev. Med. Child Neurol.* 44, 508–516. doi: 10.1111/j.1469-8749.2002.tb00321.x

Breeman, L. D., Jaekel, J., Baumann, N., Bartmann, P., and Wolke, D. (2017). Neonatal predictors of cognitive ability in adults born very preterm: a prospective cohort study. *Dev. Med. Child Neurol.* 59, 477–483. doi: 10.1111/dmcn.13380

Constable, R. T., Ment, L. R., Vohr, B. R., Kesler, S. R., Fulbright, R. K., Lacadie, C., et al. (2008). Prematurely born children demonstrate white matter microstructural differences at 12 years of age, relative to term control subjects: an investigation of group and gender effects. *Pediatrics* 121, 306–316. doi: 10.1542/peds.2007-0414

Crawford, J. R., Anderson, V., Rankin, P. M., and Macdonald, J. (2010). An index-based short-form of the WISC-IV with accompanying analysis of the reliability and abnormality of differences. *Br. J. Clin. Psychol.* 49, 235–258. doi: 10.1348/014466509X455470

David, W. (2006). *Wechsler Preschool and Primary Scale of Intelligence*, 2nd Edn. Stockholm: Harcourt Assessment.

David, W. (2007). *Wechsler Intelligence Scale for Children*, 4th Edn. Stockholm: Harcourt Assessment.

Dean, D. C., Planalp, E. M., Wooten, W., Schmidt, C. K., Kecskemeti, S. R., Frye, C., et al. (2018). Investigation of brain structure in the 1-month infant. *Brain Struct. Funct.* 223, 1953–1970. doi: 10.1007/s00429-017-1600-2

Doyle, L. W., and Anderson, P. J. (2009). Long-term outcomes of bronchopulmonary dysplasia. *Semin. Fetal Neonatal Med.* 14, 391–395. doi: 10.1016/j.siny.2009.08.004

Doyle, L. W., and Anderson, P. J. (2018). Stability of general cognition in children born extremely preterm as they grow older: good or bad news? *Arch. Dis. Child Fetal Neonatal Educ.* 103, F299–F300. doi: 10.1136/archdischild-2017-313987

Doyle, L. W., Cheong, J. L., Burnett, A., Roberts, G., Lee, K. J., Anderson, P. J., et al. (2015). Biological and social influences on outcomes of extreme-preterm/low-birth weight adolescents. *Pediatrics* 136, e1513–e1520. doi: 10.1542/peds.2015-2006

Fuster, J. M. (2005). *Cortex and Mind*. Oxford: Oxford University Press. doi: 10.1093/acprof:oso/9780195300840.001.0001

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Haynes, R. L., Xu, G., Folkerth, R. D., Trachtenberg, F. L., Volpe, J. J., and Kinney, H. C. (2011). Potential neuronal repair in cerebral white matter injury in the human neonate. *Pediatr. Res.* 69, 62–67. doi: 10.1203/PDR.0b013e3181ff3792

Hintz, S. R., Kendrick, D. E., Vohr, B. R., Kenneth Poole, W., Higgins, R. D., and Nichd Neonatal Research Network. (2006). Gender differences in neurodevelopmental outcomes among extremely preterm, extremely-low-birthweight infants. *Acta Paediatr.* 95, 1239–1248. doi: 10.1080/08035250600599727

Hofstetter, A. O., Legnevall, L., Herlenius, E., and Katz-Salamon, M. (2008). Cardiorespiratory development in extremely preterm infants: vulnerability to infection and persistence of events beyond term-equivalent age. *Acta Paediatr.* 97, 285–292. doi: 10.1111/j.1651-2227.2007.00618.x

Janvier, A., Khairy, M., Kokkotis, A., Cormier, C., Messmer, D., and Barrington, K. J. (2004). Apnea is associated with neurodevelopmental impairment in very low birth weight infants. *J. Perinatol.* 24, 763–768. doi: 10.1038/sj.jp.7211182

Johnson, S., Fawke, J., Hennessy, E., Rowell, V., Thomas, S., Wolke, D., et al. (2009). Neurodevelopmental disability through 11 years of age in children born before 26 weeks of gestation. *Pediatrics* 124, e249–e257. doi: 10.1542/peds.2008-3743

Liang, K.-Y., and Zeger, S. L. (1986). Longitudinal data analysis using generalized linear models. *Biometrika* 73, 13–22. doi: 10.1093/biomet/73.1.13

Linsell, L., Johnson, S., Wolke, D., O'reilly, H., Morris, J. K., Kurinczuk, J. J., et al. (2018). Cognitive trajectories from infancy to early adulthood following birth before 26 weeks of gestation: a prospective, population-based cohort study. *Arch. Dis. Child.* 103, 363–370. doi: 10.1136/archdischild-2017-313414

Linsell, L., Malouf, R., Morris, J., Kurinczuk, J. J., and Marlow, N. (2015). Prognostic factors for poor cognitive development in children born very preterm or with very low birth weight: a systematic review. *JAMA Pediatr.* 169, 1162–1172. doi: 10.1001/jamapediatrics.2015.2175

Luttikhuizen dos Santos, E. S., De Kieviet, J. F., Konigs, M., Van Elburg, R. M., and Oosterlaan, J. (2013). Predictive value of the Bayley scales of infant development on development of very preterm/very low birth weight children: a meta-analysis. *Early Hum. Dev.* 89, 487–496. doi: 10.1016/j.earlhumdev.2013.03.008

Mangin, K. S., Horwood, L. J., and Woodward, L. J. (2017). Cognitive development trajectories of very preterm and typically developing children. *Child Dev.* 88, 282–298. doi: 10.1111/cdev.12585

Manley, B. J., Roberts, R. S., Doyle, L. W., Schmidt, B., Anderson, P. J., Barrington, K. J., et al. (2015). Social variables predict gains in cognitive scores across the preschool years in children with birth weights 500 to 1250 grams. *J. Pediatr.* 166, 870–876.e2. doi: 10.1016/j.jpeds.2014.12.016

Marlow, N., Wolke, D., Bracewell, M. A., and Samara, M. (2005). Neurologic and developmental disability at six years of age after extremely preterm birth. *N. Engl. J. Med.* 352, 9–19. doi: 10.1056/NEJMoa041367

Ment, L. R., Vohr, B., Allan, W., Katz, K. H., Schneider, K. C., Westerveld, M., et al. (2003). Change in cognitive function over time in very low-birth-weight infants. *JAMA* 289, 705–711. doi: 10.1001/jama.289.6.705

Moore, T., Hennessy, E. M., Myles, J., Johnson, S. J., Draper, E. S., Costeloe, K. L., et al. (2012). Neurological and developmental outcome in extremely

preterm children born in England in 1995 and 2006: the EPICure studies. *BMJ* 345:e7961. doi: 10.1136/bmj.e7961

Mürner-Lavanchy, I. M., Doyle, L. W., Schmidt, B., Roberts, R. S., Asztalos, E. V., Costantini, L., et al. (2018). Neurobehavioral outcomes 11 years after neonatal caffeine therapy for apnea of prematurity. *Pediatrics* 141:e20174047. doi: 10.1542/peds.2017-4047

Murray, A. L., Scratch, S. E., Thompson, D. K., Inder, T. E., Doyle, L. W., Anderson, J. F., et al. (2014). Neonatal brain pathology predicts adverse attention and processing speed outcomes in very preterm and/or very low birth weight children. *Neuropsychology* 28, 552–562. doi: 10.1037/neu0000071

Nguyen, T. N., Spencer-Smith, M., Haebich, K. M., Burnett, A., Scratch, S. E., Cheong, J. L. Y., et al. (2018). Language trajectories of children born very preterm and full term from early to late childhood. *J. Pediatr.* 202, 81–91e1. doi: 10.1016/j.jpeds.2018.06.036

Roberts, G., Anderson, P. J., Doyle, L. W., and Victorian Infant Collaborative Study Group. (2010). The stability of the diagnosis of developmental disability between ages 2 and 8 in a geographic cohort of very preterm children born in 1997. *Arch. Dis. Child.* 95, 786–790. doi: 10.1136/adc.2009.160283

Rose, S. A., Feldman, J. F., and Jankowski, J. J. (2009). Information processing in toddlers: continuity from infancy and persistence of preterm deficits. *Intelligence* 37, 311–320. doi: 10.1016/j.intell.2009.02.002

Rose, S. A., Feldman, J. F., Jankowski, J. J., and Van Rossem, R. (2011). Basic information processing abilities at 11 years account for deficits in IQ associated with preterm birth. *Intelligence* 39, 198–209. doi: 10.1016/j.intell.2011.03.003

Rose, S. A., Feldman, J. F., Jankowski, J. J., and Van Rossem, R. (2012). Information processing from infancy to 11 years: continuities and prediction of IQ. *Intelligence* 40, 445–457. doi: 10.1016/j.intell.2012.05.007

Schmidt, B. (2005). Methylxanthine therapy for apnea of prematurity: evaluation of treatment benefits and risks at age 5 years in the international caffeine for apnea of prematurity (CAP) trial. *Biol. Neonate* 88, 208–213. doi: 10.1159/000087584

Schmidt, B., Anderson, P. J., Doyle, L. W., Dewey, D., Grunau, R. E., Asztalos, E. V., et al. (2012). Survival without disability to age 5 years after neonatal caffeine therapy for apnea of prematurity. *JAMA* 307, 275–282. doi: 10.1001/jama.2011.2024

Schmidt, B., Roberts, R. S., Anderson, P. J., Asztalos, E. V., Costantini, L., Davis, P. G., et al. (2017). Academic performance, motor function, and behavior 11 years after neonatal caffeine citrate therapy for apnea of prematurity: an 11-year follow-up of the CAP randomized clinical trial. *JAMA Pediatr.* 171, 564–572. doi: 10.1001/jamapediatrics.2017.0238

Schmidt, B., Roberts, R. S., Davis, P. G., Doyle, L. W., Asztalos, E. V., Opie, G., et al. (2015). Prediction of late death or disability at age 5 years using a count of 3 neonatal morbidities in very low birth weight infants. *J. Pediatr.* 167, 982–986.e2. doi: 10.1016/j.jpeds.2015.07.067

Schmidt, B., Roberts, R. S., Davis, P., Doyle, L. W., and Steering Committee of the Caffeine for Apnea of Prematurity (CAP) Trial. (2011). Archimedes: does caffeine treatment for apnoea of prematurity improve neurodevelopmental outcome in later life? *Arch Dis Child.* 96:784. doi: 10.1136/adc.2010.206698

Schmidt, B., Roberts, R. S., Davis, P., Doyle, L. W., Barrington, K. J., Ohlsson, A., et al. (2006). Caffeine therapy for apnea of prematurity. *N. Engl. J. Med.* 354, 2112–2121. doi: 10.1056/NEJMoa054065

Skiold, B., Alexandrou, G., Padilla, N., Blennow, M., Vollmer, B., and Aden, U. (2014). Sex differences in outcome and associations with neonatal brain morphology in extremely preterm children. *J. Pediatr.* 164, 1012–1018. doi: 10.1016/j.jpeds.2013.12.051

Spencer-Smith, M. M., Spittle, A. J., Lee, K. J., Doyle, L. W., and Anderson, P. J. (2015). Bayley-III cognitive and language scales in preterm children. *Pediatrics* 135, e1258–e1265. doi: 10.1542/peds.2014-3039

Stalnacke, J., Lundequist, A., Bohm, B., Forssberg, H., and Smedler, A. C. (2015). Individual cognitive patterns and developmental trajectories after preterm birth. *Child Neuropsychol.* 21, 648–667. doi: 10.1080/09297049.2014.958071

Thompson, D. K., Kelly, C. E., Chen, J., Beare, R., Alexander, B., Seal, M. L., et al. (2018). Early life predictors of brain development at term-equivalent age in infants born across the gestational age spectrum. *Neuroimage* 185, 813–824. doi: 10.1016/j.neuroimage.2018.04.031

Volpe, J. J. (1980). Evaluation of neonatal periventricular-intraventricular hemorrhage. A major advance. *Am. J. Dis. Child* 134, 1023–1025. doi: 10.1001/archpedi.1980.02130230003001

Volpe, J. J., Kinney, H. C., Jensen, F. E., and Rosenberg, P. A. (2011). Reprint of "the developing oligodendrocyte: key cellular target in brain injury in the premature infant". *Int. J. Dev. Neurosci.* 29, 565–582. doi: 10.1016/j.ijdevneu.2011.07.008

Wong, H. S., Santhakumaran, S., Cowan, F. M., Modi, N., and Medicines for Neonates Investigator Group. (2016). Developmental assessments in preterm children: a meta-analysis. *Pediatrics* 138:e20160251. doi: 10.1542/peds.2016-0251

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Stålnacke, Tessma, Böhm and Herlenius. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 02 April 2019
doi: 10.3389/fphys.2019.00324

CXCR2 Blockade Mitigates Neural Cell Injury Following Preclinical Chorioamnionitis

Tracylyn R. Yellowhair[1], Jessie C. Newville[2], Shahani Noor[2], Jessie R. Maxwell[1,2], Erin D. Milligan[2], Shenandoah Robinson[3] and Lauren L. Jantzie[1,2†]*

[1] Department of Pediatrics, School of Medicine, The University of New Mexico, Albuquerque, NM, United States, [2] Department of Neurosciences, School of Medicine, The University of New Mexico, Albuquerque, NM, United States, [3] Division of Pediatric Neurosurgery, Department of Neurosurgery, The Johns Hopkins University School of Medicine, Baltimore, MD, United States

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Courtney Anne McDonald,
Hudson Institute of Medical Research,
Australia
Madison Claire Badawy Paton,
Cerebral Palsy Alliance Research
Institute, Australia

***Correspondence:**
Lauren L. Jantzie
LJantzie@jhmi.edu

†Present address:
Lauren L. Jantzie,
Division of Neonatology, Department
of Pediatrics, The Johns Hopkins
University School of Medicine,
Baltimore, MD, United States

Specialty section:
This article was submitted to
Embryonic and Developmental
Physiology,
a section of the journal
Frontiers in Physiology

Received: 15 November 2018
Accepted: 11 March 2019
Published: 02 April 2019

Citation:
Yellowhair TR, Newville JC,
Noor S, Maxwell JR, Milligan ED,
Robinson S and Jantzie LL (2019)
CXCR2 Blockade Mitigates Neural
Cell Injury Following Preclinical
Chorioamnionitis.
Front. Physiol. 10:324.
doi: 10.3389/fphys.2019.00324

Minimizing central nervous system (CNS) injury from preterm birth depends upon identification of the critical pathways that underlie essential neurodevelopmental and CNS pathophysiology. While chorioamnionitis (CHORIO), is a leading cause of preterm birth, the precise mechanism linking prenatal brain injury and long-term CNS injury is unknown. The chemokine (C-X-C motif) ligand 1 (CXCL1) and its cognate receptor, CXCR2, are implicated in a variety of uterine and neuropathologies, however, their role in CNS injury associated with preterm birth is poorly defined. To evaluate the putative efficacy of CXCR2 blockade in neural repair secondary to CHORIO, we tested the hypothesis that transient postnatal CXCR2 antagonism would reduce neutrophil activation and mitigate cerebral microstructural injury in rats. To this end, a laparotomy was performed on embryonic day 18 (E18) in Sprague Dawley rats, with uterine arteries transiently occluded for 60 min, and lipopolysaccharide (LPS, 4 μg/sac) injected into each amniotic sac. SB225002, a CXCR2 antagonist (3 mg/kg), was administered intraperitoneally from postnatal day 1 (P1)-P5. Brains were collected on P7 and P21 and analyzed with western blot, immunohistochemistry and *ex vivo* diffusion tensor imaging (DTI). Results demonstrate that transient CXCR2 blockade reduced cerebral neutrophil activation (myeloperoxidase expression/MPO) and mitigated connexin43 expression, indicative of reduced neuroinflammation at P7 ($p < 0.05$ for all). CXCR2 blockade also reduced alpha II-spectrin calpain-mediated cleavage, improved pNF/NF ratio, and minimized Iba1 and GFAP expression consistent with improved neuronal and axonal health and reduced gliosis at P21. Importantly, DTI revealed diffuse white matter injury and decreased microstructural integrity following CHORIO as indicated by lower fractional anisotropy (FA) and elevated radial diffusivity (RD) in major white matter tracts ($p < 0.05$). Early postnatal CXCR2 blockade also reduced microstructural abnormalities in white matter and hippocampus at P21 ($p < 0.05$). Together, these data indicate that transient postnatal blockade of CXCR2 ameliorates perinatal abnormalities in inflammatory signaling, and facilitates neural repair

following CHORIO. Further characterization of neuroinflammatory signaling, specifically via CXCL1/CXCR2 through the placental-fetal-brain axis, may clarify stratification of brain injury following preterm birth, and improve use of targeted interventions in this highly vulnerable patient population.

Keywords: preterm, chemokine, CXCL1, diffusion tensor imaging, neutrophil, white matter, alpha-II spectrin

INTRODUCTION

Perinatal brain injury (PBI) is a major contributor to long-term disability in children across the globe (Blencowe et al., 2012, 2013; Kochanek et al., 2012). For a large proportion of infants with PBI, central nervous system (CNS) injury begins *in utero* secondary to inflammation (chorioamnionitis/CHORIO) and/or hypoxia-ischemia (HI) with placental insufficiency (Dammann and Leviton, 1997, 2014; Goldenberg et al., 2000; Lee J. et al., 2013; Lee S.M. et al., 2013; Chau et al., 2014; Fant et al., 2014). Specifically defined as infection/inflammation of the amniotic fluid, membranes, and placenta, concomitant with neutrophil infiltration into the choriodecidua along a chemotactic gradient of pro-inflammatory chemokines, (Yanowitz et al., 2002; Lee J. et al., 2013; Lee S.M. et al., 2013; Kallapur et al., 2014; Kim et al., 2015) CHORIO creates a toxic *in utero* microenvironment that limits oxygen exchange and propagates inflammation during critical periods of neurodevelopment (Redline, 2009, 2013; Galinsky et al., 2013; Anblagan et al., 2016). Typically, infants with PBI present with injury to major white and gray matter structures, leading to reduced connectivity of developing networks. Subsequently, diverse functional deficits ensue with impairment in multiple motor, cognitive and emotional realms, including educational under underachievement in childhood (Counsell et al., 2008; Boardman et al., 2010).

While CHORIO is implicated in preterm CNS injury, the molecular mechanisms meadiating inflammation in the placental-fetal-brain axis that causes PBI remains a gap in knowledge. Specifically, the overlap between placental and CNS physiology and the bi-directional cross talk between the developing immune system and neurodevelopment is relatively unknown. In pregnancy, the physiologic roles for chemokines are well described and dysregulated cytokine production due to infection has tremendous impact on the developing fetus (Hagberg and Mallard, 2005; Bastek et al., 2011; Hagberg et al., 2015). Notably, the chemokine (C-X-C motif) ligand 1 (CXCL1) and its cognate receptor (CXCR2) have been clinically implicated in the pathophysiology of CHORIO (Hsu et al., 1998; Lockwood et al., 2006; Bergeron et al., 2016). CXCL1 provides a chemotactic gradient for neutrophil infiltration to the maternal-fetal interface, and is extensively upregulated with intrauterine inflammation (Hsu et al., 1998; Lockwood et al., 2006). CXCL1 is also a major player in pregnancy failure from CHORIO (Saini et al., 2011; Mizugishi et al., 2015). Indeed, the severity of pathologic placental inflammation correlates positively with CXCL1 levels in newborns with CHORIO and funisitis, (Bry et al., 2015) and CXCL1 is upregulated in amniotic fluid, umbilical cord, and maternal plasma in both term and preterm babies with amniotic infection (Cohen et al., 1996). Pregnant women with intra-amniotic infection have significantly higher amniotic fluid concentrations of CXCL1, (Hsu et al., 1998) and high CXCL1 levels correlate with maternal and newborn peripheral white blood cell counts (Cohen et al., 1996).

In the brain, chemokine receptors play a crucial role in the onset, regulation, and propagation of inflammation. They are also essential in cellular communication, neuronal survival, and neural transmission (Reaux-Le Goazigo et al., 2013; Xu et al., 2017). CXCR2 is one of the most well characterized chemokine receptors, and is located at the cell surface and in the cytoplasm (Semple et al., 2010b; Veenstra and Ransohoff, 2012; Cao et al., 2014; Xu et al., 2017). CXCL1 is the dominant CXCR2 ligand expressed in the inflamed CNS, and its levels are directly proportional to its function (Kielian et al., 2001; Carlson et al., 2008; Kerstetter et al., 2009; Liu et al., 2010; Roy et al., 2012; Veenstra and Ransohoff, 2012). Signaling through CXCR2 is a non-redundant driving force for neutrophil recruitment from blood (Semple et al., 2010a). CXCR2 is also expressed on oligodendrocyte progenitors (OPCs) and microglia in the fetal brains as early as 19–22 weeks gestation (Filipovic et al., 2003), and CXCR2 activation by CXCL1 on OPCs regulates their proliferation and migration (Robinson et al., 1998; Robinson and Franic, 2001). Additionally, CXCR2 is constitutively expressed on other neural cells including neurons, astrocytes, (Filipovic et al., 2003) and monocytes (Valles et al., 2006; Lindner et al., 2008; Veenstra and Ransohoff, 2012). Multiple reports have suggested a role for aberrant CXCL1/CXCR2 signaling in adult CNS injury including stroke, traumatic brain injury (TBI), temporal lobe epilepsy, (Xu et al., 2017) neuropathic nociception, (Abbadie et al., 2009; Yang et al., 2016) central sensitization, (Zhang et al., 2013; Manjavachi et al., 2014) and mechanical hypersensitivity (Chen et al., 2018). Despite the wealth of scientific knowledge of CXCL1/CXCR2 pathophysiology in the mature CNS, their specific role in the pathophysiology in PBI is undefined.

Previously, we published that upregulation of CXCL1 commencing *in utero* negatively affects the fetal microenvironment and trajectory of CNS development (Jantzie et al., 2014a, 2018; Maxwell et al., 2015; Yellowhair et al., 2018). Specifically, CXCL1/CXCR2 signaling is increased following CHORIO in rat placenta, fetal and neonatal circulation and brain over an extended developmental time course, concomitant with increased numbers of placental and cerebral CXCR2-positive neutrophils, and other markers of neuroinflammation (Yellowhair et al., 2018). Thus, to evaluate the putative efficacy of CXCR2 blockade in neural repair following CHORIO, we tested the hypothesis that transient postnatal CXCR2 antagonism would reduce neutrophil activation, mitigate inflammation and neural injury, and preserve brain diffusion and microstructure

following CHORIO in rats. The goal of this investigation was to connect aberrant CXCL1/CXCR2 signaling to PBI secondary to CHORIO, and putatively define novel targets for directed therapies for infants at high risk for PBI from CHORIO and related etiologies.

MATERIALS AND METHODS

Animals

All procedures were performed consistent with National Research Council guidelines, and with the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico Health Sciences Center. ARRIVE guidelines were followed.

In Utero Chorioamnionitis (CHORIO)

We used an established model of CHORIO that yields deficits in the mature CNS that mimic those of preterm survivors (Jantzie et al., 2014a, 2018; Maxwell et al., 2015; Yellowhair et al., 2018). Specifically, pregnant Sprague Dawley rats underwent abdominal laparotomy on embryonic day 18 (E18), consistent with previous reports (Jantzie et al., 2013, 2014a,b, 2015a,b, 2018; Maxwell et al., 2015; Yellowhair et al., 2018). Uterine arteries were transiently occluded for 60 min, to induce placental insufficiency, followed by an intra-amniotic injection of lipopolysaccharide (LPS 0111:B4, 4 μg/sac; Sigma-Aldrich, St. Louis, MO, United States). Laparotomies were closed, and the rat pups were born at term on embryonic day 22 (E22). Sham dams underwent anesthesia for an equivalent duration of time without further intervention. Pups were then euthanized on postnatal day (P) 7 or P21 for biochemical or *ex vivo* magnetic resonance imaging (MRI) analyses. Previously, we reported the placental pathology, Fetal Inflammatory Response Syndrome (FIRS), neuroinflammatory responses, as well as MRI outcomes and the long-term cognitive and motor functional abnormalities in this model (Jantzie et al., 2014a, 2018; Maxwell et al., 2015; Yellowhair et al., 2018). For each experiment described, equal numbers of male and female pups were used in each assay, and data represents true n (individual pups) from at least four different dams per condition. A summary diagram of our experimental method is provided as **Figure 1**.

Neonatal Administration of SB225002, a CXCR2 Antagonist

The selective, competitive CXCR2 antagonist SB225002 (Cayman Chemical, Ann Arbor, MI, United States) was used to block CXCR2 in rats following CHORIO. Previously, SB225002 has been reported to be safe in the developing CNS and has been widely used in adult models of CNS injury (Cao et al., 2014; Wang et al., 2016; Xu et al., 2017). Accordingly, CHORIO rat pups of both sexes were randomly assigned to treatment with SB225002 3 mg/kg (Cao et al., 2014; Wang et al., 2016; Xu et al., 2017) intraperitoneally (i.p.) from P1–P5, consistent with prior reports of translatable neonatal neurorepair dosing intervals with other compounds such as erythropoietin and melatonin (**Figure 1**; Jantzie et al., 2013, 2014b, 2015a,b, 2018) SB225002 was prepared by dissolving 25 mg of crystalline solid in 1 mL of DMSO. It was then aliquoted and stored at −20°C. On the day of experimentation, frozen SB225002 stock was further diluted to a working concentration of 0.5 mg/mL in 30% DMSO and 70% normal saline, after which a 3 mg/kg dose was prepared for each pup using normal saline. The total injection volume for each injection was 100 μl. Sham rat pups received a vehicle injection of 30% DMSO and 70% normal saline solution only.

Western Blot

Microdissected cortical samples from sham, CHORIO, or CHORIO rats treated with CXCR2 antagonist at P7 or P21 were homogenized and sonicated, and centrifuged at 4200 × *g* for 10 min consistent with prior reports (Jantzie et al., 2015a,b,c, 2016). Protein concentration in the whole cell fraction was determined with a Bradford assay (Bio-Rad, Hercules, CA, United States). Thirty micrograms of protein were loaded on 4–20% Tris–HCl gels or 4–12% bis-tris HCl gels (Bio-Rad), separated by electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat dry milk in TBST and incubated with primary antibody overnight at 4 degrees. A species appropriate horseradish-peroxidase-conjugated secondary antibody (Thermo, Grand Island, NY, United States) was applied, and after washing, detected with chemiluminescence (Thermo) using a LAS 4000 imager (GE, Healthcare, PA). Primary antibodies against the following targets were used consistent with prior publications: alpha-II spectrin (Santa Cruz, Dallas, TX, 1:100), myeloperoxidase (MPO) (AbCam, Cambridge, MA, 1:500, connexin43 (CX43, Cell Signaling, Danvers, MA, 1:500), phosphoneurofilament (pNF, Millipore, Temecula, CA, 1:500) or neurofilament (NF, SMI-312, Covance, Princeton, NJ, 1:1000) (Jantzie et al., 2016; Yellowhair et al., 2018). Blots were imaged using an ImageQuant LAS 4000 (GE) and bands of interest were quantified using ImageQuant Software (GE) normalized to the loading control, actin (Sigma, St. Louis, MO, 1:5000). At least two blots were used to assay each protein. Data was then normalized to the sham group consistent with previous reports (Veenstra and Ransohoff, 2012; Jantzie et al., 2014b, 2015a, 2016).

Immunohistochemistry

On P21 rats were deeply anesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde. Brains were then collected, and post-fixed in paraformaldehyde. After immersion in 30% sucrose solution, 20 μm, frozen, slide mounted, coronal sections were obtained and collected using a cryostat (Leica, Buffalo Grove, IL, United States). Slides were then washed and incubated with 0.3% hydrogen peroxide, followed by blocking solution containing 10% normal goat serum in phosphate buffered solution (PBS). Primary antibodies against glial fibrillary acidic protein (GFAP, Dako 1:500, Carpinteria, CA, United States) or ionized calcium binding adaptor 1 (Iba1, Wako, 1:500, United States), in blocking solution containing 0.1% Trition-X100 were incubated on sections overnight at 4°C. The next day, sections were rinsed, and incubated with species-appropraite biotinylated secondary antibodies for 1 h.

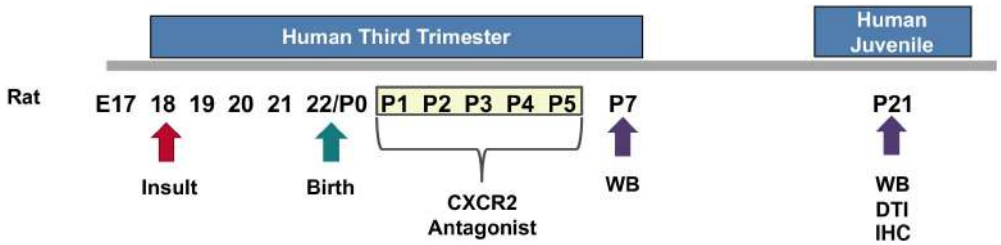


FIGURE 1 | Experimental Design. On embryonic day 18 (E18), a prenatal chorioamnionitis insult was induced in pregnant Sprague Dawley rats. Rat pups were born on E22. SB225002, a CXCR2 antagonist, or vehicle, was administered during a critical neonatal window from postnatal day 1 (P1) through P5 (3 mg/kg i.p.). Pup brains were collected and analyzed on P7 or P21 for western blot analysis (WB), *ex vivo* diffusion tensor imaging (DTI), and immunohistochemistry (IHC).

This was followed by incubation in VECTASTAIN (Vector Labs, Burlingame, CA, United States) and 3,3′-diaminobenzidine (DAB). Sections were then processed for dehydration, cleared in xylenes and cover-slipped in Permount (Millipore Sigma, St. Louis, MO, United States). Appropriate negative controls without primary antibodies were run in parallel. Using bright-field illumination, representative images were photographed on an upright Leica microscope.

Stereological Estimates

All P21 sections were coded prior to analyses by a blinded observer and stereology performed consistent with previously published methodology (Jantzie et al., 2014a, 2015a; Robinson et al., 2017a). Estimates of the load of each antigen were obtained from 20 μm coronal sections using a thin section modification of the optical fractionator method (Gundersen et al., 1988; Mouton et al., 2002). Specifically, object area fraction and volume probes (Cavalieri's method) were used to calculate load and to quantify the amount of Iba1-positive microglia and GFAP-positive astrocytes in the fimbria. At the completion of the stereological analyses, the samples were decoded, and mean and SEM of load were calculated.

Diffusion Tensor Imaging (DTI)

Ex vivo MRI using diffusion sequences was performed on a Bruker BioSpec 7T 70/30 Ultra Shield Refrigerated (USR) nuclear MRI system, consistent with prior published methods (Robinson et al., 2016, 2017b; Yellowhair et al., 2018). Briefly, P21 rats were deeply anesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde. Brains were removed from the skull and post-fixed in 4% paraformaldehyde for 1 week and embedded in 2% agarose containing 3 mM sodium azide for immediate *ex vivo* MR imaging. Echo-planar diffusion tensor imaging (EP-DTI) of twenty contiguous coronal 1 mm slices were obtained with a FOV (field-of-view) of 3.00 cm and an MTX of 256. Brain regions of interest (ROI) in major white matter tracts (corpus callosum and external capsule) and gray matter (hippocampus and thalamus), were traced by an observer blinded to experimental conditions and analyzed using Bruker's ParaVision 5.1 imaging software. Fractional anisotropy (FA), axial (λ1) and radial [(λ2+λ3)/2] diffusivity eigenvectors were measured and calculated. For bilateral neuroanatomical ROIs, metrics were acquired on each side and averaged per ROI. Directionally encoded diffusion color maps and color-coded FA maps were created.

Statistical Analyses

Data are represented as mean ± the standard error of the mean (SEM). Parametric statistical differences between three groups (sham, CHORIO and CHORIO+CXCR2 antagonist) were established using a two-way ANOVA with Bonferroni *post hoc* correction to discern the effects of injury and treatment. $p < 0.05$ was considered statistically significant.

RESULTS

Transient Blockade of CXCR2 Attenuates Neutrophil Activation and Reduces Connexin43 Expression

To first establish putative beneficial effect of CXCR2 blockade on neutrophil activation, a common cellular mediator of inflammation throughout the placental-fetal-brain axis, we examined cerebral myeloperoxidase (MPO) protein expression on P7, 48 h following the last dose of SB225002. As expected, vehicle-treated CHORIO pups had increased MPO expression compared shams (n = 10–15, $p < 0.05$, **Figure 2A**). Notably, treatment with SB225002, the CXCR2 antagonist, mitigated neutrophil activation in CHORIO pups and restored MPO protein expression levels to that observed in sham pups ($p < 0.01$, **Figure 2A**).

As CXCR2 has been documented to be important in intracellular signaling and communication under normal and inflamed conditions, we also examined the effect of CXCR2 blockade on connexin43 expression, a hemichannel protein that is present in placenta and brain, including on astrocytes and other immune cells (Dunk et al., 2012; Theodoric et al., 2012; Chen et al., 2014; Yin et al., 2018). Indeed, connexin43 is a gap junction protein intimately connected to CXCR2 activation, astrocyte activation, and excitotoxicity (Theodoric et al., 2012; Chen et al., 2014). Consistent with increased neuroinflammatory signal transduction in our model of CHORIO, (Jantzie et al., 2014a; Yellowhair et al., 2018) connexin43 protein expression was significantly elevated in the brains of vehicle-treated CHORIO pups at P7 (n = 7–9, $p < 0.01$, **Figure 2B**). Similar to the

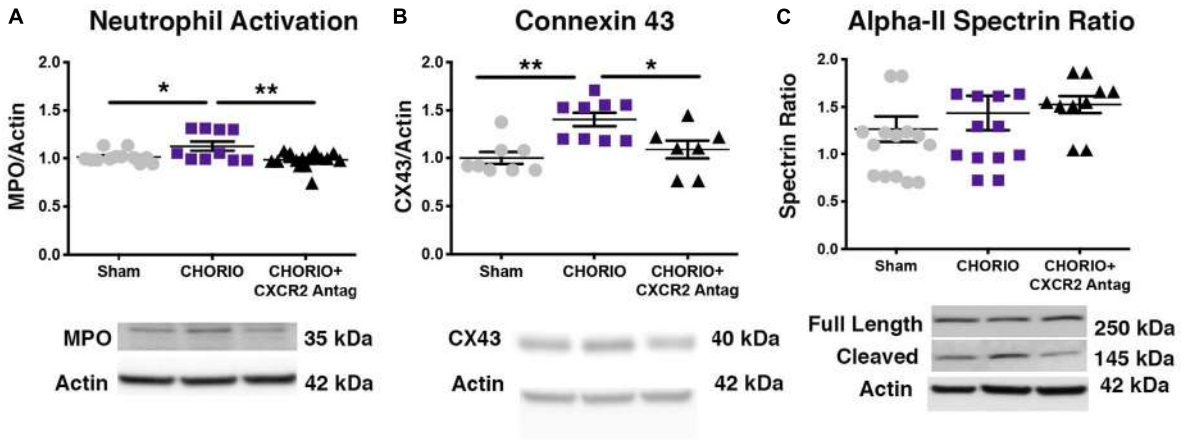


FIGURE 2 | CXCR2 Antagonism Reduces Neutrophil Activation and Connexin43 Expression on Postnatal Day 7 (P7). Following prenatal chorioamnionitis, transient postnatal administration of the CXCR2 antagonist, SB225002, reduced neutrophil reactivity (myeloperoxidase, MPO, **A**) and mitigated connexin43 expression (CX43, **B**) in the cortex on P7, consistent with reduced cellular neuroinflammation. At P7, neither CHORIO or CXCR2 antagonism changed cleaved alpha-II spectrin ratio, a marker of neuronal health **(C)**. (n = 7–15, two-way ANOVA with Bonferroni's *post hoc* correction, *$p < 0.05$, **$p < 0.01$).

reduction in MPO observed with CXCR2 antagonism, treatment with SB225002 also significantly reduced connexin43 expression at P7 ($p < 0.05$, **Figure 2B**). We also examined alpha-II spectrin at P7, a neuron specific cytoskeletal protein and target of calpain (Jantzie et al., 2014b, 2016; Schober et al., 2014). Consistent with our previous reports, we found no effect of CHORIO or SB225002 administration at P7 on the ratio of full length alpha-II spectrin to cleaved alpha-II spectrin (n = 10–16 p = ns, **Figure 2C**).

CXCR2 Antagonism Attenuates CHORIO-Induced Gliosis and Neural Injury

Given the acute effect of CXCR2 blockade at P7 on neutrophil activation and reduced connexin43 expression, we examined global markers of axons, astrocytes, microglia and assessed neuronal health at P21 to establish longer term effects of CXCR2 blockade (**Figure 3**). P21 in rats is equivalent to a young, human juvenile and represents a timepoint 4 weeks following *in utero* insult and just over 2 weeks following the last dose of SB225002 (Semple et al., 2013; Jantzie and Robinson, 2015). Notably, transient neonatal antagonism of CXCR2 with SB225002 significantly improved the ratio of pNF to NF compared to CHORIO pups treated with vehicle, consistent with improved axonal health in treated pups and similar levels of these key axonal proteins as shams (n = 9–11, $p < 0.01$, **Figure 3A**). Given the beneficial effect of CXCR2 antagonism on axons, we then examined the alpha-II spectrin ratio in treated and untreated pups at P21. Importantly, the spectrin ratio in vehicle-treated CHORIO pups was elevated compared to shams (n = 6–7, $p < 0.01$, **Figure 3B**), consistent with elevated levels of calpain protease activity and neuronal cytoskeletal breakdown. Notably, treatment with the CXCR2 antagonist SB225002, mitigated the increase in spectrin cleavage ratio and normalized alpha-II spectrin cleavage to sham levels (n = 6–11, $p < 0.05$, **Figure 3B**). We also examined microglia and astrocytes at this time point. Immunoreactivity for microglia (Iba1, **Figure 3C**) and astrocytes (GFAP) (**Figure 3D**) was augmented at P21 in the fimbria of CHORIO animals compared to Sham, an effect that was ameliorated by CXCR2 antagonism. Using stereological principles, we quantified Iba1 and GFAP load (**Table 1**). These analyses confirmed significant increases in Iba1 and GFAP load induced by CHORIO that was significantly mitaged by CXCR2 antagonism. Together, these data indicated that transient CXCR2 antagonism attenuates gliosis, and reduces axonal and neuronal injury following CHORIO.

CXCR2 Blockade During a Critical Postnatal Window Mitigates the Effects of CHORIO and Protects the Developing Brain

Given the biochemical evidence of reduced neuroinflammation and neural health at P7, and at P21, we next performed DTI analyses to examine the effects of CXCR2 antagonism on white and gray matter microstructure using a translational imaging outcome measure. We began by creating color coded fractional anisotropy (FA) maps and directionally-encoded color diffusion maps (**Figure 4**). Both color FA and the directionally encoded color diffusion maps depict loss of structural integrity in major white matter tracts, including the corpus callosum, fimbria, external and internal capsule, and demonstrate subsequent improvement with CXCR2 antagonism (**Figure 4**). Indeed, quantification of diffusion metrics and scalars in corpus callosum (**Figures 5A–C**) and external capsule FA (**Figures 5D–F**), confirms significant loss of microstructural integrity in CHORIO pups that is recovered with CXCR2 antagonism. These changes in FA are also associated with injury-induced elevations in radial diffusivity (RD). Notably, CXCR2 antagonism ameliorated CHORIO-induced elevations in RD in both the corpus callosum and external capsule

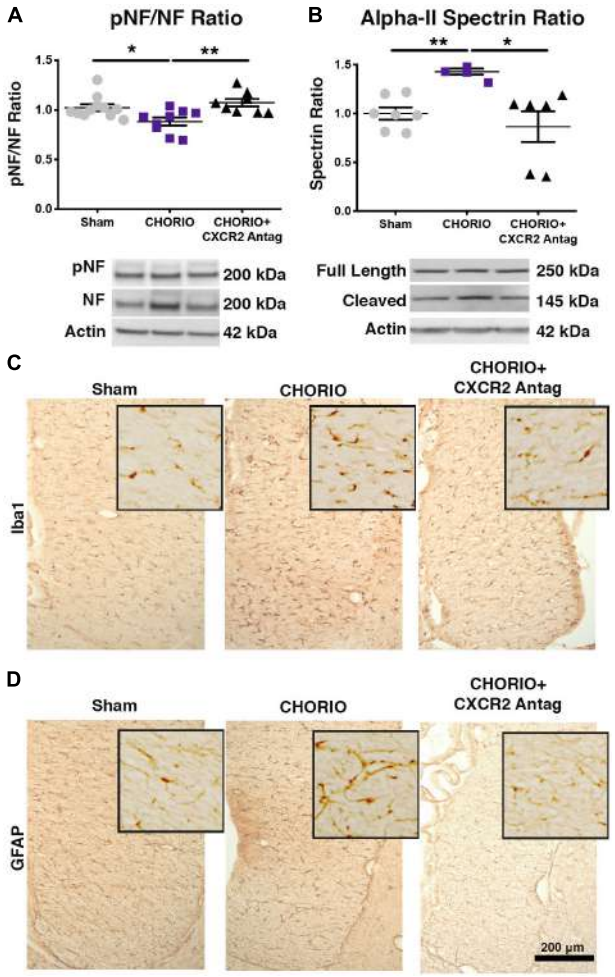


FIGURE 3 | CXCR2 Antagonism Improves Axonal and Neuronal Health Through Postnatal Day 21 (P21). Following prenatal chorioamnionitis (CHORIO), transient postnatal administration of the CXCR2 antagonist, SB225002, mitigated injury-induced alterations in cortical phosphoneurofilament to neurofilament ratio (pNF/NF, **A**), consistent with improved axonal health. Similarly, transient CXCR2 blockade also reduced pathological augmentation of alpha-II spectrin cleavage in the cortex **(B)**, consistent with reduced calpain activity and improved neuronal health. Immunoreactivity for microglia (Iba1, **C**) and astrocytes (GFAP, **D**) is augmented at P21 in CHORIO fimbria compared to Sham, an effect that is ameliorated by CXCR2 antagonism. Scale bar = 200 µm. (n = 6–11/group for immunoblot, n = 3 for immunohistochemistry, two-way ANOVA with Bonferroni's *post hoc* correction, $^*p < 0.05$, $^{**}p < 0.01$).

TABLE 1 | Stereological Estimates of Iba1 and GFAP Load in the Fimbria.

	Sham	CHORIO	CHORIO+CXCR2 ANTAG
Iba1 (μm^3)	$4.9+1.2 \times 10^6$	$14.5+1.1 \times 10^{6**}$	$7.2+0.3 \times 10^{6*}$
GFAP (μm^3)	$4.1+0.7 \times 10^6$	$15.1+1.7 \times 10^{6*}$	$7.7+1.5 \times 10^{6*}$

Asterix(s) in CHORIO column indicates statistical difference from Sham group. Asterix in CHORIO+CXCR2 ANTAG column indicates statistical difference from CHORIO group. $^*p < 0.05$, $^{**}p < 0.01$.

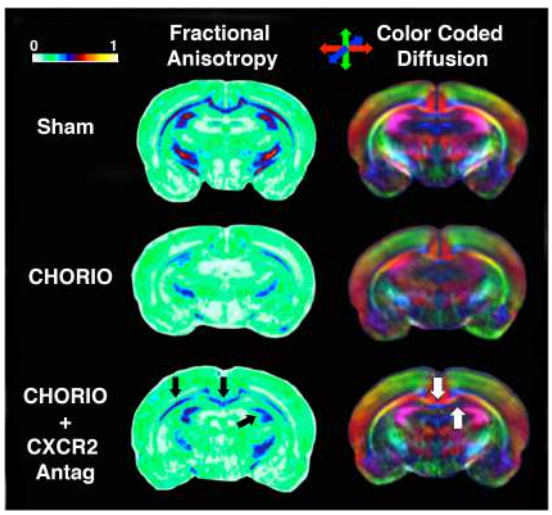


FIGURE 4 | CXCR2 Antagonism Mitigates Abnormalities in Cerebral Diffusion. Following prenatal chorioamnionitis (CHORIO), transient postnatal administration of the CXCR2 antagonist, SB225002, attenuated losses of fractional anisotropy (FA) in major white matter tracts (left, black arrows) on postnatal day 21 (P21). Similarly, directionally encoded color maps (right) reveal loss of diffusion in white and gray matter, including the corpus callosum and anterior hippocampus (white arrows). Color coded FA legend (left) indicates the degree of anisotropy from 0 to 1, with 0 being unrestricted and 1 fully restricted. Directionally color encoded arrows (right) indicate horizontal diffusion (red), anterior to posterior diffusion (blue), and superior-inferior diffusion (green).

(**Figures 5C,F**), and restored axial diffusivity (AD) in the corpus callosum (**Figure 5B**), consistent with improved white matter and axonal health, and improved structural coherence and directional diffusion. We also examined gray matter microstructure using DTI. Similar to the white matter regions, we found injury induced decreases in hippocampal and thalamic FA in CHORIO pups compared to shams (n = 5–6/group, $p < 0.01$, **Figure 6**). Also consistent with white matter regions, administration of SB225002, restored FA in the hippocampus, indicative of improved gray matter microstructure. No changes in RD and AD were noted in the gray matter (data not shown).

DISCUSSION

Chemokines, a family of small molecular weight chemotactic cytokines, (Ben-Baruch et al., 1995; Luster, 1998; Kielian et al., 2001) are classically defined by their ability to induce directional migration and activation of leukocytes to areas of inflammation in the body (Kielian et al., 2001; Semple et al., 2010b; Veenstra and Ransohoff, 2012). Chemokine signaling is also integral to development of multiple placental and neural cell lineages. CXCL1 is an ERL (glutamic acid-arginine-leucine) CXC chemokine defined by potent CXCR2 receptor-dependent neutrophil chemoattractant activity, whereas CXC chemokines lacking the ERL motif are inactive toward neutrophils (Murphy, 1997; Biondo et al., 2014). CXCL1 is

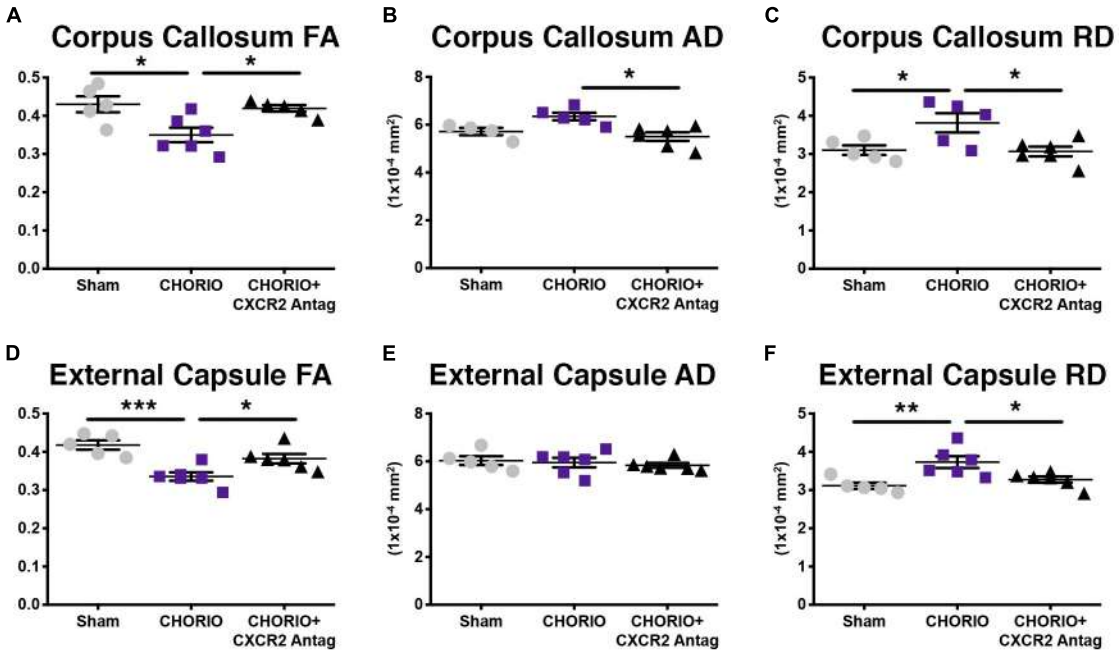


FIGURE 5 | CXCR2 Antagonism Attenuates White Matter Microstructural Injury. Following prenatal chorioamnionitis (CHORIO), transient postnatal administration of the CXCR2 antagonist, SB225002, attenuated injury induced changes in fractional anisotropy **(A)** in the corpus callosum at postnatal day 21 (P21), together with mitigating changes in axial (AD, **B**) and radial (RD) diffusion **(C)**. Similarly, CHORIO animals had reduced FA **(D)** and increased RD **(F)** in the external capsule. Treatment with a CXCR2 antagonist significantly improved both FA and RD. No changes were observed in AD **(E)**. (n = 5–6, two-way ANOVA with Bonferroni's *post hoc* correction, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

the dominant CXCR2 ligand expressed in the inflamed CNS (Roy et al., 2012). CXCL1 transcripts are 4-fold more abundant in the brain than those of CXCL2 and its levels are directly proportional to its function (Roy et al., 2012). Upregulation of CXCL1/CXCR2 signaling is central to neuroinflammation following exposure to bacterial endotoxin, (Kielian et al., 2001; Carlson et al., 2008; Kerstetter et al., 2009; Liu et al., 2010; Roy et al., 2012) and CXCL1 is rapidly upregulated in traumatic brain injury (TBI), Alzheimer's disease, multiple sclerosis, chronic pain and stroke, and is followed by neural cell specific increases in CXCR2 expression (Valles et al., 2006; Lindner et al., 2008; Kerstetter et al., 2009; Liu et al., 2010, 2015; Cao et al., 2014; Connell et al., 2015; Ryu et al., 2015). Indeed, CXCR2 expression is essential for cerebral endothelial activation and leukocyte recruitment, (Wu et al., 2015) with CXCR2 antagonism or CXCL1 deficiency mitigating neutrophil infiltration and recruitment into brain parenchyma (Wu et al., 2015). In alignment with these data and with clinical literature confirming that PBI in preterm infants often originates *in utero*, the present investigation supports the hypothesis that CXCL1/CXCR2 signaling negatively impacts brain development. Importantly, here we show CXCR2 blockade reversed injury-induced CNS elevations in neuron-specific alpha-II spectrin cleavage, an established biomarker of PBI (Jantzie et al., 2014b, 2016). CXCR2 blockade also mitigated connexin43 expression, a hemichannel and gap junction protein connected to CXCR2 activation, astrocyte activation, and excitotoxicity (Theodoric et al., 2012; Chen et al., 2014; Yin et al., 2018). Interestingly, activated astrocytes can release CXCL1 and facilitate CXCR2 signal transduction via connexin43 to enhance and feed forward neuroinflammation (Cao et al., 2014; Chen et al., 2014, 2018). Similarly, here CXCR2 blockade attenuated white matter loss and axonal injury and mitigated CHORIO-induced increases in Iba1 and GFAP expression, and provided sustained protection to white and gray matter microstructure 4 weeks following *in utero* exposure to CHORIO. These data validate CXCR2 blockade and a putative functional relationship between CXCL1/CXCR2 and neural injury *in vivo*, and support the hypothesis that CXCL1/CXCR2 signaling is a prominent mediator of inflammation through the placental-fetal-brain axis. Moreover, we provide first evidence of the primacy of excess CXCR2 activation in white and gray matter neural injury that hallmarks PBI.

Prior preclinical reports confirm that CXCR2 blockade may be beneficial in the mature CNS. CXCR2 is dysregulated on OPCs and monocytes/microglia during demyelination (Lindner et al., 2008). On neurons, CXCL1 induction sustains late-phase neuropathic pain by activating CXCR2, and modulates synaptic transmission (Chen et al., 2014). Blockade of CXCR2 with the same inhibitor used here, SB225002, reverses allodynia and suppresses injury-induced increases in neuronal firing frequency, confirming a role for CXCL1/CXCR2 in synaptic plasticity (Chen et al., 2014). In TBI and stroke, cerebral CXCL1/CXCR2

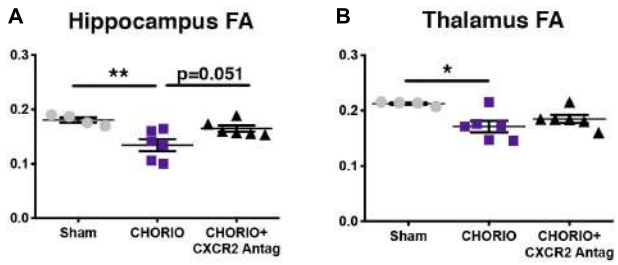


FIGURE 6 | CXCR2 Antagonism Improves Gray Matter Microstructural Integrity. Following prenatal chorioamnionitis (CHORIO), transient postnatal administration of the CXCR2 antagonist, SB225002, attenuated injury induced changes in fractional anisotropy (FA) in the hippocampus **(A)** at postnatal day 21 (P21). While CHORIO reduced FA in the thalamus, no difference was observed with CXCR2 antagonism in the thalamus **(B)**. (n = 5–6, two-way ANOVA with Bonferroni's *post hoc* correction, $^{*}p < 0.05$, $^{**}p < 0.01$).

levels determine the magnitude of neutrophil infiltration, subsequent neuronal loss, and infarct volume (Semple et al., 2010a; Hennessy et al., 2015). Here, we show that CXCR2 blockade during a critical postnatal window reverses some of the key biochemical and imaging hallmarks of CHORIO through P21 and protects the developing brain from excess CXCR2 signaling. Together, with high-resolution DTI confirming that CXCR2 blockade also reduced microstructural white and gray matter injury and resolves pathological changes in diffusion, these data emphasize the role of CXCL1/CXCR2 signaling in PBI defined by *in utero* inflammation and for the first time report the putative efficacy of transient CXCR2 blockade in the developing brain. Interestingly, numerous studies in adult animals emphasize that blocking chemokine receptors such as CXCR2, rather than individual ligands such as CXCL1, is most effective for neural repair (Semple et al., 2010a,b; Veenstra and Ransohoff, 2012). While commonly used, CXCR2 knockout (KO) mice have drawbacks including lymph node enlargement and myelination defects, (Tsai et al., 2002; Cardona et al., 2008; Semple et al., 2010a,b) emphasizing the fundamental role for CXCR2 in normal physiology and neurodevelopment. We hypothesized that rather than complete CXCR2 silence throughout development, transient loss of excessive CXCR2 signaling following CHORIO would mitigate brain injury. Additional dose-response and duration-response studies beyond the scope here are needed to clarify the optimal dosing regimen for SB225002. Indeed, a gradient effect may be promising for therapeutics targeting CXCL1/CXCR2 because it suggests partial receptor inhibition may be sufficient to attenuate neutrophil activation and improve neural health without complete cessation of essential biological processes that may cause additional detrimental effects in the developing injured brain (Abdulkadir et al., 2010; Semple et al., 2010a).

Infants with CHORIO have elevated neutrophil and monocyte counts compared to infants without intra-amniotic infection, (Weitkamp et al., 2016) emphasizing compartment-specific modulation of cytokines and immune cell diversity in CHORIO. Previously, we have shown that elevated placental CXCL1 is associated with acute neutrophilia and immune cell activation in the placenta, and with neutrophilia, immune cell activation and microgliosis in the brain (Jantzie et al., 2014a; Maxwell et al., 2015; Yellowhair et al., 2018). Using flow cytometry, we also demonstrated increased $CXCR2^{+}$ neutrophils in the placenta and brain following CHORIO (Yellowhair et al., 2018). Here, we show that CXCR2 blockade attenuates neutrophil activation in the brain at P7. Together with prior publications confirming a sustained neuroinflammatory response following CHORIO, hallmarked by upregulated CXCL1, and increased microglia and macrophages, (Jantzie et al., 2014a; Maxwell et al., 2015; Yellowhair et al., 2018) these data indicate that CXCR2 antagonism reduces GFAP and Iba1 immunoreactivity consistent with changes to microglia and astrocytes. While future investigations will examine microglial activation state and regional and temporal changes in glial morphology and number, these data are consistent with earlier reports demonstrating that cerebral CXCL1 and CXCR2 levels determine the magnitude of neutrophil infiltration, subsequent neuronal loss, gliosis and infarct volume in stroke and TBI (Semple et al., 2010a; Hennessy et al., 2015). Typically, neutrophil influx is a secondary response after injury, and further exacerbates acute endogenous brain inflammation mediated by microglia (Gelderblom et al., 2009; Jellema et al., 2013). Indeed, limiting neutrophil and macrophage infiltration improves neuropathological and functional outcomes in models of stroke and TBI (Semple et al., 2010a; Morganti et al., 2015). In preterm sheep with hypoxia-ischemia, neutrophils invade vulnerable brain regions, including hippocampus, periventricular and subcortical white matter, (Jellema et al., 2013) with mobilization and recruitment accompanied by prominent microgliosis (Raivich et al., 1999; Valles et al., 2006). Indeed, CXCR2 expression on neural cells is essential for cerebral endothelial activation and leukocyte recruitment, (Wu et al., 2015) with CXCR2 antagonism or CXCL1 deficiency mitigating neutrophil infiltration and recruitment into brain parenchyma, as well as microgliosis (Wu et al., 2015).

Despite including both sexes in all outcome measures of this study, we were underpowered to detect sex differences. Future investigations beyond the scope of the present study should include assessments at later time points with translational measures of behavior and function consistent with previous reports (Jantzie et al., 2018; Robinson et al., 2018). Another limitation is the dosing regimen of SB225002, and the duration and timing of dose-response will be the focus of future studies, as well as a complete neuropathological examination and with spatiotemporal regional assessment of oligodendrocyte, neuron, astrocyte and microglial number. Glial activation state and morphology should also be assessed rigorously. In conclusion, this is the first report that transient postnatal blockade of CXCR2 modulates PBI pathophysiology and attenuates neural injury following CHORIO. Moreover, we provide the first evidence of the primacy of excess CXCR2 activation in white and gray matter neural injury that hallmarks PBI. While blocking microglial activation, neutrophil activation and inflammatory signaling is beneficial after CHORIO, the homeostatic control of

chemokine signaling during development, injury, and repair cannot be overemphasized. The blockade of receptors essential for normal physiological properties, such as CXCR2, warrants further investigation.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

LJ conceptualized the hypothesis, and supervised the experiments. TY, JN, SN, JM, EM, SR, and LJ designed and performed the experiments. SR and LJ interpreted the data. LJ and TY wrote the manuscript. All authors contributed to manuscript revision and approved the final version.

FUNDING

This study was supported by generous funding from the National Institutes of Health 1R01HL139492 to LJ and 1S10OD021598 for 7T MRI resources at the University of New Mexico.

ACKNOWLEDGMENTS

The authors are most grateful for the exceptional MRI expertise of Yirong Yang, Ph.D.

REFERENCES

Abbadie, C., Bhangoo, S., De Koninck, Y., Malcangio, M., Melik-Parsadaniantz, S., and White, F. A. (2009). Chemokines and pain mechanisms. *Brain Res. Rev.* 60, 125–134. doi: 10.1016/j.brainresrev.2008.12.002

Abdulkadir, A. A., Kimimasa, T., Bell, M. J., Macpherson, T. A., Keller, B. B., and Yanowitz, T. D. (2010). Placental inflammation and fetal hemodynamics in a rat model of chorioamnionitis. *Pediatr. Res.* 68, 513–518. doi: 10.1203/PDR.0b013e3181f851ed

Anblagan, D., Pataky, R., Evans, M. J., Telford, E. J., Serag, A., Sparrow, S., et al. (2016). Association between preterm brain injury and exposure to chorioamnionitis during fetal life. *Sci. Rep.* 6:37932. doi: 10.1038/srep37932

Bastek, J. A., Brown, A. G., Anton, L., Srinivas, S. K., D'Addio, A., and Elovitz, M. A. (2011). Biomarkers of inflammation and placental dysfunction are associated with subsequent preterm birth. *J. Matern. Fetal Neonat. Med.* 24, 600–605. doi: 10.3109/14767058.2010.511340

Ben-Baruch, A., Michiel, D. F., and Oppenheim, J. J. (1995). Signals and receptors involved in recruitment of inflammatory cells. *J. Biol. Chem.* 270, 11703–11706. doi: 10.1074/jbc.270.20.11703

Bergeron, J., Gerges, N., Guiraut, C., Grbic, D., Allard, M. J., Fortier, L. C., et al. (2016). Activation of the IL-1beta/CXCL1/MMP-10 axis in chorioamnionitis induced by inactivated group B *Streptococcus*. *Placenta* 47, 116–123. doi: 10.1016/j.placenta.2016.09.016

Biondo, C., Mancuso, G., Midiri, A., Signorino, G., Domina, M., Lanza Cariccio, V., et al. (2014). The interleukin-1beta/CXCL1/2/neutrophil axis mediates host protection against group B streptococcal infection. *Infect. Immun.* 82, 4508–4517. doi: 10.1128/IAI.02104-2114

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., et al. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 379, 2162–2172. doi: 10.1016/S0140-6736(12)60820-4

Blencowe, H., Lee, A. C., Cousens, S., Bahalim, A., Narwal, R., Zhong, N., et al. (2013). Preterm birth-associated neurodevelopmental impairment estimates at regional and global levels for 2010. *Pediatr. Res.* 74(Suppl. 1), 17–34. doi: 10.1038/pr.2013.204

Boardman, J. P., Craven, C., Valappil, S., Counsell, S. J., Dyet, L. E., Rueckert, D., et al. (2010). A common neonatal image phenotype predicts adverse neurodevelopmental outcome in children born preterm. *Neuroimage* 52, 409–414. doi: 10.1016/j.neuroimage.2010.04.261

Bry, K. J., Jacobsson, B., Nilsson, S., and Bry, K. (2015). Gastric fluid cytokines are associated with chorioamnionitis and white blood cell counts in preterm infants. *Acta paediatr.* 104, 575–580. doi: 10.1111/apa.12947

Cao, D. L., Zhang, Z. J., Xie, R. G., Jiang, B. C., Ji, R. R., and Gao, Y. J. (2014). Chemokine CXCL1 enhances inflammatory pain and increases NMDA receptor activity and COX-2 expression in spinal cord neurons via activation of CXCR2. *Exp. Neurol.* 261, 328–336. doi: 10.1016/j.expneurol.2014.05.014

Cardona, A. E., Sasse, M. E., Liu, L., Cardona, S. M., Mizutani, M., Savarin, C., et al. (2008). Scavenging roles of chemokine receptors: chemokine receptor deficiency is associated with increased levels of ligand in circulation and tissues. *Blood* 112, 256–263. doi: 10.1182/blood-2007-10-118497

Carlson, T., Kroenke, M., Rao, P., Lane, T. E., and Segal, B. (2008). The Th17-ELR+ CXC chemokine pathway is essential for the development of central nervous system autoimmune disease. *J. Exp. Med.* 205, 811–823. doi: 10.1084/jem.20072404

Chau, V., McFadden, D. E., Poskitt, K. J., and Miller, S. P. (2014). Chorioamnionitis in the pathogenesis of brain injury in preterm infants. *Clin. Perinatol.* 41, 83–103. doi: 10.1016/j.clp.2013.10.009

Chen, G., Luo, X., Qadri, M. Y., Berta, T., and Ji, R. R. (2018). Sex-dependent glial signaling in pathological pain: distinct roles of spinal microglia and astrocytes. *Neurosci. Bull.* 34, 98–108. doi: 10.1007/s12264-017-0145-y

Chen, G., Park, C. K., Xie, R. G., Berta, T., Nedergaard, M., and Ji, R. R. (2014). Connexin-43 induces chemokine release from spinal cord astrocytes to maintain late-phase neuropathic pain in mice. *Brain* 137, 2193–2209. doi: 10.1093/brain/awu140

Cohen, J., Ghezzi, F., Romero, R., Ghidini, A., Mazor, M., Tolosa, J. E., et al. (1996). GRO alpha in the fetomaternal and amniotic fluid compartments during pregnancy and parturition. *Am. J. Reprod. Immunol.* 35, 23–29. doi: 10.1111/j.1600-0897.1996.tb00004.x

Connell, B., Gordon, J., and Saleh, T. (2015). ELR-CXC chemokine antagonism is neuroprotective in a rat model of ischemic stroke. *Neurosci. Lett.* 606, 117–122. doi: 10.1016/j.neulet.2015.08.041

Counsell, S. J., Edwards, A. D., Chew, A. T., Anjari, M., Dyet, L. E., Srinivasan, L., et al. (2008). Specific relations between neurodevelopmental abilities and white matter microstructure in children born preterm. *Brain* 131, 3201–3208. doi: 10.1093/brain/awn268

Dammann, O., and Leviton, A. (1997). Maternal intrauterine infection, cytokines, and brain damage in the preterm newborn. *Pediatr. Res.* 42, 1–8. doi: 10.1203/00006450-199707000-199707001

Dammann, O., and Leviton, A. (2014). Intermittent or sustained systemic inflammation and the preterm brain. *Pediatr. Res.* 75, 376–380. doi: 10.1038/pr.2013.238

Dunk, C. E., Gellhaus, A., Drewlo, S., Baczyk, D., Potgens, A. J., Winterhager, E., et al. (2012). The molecular role of connexin 43 in human trophoblast cell fusion. *Biol. Reprod.* 86:115. doi: 10.1095/biolreprod.111.096925

Fant, M. E., Fuentes, J., Kong, X., and Jackman, S. (2014). The nexus of prematurity, birth defects, and intrauterine growth restriction: a role for plac1-regulated pathways. *Front. Pediatr.* 2:8. doi: 10.3389/fped.2014.00008

Filipovic, R., Jakovcevski, I., and Zecevic, N. (2003). GRO-alpha and CXCR2 in the human fetal brain and multiple sclerosis lesions. *Dev. Neurosci.* 25, 279–290. doi: 10.1159/000072275

Galinsky, R., Polglase, G. R., Hooper, S. B., Black, M. J., and Moss, T. J. (2013). The consequences of chorioamnionitis: preterm birth and effects on development. *J. Pregnancy* 2013:412831. doi: 10.1155/2013/412831

Gelderblom, M., Leypoldt, F., Steinbach, K., Behrens, D., Choe, C. U., Siler, D. A., et al. (2009). Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke* 40, 1849–1857. doi: 10.1161/STROKEAHA.108.534503

Goldenberg, R. L., Hauth, J. C., and Andrews, W. W. (2000). Intrauterine infection and preterm delivery. *N. Engl. J. Med.* 342, 1500–1507. doi: 10.1056/NEJM200005183422007

Gundersen, H. J., Bagger, P., Bendtsen, T. F., Evans, S. M., Korbo, L., Marcussen, N., et al. (1988). The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* 96, 857–881. doi: 10.1111/j.1699-0463.1988.tb00954.x

Hagberg, H., and Mallard, C. (2005). Effect of inflammation on central nervous system development and vulnerability. *Curr. Opin. Neurol.* 18, 117–123. doi: 10.1097/01.wco.0000162851.44897.8f

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Hennessy, E., Griffin, E. W., and Cunningham, C. (2015). Astrocytes are primed by chronic neurodegeneration to produce exaggerated chemokine and cell infiltration responses to acute stimulation with the cytokines IL-1beta and TNF-alpha. *J. Neurosci.* 35, 8411–8422. doi: 10.1523/JNEUROSCI.2745-14.2015

Hsu, C. D., Meaddough, E., Aversa, K., and Copel, J. A. (1998). The role of amniotic fluid L-selectin, GRO-alpha, and interleukin-8 in the pathogenesis of intraamniotic infection. *Am. J. Obstetr. Gynecol.* 178, 428–432. doi: 10.1016/S0002-9378(98)70414-4

Jantzie, L. L., Corbett, C. J., Berglass, J., Firl, D. J., Flores, J., Mannix, R., et al. (2014a). Complex pattern of interaction between in utero hypoxia-ischemia and intra-amniotic inflammation disrupts brain development and motor function. *J. Neuroinflamm.* 11:131. doi: 10.1186/1742-2094-11-131

Jantzie, L. L., Corbett, C. J., Firl, D. J., and Robinson, S. (2015a). Postnatal erythropoietin mitigates impaired cerebral cortical development following subplate loss from prenatal hypoxia-ischemia. *Cereb. Cortex* 25, 2683–2695. doi: 10.1093/cercor/bhu066

Jantzie, L. L., Getsy, P., Denson, J. L., Firl, D. J., Wilson, C. G., and Robinson, S. (2015b). Prenatal hypoxia-ischemia induces potassium chloride cotransporter 2 loss and abnormalities in inhibitory tone. *Front. Cell Neurosci.* 3:347.

Jantzie, L. L., Talos, D. M., Jackson, M. C., Park, H. K., Graham, D. A., Lechpammer, M., et al. (2015c). Developmental expression of N-Methyl-d-aspartate (n.d.) receptor subunits in human white and gray matter: potential mechanism of increased vulnerability in the immature brain. *Cereb. Cortex* 25, 482–495. doi: 10.1093/cercor/bht246

Jantzie, L. L., Getsy, P. M., Firl, D. J., Wilson, C. G., Miller, R. H., and Robinson, S. (2014b). Erythropoietin attenuates loss of potassium chloride co-transporters following prenatal brain injury. *Mol. Cell. Neurosci.* 61, 152–162. doi: 10.1016/j.mcn.2014.06.009

Jantzie, L. L., Miller, R. H., and Robinson, S. (2013). Erythropoietin signaling promotes oligodendrocyte development following prenatal systemic hypoxic-ischemic brain injury. *Pediatr. Res.* 74, 658–667. doi: 10.1038/pr.2013.155

Jantzie, L. L., Oppong, A. Y., Conteh, F. S., Yellowhair, T. R., Kim, J., Fink, G., et al. (2018). Extended neonatal erythropoietin and melatonin combinatorial treatment provides enduring repair of functional deficits in a rat model of cerebral palsy. *Front. Neurol.* 13:233. doi: 10.3389/fneur.2018.00233

Jantzie, L. L., and Robinson, S. (2015). Preclinical models of encephalopathy of prematurity. *Dev. Neurosci.* 37, 277–288. doi: 10.1159/000371721

Jantzie, L. L., Winer, J. L., Corbett, C. J., and Robinson, S. (2016). Erythropoietin modulates cerebral and serum degradation products from excess calpain activation following prenatal hypoxia-ischemia. *Dev. Neurosci.* 38, 15–26. doi: 10.1159/000441024

Jellema, R. K., Lima Passos, V., Zwanenburg, A., Ophelders, D. R., De Munter, S., Vanderlocht, J., et al. (2013). Cerebral inflammation and mobilization of the peripheral immune system following global hypoxia-ischemia in preterm sheep. *J. Neuroinflamm.* 10:13. doi: 10.1186/1742-2094-10-13

Kallapur, S. G., Presicce, P., Rueda, C. M., Jobe, A. H., and Chougnet, C. A. (2014). Fetal immune response to chorioamnionitis. *Semin. Reprod. Med.* 32, 56–67. doi: 10.1055/s-0033-1361823

Kerstetter, A. E., Padovani-Claudio, D. A., Bai, L., and Miller, R. H. (2009). Inhibition of CXCR2 signaling promotes recovery in models of multiple sclerosis. *Exp. Neurol.* 220, 44–56. doi: 10.1016/j.expneurol.2009.07.010

Kielian, T., Barry, B., and Hickey, W. F. (2001). CXC chemokine receptor-2 ligands are required for neutrophil-mediated host defense in experimental brain abscesses. *J. Immunol.* 166, 4634–4643. doi: 10.4049/jimmunol.166.7.4634

Kim, C. J., Romero, R., Chaemsaithong, P., Chaiyasit, N., Yoon, B. H., and Kim, Y. M. (2015). Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. *Am. J. Obstetr. Gynecol.* 213, S29–S52. doi: 10.1016/j.ajog.2015.08.040

Kochanek, K. D., Kirmeyer, S. E., Martin, J. A., Strobino, D. M., and Guyer, B. (2012). Annual summary of vital statistics: 2009. *Pediatrics* 129, 338–348. doi: 10.1542/peds.2011-3435

Lee, J., Kim, J. S., Park, J. W., Park, C. W., Park, J. S., Jun, J. K., et al. (2013). Chronic chorioamnionitis is the most common placental lesion in late preterm birth. *Placenta* 34, 681–689. doi: 10.1016/j.placenta.2013.04.014

Lee, S. M., Park, J. W., Kim, B. J., Park, C. W., Park, J. S., Jun, J. K., et al. (2013). Acute histologic chorioamnionitis is a risk factor for adverse neonatal outcome in late preterm birth after preterm premature rupture of membranes. *PloS One* 8:e79941. doi: 10.1371/journal.pone.0079941

Lindner, M., Trebst, C., Heine, S., Skripuletz, T., Koutsoudaki, P. N., and Stangel, M. (2008). The chemokine receptor CXCR2 is differentially regulated on glial cells in vivo but is not required for successful remyelination after cuprizone-induced demyelination. *Glia* 56, 1104–1113. doi: 10.1002/glia.20682

Liu, H., Wang, J., Wang, J., Wang, P., and Xue, Y. (2015). Paeoniflorin attenuates Abeta1-42-induced inflammation and chemotaxis of microglia in vitro and inhibits NF-kappaB- and VEGF/Flt-1 signaling pathways. *Brain Res.* 1618, 149–158. doi: 10.1016/j.brainres.2015.05.035

Liu, L., Darnall, L., Hu, T., Choi, K., Lane, T. E., and Ransohoff, R. M. (2010). Myelin repair is accelerated by inactivating CXCR2 on nonhematopoietic cells. *J. Neurosci.* 30, 9074–9083. doi: 10.1523/JNEUROSCI.1238-10.2010

Lockwood, C. J., Arcuri, F., Toti, P., Felice, C. D., Krikun, G., Guller, S., et al. (2006). Tumor necrosis factor-alpha and interleukin-1beta regulate interleukin-8 expression in third trimester decidual cells: implications for the genesis of chorioamnionitis. *Am. J. Pathol.* 169, 1294–1302. doi: 10.2353/ajpath.2006.060185

Luster, A. D. (1998). Chemokines–chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* 338, 436–445. doi: 10.1056/NEJM199802123380706

Manjavachi, M. N., Costa, R., Quintao, N. L., and Calixto, J. B. (2014). The role of keratinocyte-derived chemokine (KC) on hyperalgesia caused by peripheral nerve injury in mice. *Neuropharmacology* 79, 17–27. doi: 10.1016/j.neuropharm.2013.10.026

Maxwell, J. R., Denson, J. L., Joste, N. E., Robinson, S., and Jantzie, L. L. (2015). Combined in utero hypoxia-ischemia and lipopolysaccharide administration in rats induces chorioamnionitis and a fetal inflammatory response syndrome. *Placenta* 36, 1378–1384. doi: 10.1016/j.placenta.2015.10.009

Mizugishi, K., Inoue, T., Hatayama, H., Bielawski, J., Pierce, J. S., Sato, Y., et al. (2015). Sphingolipid pathway regulates innate immune responses at the fetomaternal interface during pregnancy. *J. Biol. Chem.* 290, 2053–2068. doi: 10.1074/jbc.M114.628867

Morganti, J. M., Jopson, T. D., Liu, S., Riparip, L. K., Guandique, C. K., Gupta, N., et al. (2015). CCR2 antagonism alters brain macrophage polarization and ameliorates cognitive dysfunction induced by traumatic brain injury. *J. Neurosci.* 35, 748–760. doi: 10.1523/JNEUROSCI.2405-14.2015

Mouton, P. R., Long, J. M., Lei, D. L., Howard, V., Jucker, M., Calhoun, M. E., et al. (2002). Age and gender effects on microglia and astrocyte numbers in brains of mice. *Brain Res.* 956, 30–35. doi: 10.1016/S0006-8993(02)03475-3

Murphy, P. M. (1997). Neutrophil receptors for interleukin-8 and related CXC chemokines. *Semin. Hematol.* 34, 311–318.

Raivich, G., Bohatschek, M., Kloss, C. U., Werner, A., Jones, L. L., and Kreutzberg, G. W. (1999). Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res. Brain Res. Rev.* 30, 77–105. doi: 10.1016/S0165-0173(99)00007-7

Reaux-Le Goazigo, A., Van Steenwinckel, J., Rostene, W., and Melik Parsadaniantz, S. (2013). Current status of chemokines in the adult CNS. *Prog. Neurobiol.* 104, 67–92. doi: 10.1016/j.pneurobio.2013.02.001

Redline, R. W. (2009). Disorders of placental circulation and the fetal brain. *Clin. Perinatol.* 36, 549–559. doi: 10.1016/j.clp.2009.06.003

Redline, R. W. (2013). Correlation of placental pathology with perinatal brain injury. *Surg. Pathol. Clin.* 6, 153–180. doi: 10.1016/j.path.2012.11.005

Robinson, S., Berglass, J. B., Denson, J. L., Berkner, J., Anstine, C. V., Winer, J. L., et al. (2017a). Microstructural and microglial changes after repetitive mild traumatic brain injury in mice. *J. Neurosci. Res.* 95, 1025–1035. doi: 10.1002/jnr.23848

Robinson, S., Corbett, C. J., Winer, J. L., Chan, L. A. S., Maxwell, J. R., Anstine, C. V., et al. (2017b). Neonatal erythropoietin mitigates impaired gait, social interaction and diffusion tensor imaging abnormalities in a rat model of prenatal brain injury. *Exp. Neurol.* 302, 1–13. doi: 10.1016/j.expneurol.2017.12.010

Robinson, S., and Franic, L. A. (2001). Chemokine GRO1 and the spatial and temporal regulation of oligodendrocyte precursor proliferation. *Dev. Neurosci.* 23, 338–345. doi: 10.1159/000048717

Robinson, S., Tani, M., Strieter, R. M., Ransohoff, R. M., and Miller, R. H. (1998). The chemokine growth-regulated oncogene-alpha promotes spinal cord oligodendrocyte precursor proliferation. *J. Neurosci.* 18, 10457–10463. doi: 10.1523/JNEUROSCI.18-24-10457.1998

Robinson, S., Winer, J. L., Berkner, J., Chan, L. A., Denson, J. L., Maxwell, J. R., et al. (2016). Imaging and serum biomarkers reflecting the functional efficacy of extended erythropoietin treatment in rats following infantile traumatic brain injury. *J. Neurosurg. Pediatr.* 17, 739–755. doi: 10.3171/2015.10.PEDS15554

Robinson, S., Winer, J. L., Chan, L. A. S., Oppong, A. Y., Yellowhair, T. R., Maxwell, J. R., et al. (2018). Extended erythropoietin treatment prevents chronic executive functional and microstructural deficits following early severe traumatic brain injury in rats. *Front. Neurol.* 9:451. doi: 10.3389/fneur.2018.00451

Roy, M., Richard, J. F., Dumas, A., and Vallieres, L. (2012). CXCL1 can be regulated by IL-6 and promotes granulocyte adhesion to brain capillaries during bacterial toxin exposure and encephalomyelitis. *J. Neuroinflamm.* 9:18. doi: 10.1186/1742-2094-9-18

Ryu, J. K., Cho, T., Choi, H. B., Jantaratnotai, N., and McLarnon, J. G. (2015). Pharmacological antagonism of interleukin-8 receptor CXCR2 inhibits inflammatory reactivity and is neuroprotective in an animal model of Alzheimer's disease. *J. Neuroinflamm.* 12:144. doi: 10.1186/s12974-015-0339-z

Saini, V., Arora, S., Yadav, A., and Bhattacharjee, J. (2011). Cytokines in recurrent pregnancy loss. *Clin. Chim. Acta* 412, 702–708. doi: 10.1016/j.cca.2011.01.002

Schober, M. E., Requena, D. F., Davis, L. J., Metzger, R. R., Bennett, K. S., Morita, D., et al. (2014). Alpha II Spectrin breakdown products in immature sprague dawley rat hippocampus and cortex after traumatic brain injury. *Brain Res.* 1574, 105–112. doi: 10.1016/j.brainres.2014.05.046

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., and Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. *Prog. Neurobiol.* 106-107, 1–16. doi: 10.1016/j.pneurobio.2013.04.001

Semple, B. D., Bye, N., Ziebell, J. M., and Morganti-Kossmann, M. C. (2010a). Deficiency of the chemokine receptor CXCR2 attenuates neutrophil infiltration and cortical damage following closed head injury. *Neurobiol. Dis.* 40, 394–403. doi: 10.1016/j.nbd.2010.06.015

Semple, B. D., Kossmann, T., and Morganti-Kossmann, M. C. (2010b). Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks. *J. Cereb. Blood Flow Metab.* 30, 459–473. doi: 10.1038/jcbfm.2009.240

Theodoric, N., Bechberger, J. F., Naus, C. C., and Sin, W. C. (2012). Role of gap junction protein connexin43 in astrogliosis induced by brain injury. *PloS One* 7:e47311. doi: 10.1371/journal.pone.0047311

Tsai, H. H., Frost, E., To, V., Robinson, S., Ffrench-Constant, C., Geertman, R., et al. (2002). The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* 110, 373–383. doi: 10.1016/S0092-8674(02)00838-3

Valles, A., Grijpink-Ongering, L., de Bree, F. M., Tuinstra, T., and Ronken, E. (2006). Differential regulation of the CXCR2 chemokine network in rat brain trauma: implications for neuroimmune interactions and neuronal survival. *Neurobiol. Dis.* 22, 312–322. doi: 10.1016/j.nbd.2005.11.015

Veenstra, M., and Ransohoff, R. M. (2012). Chemokine receptor CXCR2: physiology regulator and neuroinflammation controller? *J. Neuroimmunol.* 246, 1–9. doi: 10.1016/j.jneuroim.2012.02.016

Wang, L. Y., Tu, Y. F., Lin, Y. C., and Huang, C. C. (2016). CXCL5 signaling is a shared pathway of neuroinflammation and blood-brain barrier injury contributing to white matter injury in the immature brain. *J. Neuroinflamm.* 13:6. doi: 10.1186/s12974-015-0474-476

Weitkamp, J. H., Guthrie, S. O., Wong, H. R., Moldawer, L. L., Baker, H. V., and Wynn, J. L. (2016). Histological chorioamnionitis shapes the neonatal transcriptomic immune response. *Early Hum. Dev.* 98, 1–6. doi: 10.1016/j.earlhumdev.2016.06.001

Wu, F., Zhao, Y., Jiao, T., Shi, D., Zhu, X., Zhang, M., et al. (2015). CXCR2 is essential for cerebral endothelial activation and leukocyte recruitment during neuroinflammation. *J. Neuroinflamm.* 12:98. doi: 10.1186/s12974-015-0316-16

Xu, T., Yu, X., Wang, T., Liu, Y., Liu, X., Ou, S., et al. (2017). The effect of CXCR2 inhibition on seizure activity in the pilocarpine epilepsy mouse model. *Brain Res. Bull.* 134, 91–98. doi: 10.1016/j.brainresbull.2017.07.003

Yang, L. H., Xu, G. M., and Wang, Y. (2016). Up-regulation of CXCL1 and CXCR2 contributes to remifentanil-induced hypernociception via modulating spinal NMDA receptor expression and phosphorylation in rats. *Neurosci. Lett.* 626, 135–141. doi: 10.1016/j.neulet.2015.12.044

Yanowitz, T. D., Jordan, J. A., Gilmour, C. H., Towbin, R., Bowen, A., Roberts, J. M., et al. (2002). Hemodynamic disturbances in premature infants born after chorioamnionitis: association with cord blood cytokine concentrations. *Pediatr. Res.* 51, 310–316. doi: 10.1203/00006450-200203000-200203008

Yellowhair, T. R., Noor, S., Maxwell, J. R., Anstine, C. V., Oppong, A. Y., Robinson, S., et al. (2018). Preclinical chorioamnionitis dysregulates CXCL1/CXCR2 signaling throughout the placental-fetal-brain axis. *Exp. Neurol.* 301, 110–119. doi: 10.1016/j.expneurol.2017.11.002

Yin, X., Feng, L., Ma, D., Yin, P., Wang, X., Hou, S., et al. (2018). Roles of astrocytic connexin-43, hemichannels, and gap junctions in oxygen-glucose deprivation/reperfusion injury induced neuroinflammation and the possible regulatory mechanisms of salvianolic acid B and carbenoxolone. *J. Neuroinflamm.* 15:97. doi: 10.1186/s12974-018-1127-1123

Zhang, Z. J., Cao, D. L., Zhang, X., Ji, R. R., and Gao, Y. J. (2013). Chemokine contribution to neuropathic pain: respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons. *Pain* 154, 2185–2197. doi: 10.1016/j.pain.2013.07.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Yellowhair, Newville, Noor, Maxwell, Milligan, Robinson and Jantzie. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 24 April 2019
doi: 10.3389/fphys.2019.00417

Glutamate Transport and Preterm Brain Injury

Silvia Pregnolato[1], Elavazhagan Chakkarapani[1], Anthony R. Isles[2] and Karen Luyt[1]*

[1]Department of Neonatal Neurology, Translational Health Sciences, Bristol Medical School, University of Bristol, Bristol, United Kingdom, [2]Behavioural Genetics Group, MRC Centre for Neuropsychiatric Genetics and Genomics, School of Medicine, Cardiff University, Cardiff, United Kingdom

Preterm birth complications are the leading cause of child death worldwide and a top global health priority. Among the survivors, the risk of life-long disabilities is high, including cerebral palsy and impairment of movement, cognition, and behavior. Understanding the molecular mechanisms of preterm brain injuries is at the core of future healthcare improvements. Glutamate excitotoxicity is a key mechanism in preterm brain injury, whereby the accumulation of extracellular glutamate damages the delicate immature oligodendrocytes and neurons, leading to the typical patterns of injury seen in the periventricular white matter. Glutamate excitotoxicity is thought to be induced by an interaction between environmental triggers of injury in the perinatal period, particularly cerebral hypoxia-ischemia and infection/inflammation, and developmental and genetic vulnerabilities. To avoid extracellular build-up of glutamate, the brain relies on rapid uptake by sodium-dependent glutamate transporters. Astrocytic excitatory amino acid transporter 2 (EAAT2) is responsible for up to 95% of glutamate clearance, and several lines of evidence suggest that it is essential for brain functioning. While in the adult EAAT2 is predominantly expressed by astrocytes, EAAT2 is transiently upregulated in the immature oligodendrocytes and selected neuronal populations during mid-late gestation, at the peak time for preterm brain injury. This developmental upregulation may interact with perinatal hypoxia-ischemia and infection/inflammation and contribute to the selective vulnerability of the immature oligodendrocytes and neurons in the preterm brain. Disruption of EAAT2 may involve not only altered expression but also impaired function with reversal of transport direction. Importantly, elevated EAAT2 levels have been found in the reactive astrocytes and macrophages of human infant post-mortem brains with severe white matter injury (cystic periventricular leukomalacia), potentially suggesting an adaptive mechanism against excitotoxicity. Interestingly, EAAT2 is suppressed in animal models of acute hypoxic-ischemic brain injury at term, pointing to an important and complex role in newborn brain injuries. Enhancement of EAAT2 expression and transport function is gathering attention as a potential therapeutic approach for a variety of adult disorders and awaits exploration in the context of the preterm brain injuries.

Keywords: preterm infant, brain injury, glutamate, excitotoxicity, inflammation, EAAT2, SLC1A2, GLT-1

OPEN ACCESS

Edited by:
Carina Mallard,
University of Gothenburg, Sweden

Reviewed by:
Pierre Gressens,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Changlian Zhu,
Third Affiliated Hospital of Zhengzhou University, China

****Correspondence:***
Silvia Pregnolato
sp16027@bristol.ac.uk

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *15 October 2018*
Accepted: *27 March 2019*
Published: *24 April 2019*

Citation:
Pregnolato S, Chakkarapani E, Isles AR and Luyt K (2019) Glutamate Transport and Preterm Brain Injury. Front. Physiol. 10:417. doi: 10.3389/fphys.2019.00417

GLOBAL SIGNIFICANCE OF PRETERM BRAIN INJURIES

Perinatal care has advanced considerably in the last century and has improved survival of many vulnerable newborns, including those born preterm. The World Health Organization estimates that 15 million newborns (1 in 10 live births) are born preterm (<37 weeks of gestation) worldwide each year (World Health Organization, 2012). Despite global improvements, the United Nations Millennium Development Goal to reduce childhood mortality by two-thirds in 2015 was not achieved globally (United Nations, 2015) and 2.7 million children died in the first month of life worldwide in 2015. Of these babies, over 900,000 died due to preterm birth complications – the leading cause of death of newborns and children under 5 years old (Liu et al., 2016). For the newborns who survive, the multi-organ damage can result in life-long disabilities. Globally, preterm birth complications represent the fourth leading cause of years of "healthy" life lost due to disability (i.e., over 102,000 DALYs), above causes such as diarrheal diseases, diabetes, and HIV (World Health Organization, 2016).

Prematurity is a major risk factor for cerebral palsy, "a group of permanent disorders of the development of movement and posture, causing activity limitation, that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain" (Bax et al., 2005; Rosenbaum et al., 2007). Cerebral palsy is the most common physical disability in childhood and is a heterogeneous diagnosis, including different clinical types and brain imaging patterns, comorbidities, and multiple causes (Stanley et al., 2000; Locatelli et al., 2010; MacLennan et al., 2015). Preterm birth is clearly an important risk factor and risk is 30 times higher in children born before 33 weeks of gestation than in those born at term (Stanley, 1992; Himpens et al., 2008; Beaino et al., 2010; Mercier et al., 2010; Tronnes et al., 2014; MacLennan et al., 2015; Stavsky et al., 2017). A recent meta-analysis estimated an increase in prevalence from 1.4/1,000 live births in children born at term (>36 weeks of gestation) to 6.8/1,000 live births in moderate to late preterm (32–36 weeks of gestation), rising to 43.2/1,000 live births in very preterm (28–31 weeks of gestation) and 82.3/1,000 live births in extremely preterm infants (<28 weeks of gestation) (Oskoui et al., 2013; Hirvonen et al., 2014). More than a third of the extremely preterm children with cerebral palsy are unable to walk (Moore et al., 2012), and many have multiple disabilities, which may further limit independence and quality of life (Litt et al., 2005; Glass et al., 2008, 2015; Soria-Pastor et al., 2008; Anderson et al., 2011; Moore et al., 2012). A systematic review of international cerebral palsy registers in high-income settings highlighted the extent of these comorbidities: around three quarters of children with cerebral palsy suffer from chronic pain; approximately half have intellectual disabilities (IQ, executive function, language ability); around a quarter have active epilepsy, hip dislocation, bladder control problems, behavioral problems, sleep disorders, and/or speech impairment; 11 and 4% have severe vision and hearing impairment, respectively (Novak et al., 2012). There are less data from low-income settings, but it is likely that comorbidities, as well as mortality, are higher (Khandaker et al., 2015). Preterm birth complications impose a considerable economic burden on the public sector, which was estimated around £2.9 billion in England and Wales in 2006 (Mangham et al., 2009). While administration of magnesium sulfate as a preventative treatment to the mother during preterm labor has been shown to reduce risk of cerebral palsy by a third in very preterm infants (Doyle et al., 2009), no postnatal therapy currently exists for preterm brain injury. This is a global health priority as the increase in both preterm birth and survival rates has not been matched by a decrease in long-term disability (Wilson-Costello et al., 2005).

NEUROIMAGING AND NEUROPATHOLOGY OF PRETERM BRAIN INJURIES

Preterm birth is associated with smaller brain volumes (Peterson et al., 2003; Inder et al., 2005; Srinivasan et al., 2007) as well as motor, cognitive, and behavioral problems at school age (Peterson et al., 2000, 2003; Abernethy et al., 2004; Nosarti et al., 2005; Gimenez et al., 2006; Anderson and Doyle, 2008; Kesler et al., 2008; Aarnoudse-Moens et al., 2009; Delobel-Ayoub et al., 2009; Soria-Pastor et al., 2009; Anderson et al., 2017). Progress in neuroimaging techniques has been key in linking childhood neurodevelopmental outcomes to perinatal brain injuries and in advancing our knowledge of the underlying neuropathology (Volpe, 2009c; Back, 2017). Both MRI-defined preterm white matter injury (periventricular leukomalacia) and preterm birth are predictive of cerebral palsy (Constantinou et al., 2007; Spittle et al., 2008, 2009, 2018; Duerden et al., 2013). In a large European population study of cerebral palsy, white matter injury was the most common feature found in over 40% of the children (Bax et al., 2006). Originally, cranial ultrasound could only detect the most severe cystic type of white matter injury (cystic periventricular leukomalacia), characterized by focal macroscopic cysts of necrotic tissue in the deep white matter (de Vries et al., 1992) and highly predictive of cerebral palsy (Leviton and Paneth, 1990; De Vries et al., 2004; Serdaroglu et al., 2004; Fetters and Huang, 2007). Necrotic white matter injury can also evolve into microscopic glial scars, which may not be visible with traditional ultrasound. These are a more common type of injury and are sufficient to cause a loss in brain volume (Volpe, 2009c; Volpe et al., 2011). With the development of MRI techniques, a diffuse type of white matter injury has increasingly been recognized in the form of diffuse disturbances of myelination in the central white matter. This has emerged as the predominant type of white matter injury, accounting for over 90% of periventricular leukomalacia cases, as well as the predominant type of preterm brain injury altogether, occurring in 50% preterm newborns (Volpe, 2008). Importantly, while rates of the more severe cystic form have declined to less than 5% with advances in perinatal care, this has not been reflected for the diffuse forms (Maalouf et al., 2001; Counsell et al.,

2003; Inder et al., 2003; Miller et al., 2003; Back et al., 2007b; Volpe, 2008). These could be seen as different manifestations of an "encephalopathy of prematurity" (Volpe, 2009c) or even as distinct pathologies (Back and Rosenberg, 2014). In the last two decades, advanced MRI techniques have highlighted that injury is not limited to the white matter but it extends to the deep grey matter, cortex, and cerebellum, all of which contribute to the volume loss (Counsell and Boardman, 2005; Ball et al., 2012). The cerebellum is gathering attention as a key target of injury. This region grows rapidly at the peak time for preterm birth and damage in the form of infarction, atrophy, and poor growth has been reported as common in very preterm infants developing cerebral palsy and long-term motor, cognitive, and behavioral impairment (Mercuri et al., 1997; Abraham et al., 2001; Bodensteiner and Johnsen, 2005; Johnsen et al., 2005; Limperopoulos et al., 2005a,b, 2007; Nosarti et al., 2008; Parker et al., 2008; Lawrence et al., 2014). Indeed, there is a relationship between cerebellar volume loss and white matter injury, pointing to the existence of a common insult, such as hypoxia-ischemia and infection/inflammation, which are known to damage the developing cerebellum (Shah et al., 2006; Volpe, 2009b; Hutton et al., 2014).

Disentangling the spatial and temporal contributions of infection/inflammation and hypoxia-ischemia will be key in understanding brain injuries across the perinatal spectrum. For example, while white matter injury is typical of the preterm newborn, it may be present in a subset of newborns born at term who experienced *in utero* hypoxic-ischemic insults (e.g., placental insufficiencies) (Mallard et al., 1998; Rees et al., 1998; Zhu et al., 2016). Indeed, newborns born at term with hypoxic-ischemic encephalopathy are also at high risk and up to 40% develop cerebral palsy (Gluckman et al., 2005; Shankaran et al., 2005; Azzopardi et al., 2009; Simbruner et al., 2010; Jacobs et al., 2011). Investigating the molecular basis for divergence between term and preterm injuries is paramount for development of age-appropriate pharmacological therapies.

PATHOGENESIS OF PRETERM BRAIN INJURIES

Brain injury is thought to be more common in preterm than term newborns for several reasons, including developmental and genetic vulnerabilities and differential exposure to adverse perinatal environments. A considerable body of *in vitro* and *in vivo* evidence points two potential triggers of injury, hypoxia-ischemia, and infection/inflammation (Volpe, 2008, 2009a; Deng, 2010; Volpe et al., 2011; Back and Rosenberg, 2014; Back, 2017). These insults are thought to interact in the vulnerable immature brain and converge onto three downstream mechanisms of injury: inflammation, glutamate excitotoxicity, and ultimately free radical attack, which directly damages cell components as well as triggering delayed cell death by apoptosis. Severity and temporal profile of hypoxia-ischemia and infection/inflammation, degree of brain maturity, comorbidities, sex, and genetic background may all contribute to individual differences in pathogenesis, clinical presentation, and individual susceptibility to injury. We will review the role of developmental vulnerabilities, infection/inflammation, and hypoxia-ischemia and bring the focus on the common downstream mechanism of glutamate excitotoxicity. We will then review the evidence linking glutamate transport to excitotoxic preterm brain injuries and highlight the current evidence supporting excitatory amino acid transporter 2 (EAAT2) as a potential therapeutic target.

Developmental Vulnerability

The brain undergoes rapid and critical developmental events during the peak time of premature brain injury (24–32 weeks), including neuronal migration, growth of axons and dendrites, synaptogenesis, development of the vascular system, and myelination. Interference with these natural trajectories determines selective cellular and regional vulnerabilities and may redirect subsequent development. Among their functions, oligodendrocytes are responsible for laying the highly specialized myelin membrane around axons and are therefore key for the development of the white matter. Myelination begins before birth and peaks in the first 2 years of postnatal life, with the intracortical fibers of the cortex being myelinated in the third decade. The process of myelination requires that oligodendrocytes first proliferate and develop into mature oligodendrocytes and then depose myelin around axons (Volpe, 2008). Around the peak time of preterm brain injury (28–32 weeks of gestation), the pre-oligodendrocyte stage still represents the majority of the oligodendrial pool in the very preterm brain (Iida et al., 1995; Back et al., 2001). Pre-oligodendrocytes are more vulnerable than mature oligodendrocytes to hypoxia-ischemia, infection/inflammation, oxidative damage, and ultimately cell death (Back et al., 1998, 2002, 2005, 2007b; Fern and Moller, 2000; Baud et al., 2004; Fragoso et al., 2004; Segovia et al., 2008; Volpe et al., 2011). Indeed, a unique feature of periventricular white matter injury is an arrest in the development of oligodendrocytes at the pre-oligodendrocyte stage, leading to the abnormal myelination patterns typically seen through MRI (Back et al., 2007b; Volpe et al., 2011). More severe necrotic injury extends to all the cell components, leading to cysts and exacerbating myelin injury via focal axonal degeneration (Laptook, 2016; Back, 2017). Concurrent developmental vulnerabilities include the limited ability of the immature brain to synthesize appropriate amounts of growth factors needed for brain development and self-protection, and an immature immune system, potentially promoting an excessive and sustained inflammatory response (Gilles et al., 2018).

Environmental Triggers of Injury: Hypoxia/Ischemia and Infection/Inflammation

Alongside the intrinsic developmental vulnerability of the immature brain, the preterm newborn is exposed to a range of potentially harmful exposures in the perinatal period. Supported by mounting experimental and epidemiological evidence, perinatal infection/inflammation leading to an overly

intense inflammatory response, or a "cytokine storm", has increasingly been recognized as a major risk factor not only for preterm birth but also for preterm white matter injury and long-term neurodisabilities (Yoon et al., 1996, 1997, 2000; Baud et al., 1999; Duggan et al., 2001; Dollner et al., 2002; Heep et al., 2003; Kaukola et al., 2004, 2006; Ellison et al., 2005; Bi et al., 2014). The preterm brain is often exposed to inflammation early during fetal development (e.g., maternal infections and chorioamnionitis) and usually for prolonged periods during postnatal life in the neonatal intensive care environment (e.g., neonatal infections, inflammatory comorbidities such as necrotizing enterocolitis), during critical phases of myelination and brain plasticity (Murphy et al., 1995; Grether and Nelson, 1997; Verma et al., 1997; Alexander et al., 1998; Dammann and Leviton, 1998, 2000, 2004; O'Shea et al., 1998; Leviton et al., 1999; Wu and Colford, 2000; Dammann et al., 2002; Rezaie and Dean, 2002; Stoll et al., 2002; Wu, 2002; Schlapbach et al., 2011; Hagberg et al., 2015; Anblagan et al., 2016). A combination of multiple inflammatory hits, antenatally and postnatally, has been shown to increase risk of brain injury and disability compared to single hits (Korzeniewski et al., 2014; van der Burg et al., 2016; Yanni et al., 2017). Indeed, pharmacological interventions targeting inflammation may have translational potential based on preclinical studies (Hagberg et al., 2015).

The role of hypoxia-ischemia in preterm brain injury is more controversial. In term newborns with hypoxic-ischemic encephalopathy, defined and acute hypoxic-ischemic events before or during birth (e.g., placental abruption, cord occlusion, and uterine rupture) are usually recognized by the clinician and represent the first step of a diagnosis of hypoxic-ischemic encephalopathy, aided by objective clinical and neuroimaging criteria. In the preterm newborn, a sentinel event is rarely recognized, and hypoxia-ischemia is generally assumed to have a more complex temporal profile, with intermittent or chronic nature (Laptook, 2016; Ohshima et al., 2016). However, it remains challenging to determine the individual contribution of hypoxia-ischemia among several coexistent factors, such as infection/inflammation, growth restriction, or hyperoxia (Gopagondanahalli et al., 2016). Physiologically, it is conceivable that the preterm brain is vulnerable to hypoxia-ischemia due to the anatomical and functional immaturity of the periventricular vasculature, which would make the periventricular white matter vulnerable to minor drops in cerebral perfusion (Takashima and Tanaka, 1978; Lou et al., 1979; De Reuck, 1984; Altman et al., 1988; Pryds, 1991; Miyawaki et al., 1998; Inage et al., 2000; Volpe, 2008; Laptook, 2016). The periventricular white matter has lower basal blood flow compared to grey matter regions in both humans (Greisen, 1986; Pryds et al., 1990) and the preterm fetal sheep (Szymonowicz et al., 1988; Gleason et al., 1989; Riddle et al., 2006). Further drops in blood flow are common in sick premature infants with respiratory disease due to lung immaturity (Soul et al., 2007). Mechanical ventilation may contribute to ischemia due to the vasoconstrictive effect of the induced cumulative hypocarbia (Shankaran et al., 2006). Perinatal hypoxic-ischemic episodes are also likely to play a key role, including ongoing placental pathologies, an overlapping risk factor for intrauterine growth restriction, low birthweight, and preterm birth. A meta-analysis recently reported an association between preterm brain injury and perinatal risk factors related to hypoxia-ischemia, including oligohydramnios, acidemia, low Apgar scores, apnea, respiratory distress syndrome, and seizures (Huang et al., 2017). However, the link between regional differences in blood flow and vulnerability to severe white matter injury is not consistent, and even in moderate ischemia, some regions of white matter are spared. This suggests that ischemia is necessary but not sufficient in isolation (Riddle et al., 2006; McClure et al., 2008; Back, 2017). Indeed, it has been suggested that more consistent evidence is needed to ascertain the specific role of hypoxic and ischemic events in preterm brain injury altogether and that future research should take into account contributions and interactions with other biological processes, including infection/inflammation and developmental vulnerability (Gilles et al., 2018). Importantly, the impact of hypoxia-ischemia on the cerebellum is also emerging, as shown by reports of volume loss and death of Purkinje cells and Bergmann glia in term newborns with hypoxic-ischemic encephalopathy and mid-late gestation fetal sheep exposed to asphyxia (Rees et al., 1997; Inage et al., 1998; Castillo-Melendez et al., 2004; Biran et al., 2012; Hutton et al., 2014). In an established mouse model of chronic hypoxia recapitulating perinatal brain injuries, damage to the cerebellum was reported in terms of a significant loss of GABAergic interneurons and a delay in dendritic arborization of Purkinje cells, followed by motor impairment and cerebellar learning deficits (Chahboune et al., 2009; Zonouzi et al., 2015; Sathyanesan et al., 2018).

Several experimental studies have shown that hypoxia-ischemia and infection/inflammation lead to worse brain and behavioral outcomes when they interact, and insults that are individually insufficient to cause injury can lead to injury when combined (Dommergues et al., 2000; Eklind et al., 2001; Lehnardt et al., 2003; Ikeda et al., 2004; Larouche et al., 2005; Favrais et al., 2007; Wang et al., 2007, 2009, 2010; Aden et al., 2010; van Tilborg et al., 2018). This has led to the multiple hit hypothesis of preterm brain injury, whereby a mild first event sensitizes the brain to subsequent insults (Leviton et al., 2013; Van Steenwinckel et al., 2014; Barnett et al., 2018). The current hypothesis is that hypoxia-ischemia triggers an inflammatory response *per se*. This additional endogenous response combined with the inflammation triggered by infection leads to a pro-inflammatory "cytokine storm," which is not matched by upregulation of anti-inflammatory cytokines and neurotrophic factors. This in turn sensitizes the brain to hypoxic-ischemic injury by enhancing glutamate excitotoxicity and damaging the blood-brain barrier (Hagberg et al., 2015). Tertiary mechanisms of injury, mediated by epigenetic modifications, may sustain the sensitization in the long term and interfere with remodeling and repair mechanisms (Dammann, 2007; Fleiss and Gressens, 2012).

A substantial body of experimental evidence suggests that glutamate excitotoxicity triggered by hypoxia-ischemia and/or

infection/inflammation plays a key role in the pathogenesis of preterm white matter injury (Hagberg et al., 2002; Johnston, 2005; Volpe, 2008; Deng, 2010; Volpe et al., 2011).

GLUTAMATE EXCITOTOXICITY IN THE PRETERM BRAIN

Glutamate Homeostasis and Dysregulation

Glutamate is the main excitatory neurotransmitter in the mammalian brain (Meldrum, 2000). It is essential for brain function, orchestrating not only fast excitatory neurotransmission but also long-lasting neuronal changes necessary for memory, learning, and cognition. It is also fundamental during brain development, due to its role in regulating formation and elimination of synapses, as well as neuronal migration, proliferation, and viability. Glutamate is abundant inside the brain cells, and most neurons and glial cells have glutamate receptors distributed across most cellular elements, highlighting the importance of glutamatergic systems for normal function (Curtis and Johnston, 1974; Watkins and Evans, 1981; Bliss and Collingridge, 1993; Newcomer et al., 2000; Platt, 2007). Stimulation of a glutamatergic neuron results in Ca^{2+}-dependent release of glutamate in the synapse by vesicular exocytosis. Extracellular glutamate binds to and activates post-synaptic ionotropic (NMDA, AMPA, and kainate receptors) and metabotropic (mGluR) glutamate receptors, stimulating the post-synaptic neurons *via* Ca^{2+} or Na^{+} influx and inducing intracellular signaling cascades that lead to physiological cellular responses, such as regulation of transcription factors and DNA replication (Nicholls and Attwell, 1990; Danbolt, 2001).

Glutamatergic transmission is terminated when glutamate transporters, expressed predominantly by astrocytes, slowly take up glutamate from the synaptic space (30 glutamate molecules per second at Vmax) (Otis and Kavanaugh, 2000; Bergles et al., 2002; Grewer and Rauen, 2005; Takahashi et al., 2015). In the preterm brain, glutamate transporters are also expressed by immature neurons and oligodendrocytes, although their significance is controversial, as reviewed below. In astrocytes, glutamate is converted to glutamine *via* glutamine synthetase. Glutamine is shuttled back into the pre-synaptic neuron, where it is converted into glutamate *via* glutaminase (**Figure 1**). The glutamate-glutamine cycle is not essential for supplying glutamate for neuronal release but is needed for normal glutamatergic transmission (Danbolt, 2001; Takahashi et al., 2015; Danbolt et al., 2016).

The ubiquity of glutamate is a double-edged sword: when homeostasis is disrupted, glutamate can turn into a potent neurotoxin. If the concentration of glutamate in the extracellular space rises above physiological levels, post-synaptic glutamate receptors are overactivated. This excessive activation, or excitotoxicity, leads to cell death *via* activation of suicide cell programs (apoptosis) (Danbolt, 2001; Sattler and Tymianski, 2001) (**Figure 1**). Since it was first proposed in the late 1960s (Olney, 1969), the concept of glutamate excitotoxicity has been implicated in several adult disorders, both acute (e.g., ischemic stroke and traumatic brain injury) and chronic (e.g., amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, major depression, and addiction) (Doble, 1999; Takahashi et al., 2015). Consistently, injection of glutamate agonists into the cortex, striatum, and

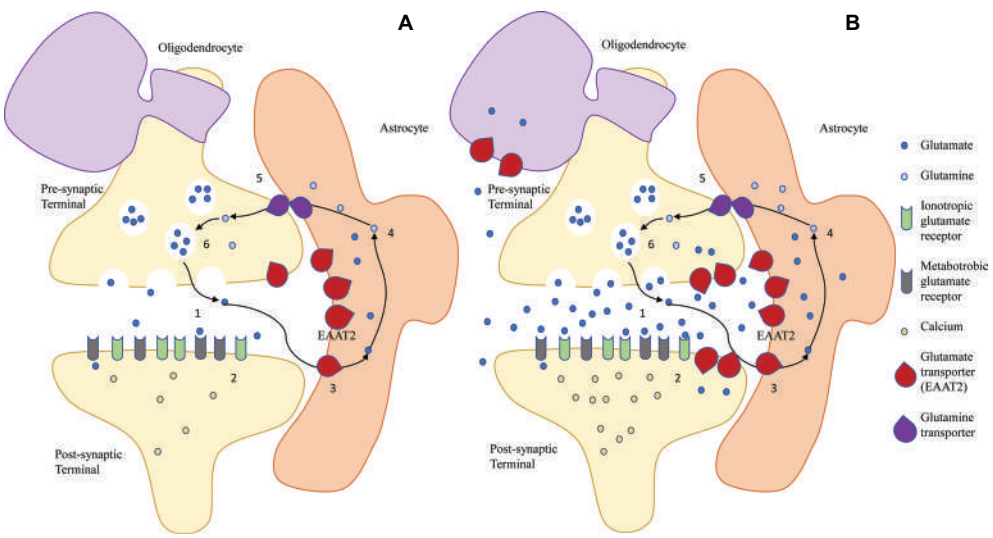


FIGURE 1 | The glutamate/glutamine cycle in **(A)** physiological conditions and **(B)** excitotoxic conditions in the immature brain. **(A)** In the mature healthy brain, glutamate is released by exocytosis from the pre-synaptic neuronal terminal into the synapse (1), and it binds to post-synaptic ionotropic (NMDA, AMPA, and kainate receptors) and metabotropic (mGluR) glutamate receptors, inducing Ca^{2+}-mediated signaling cascades that result in cellular responses (2). Extracellular glutamate is taken up primarily by astroglial EAAT2 (3) and converted to glutamine (4), which is shuttled back to the pre-synaptic terminal *via* glutamine transporters (5). Here, glutamine is converted back to glutamate (6). **(B)** During excitotoxicity, a combination of increased neuronal release and decreased astroglial uptake lead to a rise of extracellular glutamate levels, leading to overactivation of the post-synaptic glutamate receptors, Ca^{2+} overload, and activation of apoptotic pathways. Reversal of transport of astroglial transporters may also contribute to the accumulation of extracellular glutamate. In the immature brain, upregulation of the glutamate transporters in underdeveloped neurons and oligodendrocytes may contribute to their selective vulnerability.

periventricular white matter of newborn rodents, rabbits, and kittens produces patterns of perinatal brain injuries similar to those seen in humans (McDonald et al., 1988; Innocenti and Berbel, 1991a,b; Marret et al., 1995; Gressens et al., 1996; Acarin et al., 1999; Follett et al., 2000). On the other hand, pharmacological inhibition of glutamate receptors before or immediately after an hypoxic-ischemic insult is neuroprotective in both preterm (Follett et al., 2004; Manning et al., 2008) and term (Hagberg et al., 1994; Follett et al., 2000) brain injuries. Indeed, one of the mechanisms through which magnesium sulfate is thought to exert neuroprotection is by preventing excitotoxic damage through NMDA receptor blockade (Lingam and Robertson, 2018).

In vivo Evidence of Glutamate Excitotoxicity

Evidence of *in vivo* disturbance of glutamate signaling has been produced for animal models of hypoxic-ischemic brain injury. In a rat model of mild white matter injury near term, a rise in extracellular glutamate is observed in the acute phase after hypoxia-ischemia, with oligodendrocytes and axons representing the major sources of extracellular glutamate and astrocytes failing to take up excess glutamate (Back et al., 2007a). Similarly, repeated umbilical cord occlusion in the near-term fetal sheep causes periventricular white matter injury, the extent of which correlates with extracellular local glutamate levels (Loeliger et al., 2003). Notably, the largest increase in glutamate occurred over the hours after the insult, a delayed increase that suggested impaired glutamate transport. In a piglet model of hypoxic-ischemic encephalopathy at term, glutamate levels in the basal ganglia were shown to change in two phases: an early increase in the first 6 hours was followed by transient and slight recovery by 12 hours, possibly due to the self-protective glutamate transport mechanisms and conversion to glutamine in astrocytes; a further increase occurred after a day, possibly through cells bursting due to reperfusion injury and reversal of glutamate transport in the late stages of disease (Dang et al., 2017). In humans, elevated glutamate levels have been reported in the cerebrospinal fluid and basal ganglia of asphyxiated newborns (Riikonen et al., 1992; Hagberg et al., 1993). Moreover, elevated glutamine levels have been found in MRI-defined punctate necrotic white matter lesions (Wisnowski et al., 2013). Glutamate is taken up into astrocytes for conversion into glutamine and shuttling back to neurons. The finding of elevated glutamine rather than glutamate may be due at least in part to the temporal lag between insult and measurement. An important limitation of *in vivo* glutamate measurements in preterm newborns is that the peak window of glutamate changes is probably missed, because magnetic resonance measurements are likely to be carried out long after the initial insults in newborns that have already become sick. As such, these findings suggest that disrupted glutamate homeostasis persists in the subacute phase in moderate necrotic white matter injury. Although a relatively small subset of the newborns with punctate lesions also had evidence of cysts, no studies to date have measured glutamatergic metabolism specifically in newborns with severe cystic white matter injury.

Glutamate Excitotoxicity Following Hypoxia-Ischemia

Glutamate homeostasis can be disrupted by an acute hypoxic-ischemic event, and the phases of the subsequent excitotoxic injury are well described. During the primary energy failure, oxygen and blood deprivation lead to impairment of ATP production due to failure of oxidative phosphorylation. Astrocytes, with their unique oxidative capacity and ability to upregulate ATP production, are central to maintaining energy metabolism during the first stage of ischemia (Dienel and Hertz, 2005). Impairment of the ATP-dependent Na^+/K^+ pumps leads to loss of the electrochemical gradient across the cell membrane. If the insult is severe, some cells may die at this early stage *via* necrosis, due to influx of ions and water, cell swelling, and bursting. Within hours, the necrotic injury due to severe energy failure leads to death of all cellular elements and develops into the white matter cysts (Back, 2017). Depolarization of the cell membrane activates Ca^{2+} channels in the pre-synaptic terminal, triggering vesicular release of glutamate in the synapse. In astrocytes, hypoxia-ischemia leads to a failure in the astrocytic glutamate uptake system, which also relies on Na^+/K^+ gradients. The combination of increased synaptic release and reduced astrocytic uptake leads to accumulation of glutamate in the synaptic space and overactivation of post-synaptic ionotropic and metabotropic glutamate receptors (Volpe, 2008). The subsequent intracellular Ca^{2+} influx triggers activation of phospholipases, endonucleases, proteases, and nitric oxide synthase, with degradation of cellular and extracellular structures, and generation of harmful free radicals and reactive oxygen and nitrogen species. Glutamate leaking outside the synapse activates extrasynaptic NMDA receptors, which, contrarily to the pro-survival action of synaptic NMDA receptors, promotes excitotoxic cell death even further (Parsons and Raymond, 2014). This excitotoxic-oxidative cascade eventually leads to cell damage or death *via* necrosis, apoptosis, and autophagy in the secondary phase of injury (Olney, 1969; Benveniste et al., 1984; McDonald and Johnston, 1990; Choi, 1992; Thornton et al., 2012; Back, 2017; Descloux et al., 2018) (**Figure 1**).

Glutamate Excitotoxicity Following Inflammation

In preterm brain injury, comorbidities stimulating inflammation are thought to contribute to disruption of glutamate homeostasis and potentiation of excitotoxicity. TNFα, for example, is one of the most studied cytokines and is emerging as a key link between inflammation and glutamate excitotoxicity (Olmos and Llado, 2014). TNFα has both neuroprotective and neurotoxic effects depending on the different signaling pathways activated by the different receptors. In fact, pharmacological inhibition or genetic deletion after a combined inflammatory and excitotoxic insult is neuroprotective (Aden et al., 2010; Kendall et al., 2011), but knocking out TNFα receptors in the mouse increases susceptibility to hypoxic-ischemic injury (Bruce et al., 1996). TNFα potentiates glutamate excitotoxicity *in vitro via* complex and interacting mechanisms involving crosstalk between neurons and glial cells and leading to vicious cycles of glutamate and cytokine release.

In neurons, TNFα increases the excitatory strength at the synapse by increasing cell surface expression of glutamate receptors and their permeability to Ca^{2+}, while also decreasing expression of inhibitory $GABA_A$ receptors (Olmos and Llado, 2014). In microglia, TNFα stimulates autocrine release of TNFα and glutamate by upregulating glutaminase and from hemichannels of gap junctions (Takeuchi et al., 2006). In astrocytes, TNFα stimulates glutamate release *via* prostaglandin E2 and exacerbates impairment of glutamate transport (Bezzi et al., 1998). Cheung et al. (1998) suggested that glutamate concentration may be key in determining the pathways of cell death, with higher glutamate concentrations preferentially triggering necrosis and lower concentrations leading to apoptosis. Either way, even transient excess of glutamate can start a number of events that ultimately cause death or damage of vulnerable cell populations (Ottersen et al., 1996).

Glutamate Excitotoxicity and Perinatal Brain Injuries

The patterns of excitotoxic injury tend to be different in the preterm and term brain. Experimental evidence suggests that the main cellular target of excitotoxic injury in the preterm brain is pre-oligodendrocytes (Volpe et al., 2011). Glutamate is highly toxic to pre-oligodendrocytes in cell culture and leads to cell death *via* free radical attack (Oka et al., 1993). The white matter in the rat is much more vulnerable to hypoxia-ischemia at preterm-equivalent age, when pre-oligodendrocytes are predominant, than at term-equivalent age, when mature oligodendrocytes are the major form (Back et al., 2002; Craig et al., 2003; Dean et al., 2011). Indeed, the patterns of hypoxic-ischemic white matter injury seem to be determined primarily by the timing of appearance (Buser et al., 2010) and spatial distribution (Riddle et al., 2006) of pre-oligodendrocytes rather than severity of ischemia itself. Pre-oligodendrocytes are strikingly more vulnerable than immature neurons of the cortex and caudate nucleus in moderate global ischemia in the preterm fetal sheep (Dean et al., 2013; McClendon et al., 2014). Immature neurons are also vulnerable, as NMDA receptors are functionally upregulated, more permeable to calcium and less sensitive to magnesium block (Jantzie et al., 2015).

In physiological conditions, the abundance of glutamate receptors in the white matter is key during early neuronal development, contributing to rapid growth and myelination. However, their abundance also confers increased vulnerability in excitotoxic conditions (Kaindl et al., 2009). Indeed, the selective vulnerability of subplate neurons compared to cortical neurons observed in a preterm model of hypoxia-ischemia has been suggested to originate from an increase of glutamate receptors in these neurons associated with early maturation (McQuillen et al., 2003). Similarly, it has been suggested that selective vulnerability of the deep grey matter and sensorimotor cortex in term hypoxic-ischemic encephalopathy could be related to peaking NMDA receptor expression and proximity to developing glutamatergic circuits (Rocha-Ferreira and Hristova, 2016). As such, developmental expression of key glutamatergic genes in the grey and white matter may contribute to the different patterns of excitotoxic injury (Volpe, 2008).

Overall, the potential sources of extracellular glutamate in the white matter include pre-oligodendrocytes, astrocytes, neurons, ependymal cells, and cells of the choroid plexus (Back and Rosenberg, 2014). While therapies targeting excitotoxicity have so far mostly focused on glutamate receptor blockade, targeting glutamate transport is gathering interest as a potential avenue for neuroprotection by counteracting glutamate accumulation in the first place (Tilleux and Hermans, 2007; Kim et al., 2011; Fontana, 2015; Takahashi et al., 2015).

GLUTAMATE TRANSPORT: FOCUS ON EAAT2/GLT-1

Maintaining the baseline extracellular glutamate concentrations in the nanomolar range is essential to avoid extracellular glutamate build-up. The brain has no known enzymatic mechanism to metabolize glutamate in the extracellular space, and simple diffusion over short distances is thought to bring only a minor contribution. Hence, the brain relies substantially on intracellular glutamate uptake, and astrocytes provide by far the largest contribution to preventing excitotoxicity through expression of glutamate transporters (Danbolt, 2001; Tzingounis and Wadiche, 2007; Vandenberg and Ryan, 2013). Given their crucial role, it is not surprising that expression of astrocytic glutamate transporters is constitutively high (Zhou and Danbolt, 2013). Crosstalk between neurons and glia relies on tightly controlled extracellular glutamate homeostasis, and it is becoming increasingly evident that neuron-glia interactions are central to both the kinetics of glutamatergic synaptic activity in physiological (Fontana, 2015) and excitotoxic conditions (Carmignoto, 2000). Glutamate is released by astrocytes in immature rat optic nerve in ischemia *in vitro* (Wilke et al., 2004). Moreover, glutamate transport has been observed in immature axons (Arranz et al., 2008), and impairment has been reported in pre-oligodendrocytes during hypoxia-ischemia, providing a potential mechanism of excitotoxic vulnerability (Oka et al., 1993; Domercq et al., 1999; Fern and Moller, 2000; Deng et al., 2003; Desilva et al., 2007, 2009). The importance of glutamate transport to the integrity of oligodendrocytes and white matter is supported by evidence of extensive excitotoxic injury in oligodendrocytes and axons with experimental inhibition of glutamate transport in the optic nerve *in vivo* (Domercq et al., 2005).

The five members of the excitatory amino acid transporter (EAAT) family carry out most of the glutamate clearance in the central nervous system (Anderson and Swanson, 2000), especially EAAT1 (SLC1A3, rodent orthologue Glast) and EAAT2 (SLC1A2, rodent orthologue Glt-1) (Bristol and Rothstein, 1996). EAAT2 is the major glutamate transporter in the forebrain, except in the cerebellum, circumventricular organs, and retina, where EAAT1 is prevalent. In physiological conditions, both EAAT1 and EAAT2 are expressed predominantly by astrocytes and localized to the cellular membrane in the adult brain (Danbolt, 2001; Roberts et al., 2014; Takahashi et al., 2015). The high concentration (1 mg/g rat brain tissue), ubiquity

(1% of total CNS protein in the adult brain), and high degree of conservation across mammalian species are all indications of physiological importance of EAAT2/Glt-1 (Danbolt, 2001; Fontana, 2015; Danbolt et al., 2016). Unsurprisingly, it is expressed at high density near glutamatergic synapses in developing hippocampal astrocytes, with density and vicinity increasing with neuronal activity (Benediktsson et al., 2012). This transmembrane transporter carries out glutamate uptake through a high affinity energy-dependent process driven by Na^+ and K^+ gradients. Specifically, glutamate and aspartate are co-transported inside the brain cells with 3 Na^+ and 1 H^+ for the antiport of 1 K^+. EAAT2 is also a selective anion channel, transporting Cl^- anions during intermediate conformations, uncoupled from the flux of glutamate (Fontana, 2015).

Several lines of evidence support the central role of EAAT2 expression/function in maintaining extracellular glutamate homeostasis. Pharmacological inhibition of glutamate transport, including EAAT2, leads to rapid extracellular glutamate increase *in vitro* (Jabaudon et al., 1999) and extended post-synaptic activation mediated by NMDA receptors (Lozovaya et al., 1999). Genetic deletion of Glt-1 *via* constitutive knockout in the mouse leads to lower body weight, seizures, acute cortical injury in the forebrain, and increased mortality from the second/third postnatal week (Tanaka et al., 1997). Brain tissue from this mouse shows much lower (5%) glutamate transport activity than wild-type, suggesting that Glt-1 is responsible for up to 95% of glutamate transport. This is confirmed by the ability of Glt-1 antibodies to remove 90% of the transport activity in forebrain tissue (Haugeto et al., 1996). Other Glt-1 knockouts have confirmed the obvious phenotype, with lower life span, lower body and brain weight, mild loss of CA1 neurons in the hippocampus, and severe focal neuronal loss in layer II of the neocortex and focal gliosis (Kiryk et al., 2008). A conditional knockout mouse with selective deletion of Glt-1 reproduces this phenotype while ruling out developmental adaptations (Zhou et al., 2014). Heterozygote knockouts, on the other hand, show halved concentrations of Glt-1, but no apparent morphological brain changes, despite an increased risk of traumatic spinal cord injury (Kiryk et al., 2008; Lepore et al., 2011). Inhibition with antisense oligonucleotides *in vitro* and *in vivo* induces a rise in extracellular glutamate, excitotoxic injury, and progressive paralysis (Rothstein et al., 1996). On the other hand, selective overexpression in astrocytes is neuroprotective during ischemia (Chao et al., 2010).

Studies of EAAT2 expression point to different patterns depending on cell type, region, developmental age, species, and methodology used (DeSilva et al., 2012). In the adult rat, Glt-1 is expressed in the forebrain, especially in the hippocampus, cortex, striatum, and thalamus as well as in fibrous astrocytes in the white matter (Lehre et al., 1995). The transporter is expressed predominantly by astrocytes but also pre-synaptic axon terminals in the rodent hippocampus and somatosensory cortex (Danbolt, 2001; Chen et al., 2004; Furness et al., 2008; Melone et al., 2009; de Vivo et al., 2010; Danbolt et al., 2016). Neuronal EAAT2 represents no more than 10–20% total EAAT2 (Furness et al., 2008; Danbolt et al., 2016), and while being implicated in adult neuropsychiatric disorders (O'Donovan et al., 2017), neuronal knockout barely affects total Glt-1 protein levels and mouse development (Petr et al., 2015). Conversely, astrocytic knockout leads to a reduction of protein levels to a fifth in the forebrain, lower body weight and increased epilepsy and mortality.

Developmental Expression of EAAT2

The scenario may be at least in part different in the preterm brain, where transient but more prominent neuronal and pre-oligodendrial expression is observed. During development, dynamic and species-specific changes in both cellular and regional expression have been observed, suggesting that glutamate transporters may be both regulated by and involved in brain development (e.g., participation in the development of the topographic organization). As expected, these changes in rodent Glt-1 expression correspond to changes in total glutamate uptake activity (Ullensvang et al., 1997). Briefly, Glt-1 expression is low until after birth, except for a transient peak of expression in developing axons and oligodendrocytes around mid-late gestation. Glt-1 is expressed *in vivo* in rat pre-oligodendrocytes, whereas it is no longer detectable in mature oligodendrocytes (DeSilva et al., 2009). Transient neuronal expression is also seen around mid-late gestation in the mouse (Sutherland et al., 1996; Yamada et al., 1998), rat (Furuta et al., 1997), and sheep (Northington et al., 1998). In the fetal rat, Glt-1 is expressed in the amygdala and hippocampus, as well as white matter tracts interconnecting neocortex, basal ganglia, and thalamus (Furuta et al., 1997). In the fetal sheep, Glt-1 is found not only in white matter tracts but also in neuronal bodies and extended to the subplate, cranial nerve nuclei, basal ganglia, and cerebellar cortex, highlighting potential species differences in cellular expression during development (Furuta et al., 1997; Northington et al., 1998, 1999). In the newborn rat at P1, Glt-1 levels are the highest in the spinal cord and moderate in the hippocampus and hypothalamus. Expression increases dramatically from the second postnatal week throughout the central nervous system, especially in the cortex, striatum, caudate nucleus, and hippocampus, reaching adult levels by weeks 4–5 (Rothstein et al., 1994; Levy et al., 1995; Shibata et al., 1996; Sutherland et al., 1996; Furuta et al., 1997; Ullensvang et al., 1997). Astrocyte selectivity is established in the postnatal period in rodents and around mid-late gestation in sheep (Furuta et al., 1997; Takasaki et al., 2008). Nonetheless, Glt-1 is still detected in immature axons at P14–17 (Arranz et al., 2008). The significant developmental changes in Glt-1 after birth may explain why the Glt-1 knockout mice seem to develop normally for the first few weeks and develop seizures and brain injury during postnatal week 3, with many dying by week 4 (Tanaka et al., 1997; Takasaki et al., 2008).

A limited number of studies have investigated developmental regulation of EAAT2 in humans. DeSilva et al. (2012) found that, among EAAT1–3, expression of EAAT2 undergoes particularly prominent maturational changes in post-mortem cortex tissue of preterm and term newborns without neurological disease, all the way into childhood. Consistent with animal

studies, EAAT2 expression is generally low until birth and is limited to glia limitans, layer I-III fine astrocytes, and some neuron populations. EAAT2 was found not only in axons but also in the cell body and dendrites of certain neuron populations from as early as 23 gestational weeks up until term and, in some cases, until 8 postnatal months. These neuron populations are layer V pyramidal neurons, layer I neurons (putative Cajal-Retzius cells), and subplate neurons (DeSilva et al., 2012). A great proportion of these neuronal populations is glutamatergic, and it has been suggested that this transient neuronal EAAT2 expression is critical for establishing and orchestrating excitatory transmission during maturation and migration of cortical neurons. Similarly, it could also provide the basis for selective vulnerability to premature excitotoxic injury due to expression of glutamate transporters, which may reverse transport and become sources of extracellular glutamate (Takasaki et al., 2008; DeSilva et al., 2012), as discussed below. This is supported by evidence of selective vulnerability of layer V pyramidal neurons and subplate neurons in human and rat preterm white matter injury (McQuillen et al., 2003; Andiman et al., 2010). The same group reported EAAT2 expression in pre-oligodendrocytes in human fetal white matter at 32 weeks of gestation, during the peak time for premature brain injury, but not at 7 months old, consistent with rat studies (Desilva et al., 2007). EAAT2 expression appeared in the astrocytes of the developing cortex at 41 postconceptional weeks, increasing steeply in the first 1.5 years (DeSilva et al., 2012). Taken together, these findings suggest that the expression of EAAT2/Glt-1 undergoes substantial changes during development and that these changes may contribute to the selective vulnerability of cellular (e.g., immature oligodendrocytes and neurons) and regional (e.g., white matter tracts, hippocampus) targets in preterm brain injury.

EAAT2 and Preterm Brain Injury

Following severe energy failure, the dissipation of the transmembrane gradient impairs astrocytic EAAT2, which relies on transmembrane Na^+/K^+ gradients. This disruption may involve both quantity and quality of transport activity, i.e., it can manifest as decreased expression and/or impairment of glutamate transport function with establishment of reverse transport. Reverse transport has an outward direction and is driven by the transmembrane gradient of excitatory amino acids independently of ATP and Ca^{2+} (Nicholls and Attwell, 1990; Szatkowski et al., 1990; Levi and Raiteri, 1993). In this scenario, glutamate transporters become themselves a major source of extracellular glutamate, potentially turning into key contributors of excitotoxic injury in any cells expressing them (Domingues et al., 2010) (**Figure 1**). While its significance to preterm brain injuries remains to be explored, the importance of reverse transport is supported by evidence that ischemic cell death in the rat striatum can be blocked by an inhibitor of reverse Glt-1 transport (Seki et al., 1999). Moreover, knockout mice lacking Glt-1 are more vulnerable to neuronal death after a short, severe episode of ischemia than wild-type mice, suggesting that Glt-1 is essential for neuroprotection when ischemia is acute; on the other hand, wild-type mice expressing Glt-1 are more vulnerable to neuronal death than mice lacking Glt-1 during extended, chronic ischemia, suggesting that Glt-1 (*via* reverse transport) becomes neurotoxic when ischemia is prolonged (Mitani and Tanaka, 2003).

Consistent with impairment of glutamate transport, a decrease in glutamate uptake is seen in the hippocampus of rat pups exposed to intrauterine hypoxia following caesarean delivery (Frizzo et al., 2010) and in the cortex, basal ganglia and thalamus of newborn piglets exposed to hypoxia (Jantzie et al., 2010). Loss of Glt-1 expression and/or function has been reported in astrocyte cultures during hypoxia (Dallas et al., 2007) as well as in the adult rat cortex and hippocampus after ischemia (Torp et al., 1995; Rao et al., 2001a,b). In a small study of term-equivalent rats, astrocytic Glt-1 was suppressed in the initial 12 hours in the ischemic core of both the hippocampus and the neocortex, recovered after 48 hours only in the hippocampus, followed by astrogliosis at 72 hours (Fukamachi et al., 2001). In a piglet model of hypoxic-ischemic encephalopathy at term, canonical suppression of Glt-1 in astrocytes of the striatum and hippocampus was accompanied by upregulation in neurons of the striatum (Martin et al., 1997b; Danbolt, 2001; Pow et al., 2004; Desilva et al., 2007, 2012). The striatum is known to be selectively vulnerable to excitotoxicity at term, and this may suggest a potential neuronal response to locally increasing extracellular glutamate levels (Martin et al., 1997a). In P6 rats, exposure to hypoxic preconditioning led to upregulation of Glt-1 in the cortex and suppression in the striatum, with no detectable changes in the hippocampus (Cimarosti et al., 2005). Glt-1 was also suppressed in the white matter in a preterm mouse model of chronic hypoxia, although this model was not subjected to ischemia and showed no sign of reactive astrogliosis (Raymond et al., 2011). Moreover, hypoxia has been found to alter the expression of Glt-1 splice variants in mouse brain and neurons of newborn pigs (Munch et al., 2003; Pow et al., 2004).

Exposure of mouse astrocytes, rat microglia, and human blood macrophages to the bacterial endotoxin lipopolysaccharide (LPS) and the pro-inflammatory cytokine TNFα has been found to enhance EAAT2 expression and glutamate uptake function *in vitro* (Rimaniol et al., 2000; Persson et al., 2005; O'Shea et al., 2006). On the other hand, TNFα suppresses both glutamate uptake and EAAT2 in a dose-dependent manner (via NF-κB) in human fetal astrocytes (Fine et al., 1996; Liao and Chen, 2001; Su et al., 2003). TNFα also selectively suppresses EAAT2 *via* NF-κB during hypoxia *in vitro* (Boycott et al., 2008).

An important finding is that EAAT2 is upregulated in the reactive astrocytes and macrophages of post-mortem human brain tissue from preterm babies with white matter injury compared to controls, suggesting a possible response to hypoxia-ischemia and/or inflammation in the preterm brain (Desilva et al., 2008). Pre-oligodendrocytes in both cases and controls expressed EAAT2, with no qualitative differences in expression, although function was not measured. Upregulation of EAAT2 in reactive astrocytes and macrophages in preterm white matter injury may be an adaptive mechanism to counteract excitotoxicity, or it could be a secondary mechanism due to gliosis. Whether in chronic white matter injury, this upregulation contributed to excitotoxicity *via* transport reversal remains to

be established. Further studies are needed to elucidate how perinatal hypoxia-ischemia and infection/inflammation affect EAAT2 homeostasis, separately and in combination. Interestingly, genome-wide gene expression analysis of reactive astrocytes in two adult mouse models of ischemic stroke and LPS-induced neuroinflammation revealed that at least half of the altered gene expression is specific on the insult, with indication that reactive astrocytes may be neuroprotective in ischemia but detrimental in neuroinflammation (Zamanian et al., 2012). Overall, candidacy of EAAT2 is supported by the fact that dysregulation is implicated in several neurological, neurodegenerative, and psychiatric disorders thought to involve glutamate excitotoxicity (i.e., transient cerebral ischemia, ischemic stroke, epilepsy, traumatic brain injury, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, chronic pain, Huntington's disease, HIV-associated cognitive disorder, glioma, major depression, schizophrenia, and addiction) (Danbolt, 2001; Beart and O'Shea, 2007; Fontana, 2015; Karki et al., 2015; Takahashi et al., 2015; Verkhratsky et al., 2016; Zhang et al., 2016; Zhou et al., 2016; Goodwani et al., 2017; O'Donovan et al., 2017; Assefa et al., 2018; Fogarty, 2018; Kim et al., 2018; Parkin et al., 2018).

A better understanding of the role of glutamate transport in preterm brain injuries will require further investigations of EAAT1 in the cerebellum. EAAT1 is highly expressed in cerebellar astrocytes, particularly Bergmann's glia (Lehre et al., 1995; Danbolt, 2001). The processes of these cells ensheath the Purkinje cell synapses, which have been suggested to be selectively vulnerable to excitotoxicity induced by hypoxia-ischemia (Harding et al., 1984; Shibata et al., 1996). Indeed, EAAT1 is developmentally upregulated from 23 weeks gestation, possibly in conjunction with the maturation of the Purkinje cells. Importantly, EAAT1 undergoes rapid changes in hypoxic-ischemic encephalopathy at term, with a decrease in the molecular layer and an increase in the Purkinje and inner granule cell layer at an early stage. This increase becomes marked at a later stage, potentially pointing to an adaptive neuroprotective mechanism against excitotoxicity (Inage et al., 1998).

Mechanisms leading to loss of expression and/or function are likely to be complex. Ying's (1997) "deleterious network hypothesis" (1997) suggests that glutamate build-up may lead to detrimental vicious cycles. For example, receptor overactivation may lead to increased energy consumption and oxidative damage, which is known to impair glutamate transporters' activity and expression, potentially leading to reverse transport with further glutamate release. Ion flux may cause cell swelling, leading to impaired energy metabolism (Danbolt, 2001). Inflammation may further potentiate the risks of excitotoxicity *via* glutamate transport suppression, including selective effects on EAAT2 (Aden et al., 2010; Kapitanovic Vidak et al., 2012). Evidence to date supports the concept of suicide loops in pre-oligodendrocytes, which could provide both the source and the target for excitotoxic injury in the preterm brain. In this context, the combination of developmental upregulation of EAAT2 and establishment of reverse transport in the context of an energy failure could increase vulnerability of pre-oligodendrocytes to excitotoxic death (Back and Rosenberg, 2014). Similarly, transient expression in neuronal populations could feed into suicide loops and explain the loss of layer V pyramidal neurons accompanying necrotic PVL (Andiman et al., 2010). This is a different mechanism to that hypothesized in the mature brain, where the sources of glutamate killing neurons are thought to be other cells, including astrocytes and excitatory terminals (Lipton and Rosenberg, 1994) or, alternatively, retrograde degeneration from axonal injury. Astrocytes may have a delayed response due to their unique ability to use glycogen as a metabolic fuel during the initial stages of energy deprivation. In this scenario, extracellular glutamate concentrations may rise significantly only after depletion of glycogen stores in astrocytes (Grewer et al., 2008), with a subsequent steep rise in extracellular glutamate and excitotoxic cell death (Gouix et al., 2009). In chronic white matter injury, upregulation of astrocytic EAAT2 may be detrimental when accompanied by establishment of reverse transport. Experimental data are needed to evaluate these hypotheses.

POTENTIAL FUTURE DEVELOPMENTS

In summary, it is plausible that both up- and downregulation of EAAT2 contribute to disease, depending on animal model, developmental stage, type and severity of the insult, and comorbidities. Regulation and dysregulation of EAAT2 may occur at the level of transcription (including epigenetic regulation), translation, trafficking, transport, and degradation (Karki et al., 2015; Takahashi et al., 2015). Accordingly, treatments aiming at restoring EAAT2 expression are a current area of research in neuroprotection, alongside enhancement of the transport function (Fontana, 2015). Ceftriaxone, a licensed β-lactam antibiotic safe and tolerable for humans, enhances EAAT2 expression and has been shown to be neuroprotective in animal models of several adult excitotoxic disorders. Although no significant effects have been seen in clinical trials for amyotrophic lateral sclerosis and adult stroke, it is already widely used for the treatment of CNS infections in newborns and would therefore be a feasible drug to explore in the context of preterm neuroprotection. Guanosine enhances EAAT2 transport function and has shown neuroprotective effects in rat models of hypoxic-ischemic encephalopathy (Moretto et al., 2005, 2009) and adult cortical focal ischemia, *via* multiple mechanisms including prevention of free radical attack and pro-inflammatory response (Hansel et al., 2014, 2015). Several other expression and function enhancers of EAAT2 are currently gathering attention as a potential therapeutic approach for a variety of adult disorders and await exploration in the context of the newborn brain (Fontana, 2015). It is currently unknown whether EAAT2 enhancers would restore glutamate uptake or exacerbate reverse transport in the preterm brain. Combination therapies targeting different mechanisms and therapeutic windows will also need exploring, including more established (i.e., magnesium sulfate) and more exploratory therapies (e.g., anti-inflammatory treatment) (Ofek-Shlomai and Berger, 2014).

Genetic risk stratification and pharmacogenomic approaches focusing on interindividual differences in treatment response

are gathering interest and, as our healthcare systems develop, the integration of genomic data in clinical care seems an increasingly achievable goal (Rehm, 2017). Exploratory studies have implicated several functional genetic variants involved in glutamate excitotoxicity and inflammation in neurodevelopmental impairment, including as a sequelae of perinatal brain injuries (O'Callaghan et al., 2009, 2012, 2013; Wu et al., 2011; Kapitanovic Vidak et al., 2012). Among these, common genetic variants altering EAAT2 expression have been reported in association with cerebral palsy and neurodevelopmental delay in very preterm newborns (Rajatileka et al., 2017). Replication in larger samples, genome-wide designs and comparison with term brain injuries are needed to consolidate and expand the finding. Identification of panels of genetic variants that collectively increase risk of injury may be integrated with other types of clinical information and help identify high-risk pregnancies. Moreover, integration of genetic information has the potential to contribute to a more personalized approach to the care of the preterm newborn, with recent studies focusing on the interactions between genetic variants and responsiveness to antenatal magnesium sulfate therapy (Costantine et al., 2012; Clark et al., 2018). EAAT2 variants remain to be evaluated in this context.

Future *in vivo* studies will need to explore whether dysregulation of the main glutamate transporter, EAAT2, is central to the pathogenesis of preterm brain injuries or if it is a secondary process and whether the different cellular effects represent destructive or compensatory mechanisms. As explained by Danbolt (2001), "as long as one variable is not extreme, it will be the combination of several factors that will determine whether the ship will sink," and several different primary events/changes may share a final common pathway. Well-designed animal model studies will be needed to provide mechanistic evidence. Human post-mortem studies can provide insights into patterns of dysregulation of expression, function, and localization specific to the different types of perinatal brain injuries, though limited by confounding factors, post-mortem artifacts, reproducibility, and sample size. Promising preliminary findings on the neuroprotective effects of EAAT2 suggest that this is certainly an avenue worth exploring.

AUTHOR CONTRIBUTIONS

KL and SP contributed to the conception and design of the review. SP wrote the first draft of the manuscript. All authors revised, read, and approved the submitted version of the manuscript.

FUNDING

This study was funded by the UK Medical Research Council: S115971–102, funding a 3.5-year PhD studentship and the UK Medical Research Council: MR/L010305/1, funding lab facilities and consumables.

REFERENCES

Aarnoudse-Moens, C. S., Weisglas-Kuperus, N., van Goudoever, J. B., and Oosterlaan, J. (2009). Meta-analysis of neurobehavioral outcomes in very preterm and/or very low birth weight children. *Pediatrics* 124, 717–728. doi: 10.1542/peds.2008-2816

Abernethy, L. J., Cooke, R. W. I., and Foulder-Hughes, L. (2004). Caudate and hippocampal volumes, intelligence, and motor impairment in 7-year-old children who were born preterm. *Pediatr. Res.* 55, 884–893. doi: 10.1203/01.PDR.0000117843.21534.49

Abraham, H., Tornoczky, T., Kosztolanyi, G., and Seress, L. (2001). Cell formation in the cortical layers of the developing human cerebellum. *Int. J. Dev. Neurosci.* 19, 53–62. doi: 10.1016/S0736-5748(00)00065-4

Acarin, L., Gonzalez, B., Hidalgo, J., Castro, A. J., and Castellano, B. (1999). Primary cortical glial reaction versus secondary thalamic glial response in the excitotoxically injured young brain: astroglial response and metallothionein expression. *Neuroscience* 92, 827–839. doi: 10.1016/S0306-4522(99)00022-6

Aden, U., Favrais, G., Plaisant, F., Winerdal, M., Felderhoff-Mueser, U., Lampa, J., et al. (2010). Systemic inflammation sensitizes the neonatal brain to excitotoxicity through a pro-/anti-inflammatory imbalance: key role of TNFalpha pathway and protection by etanercept. *Brain Behav. Immun.* 24, 747–758. doi: 10.1016/j.bbi.2009.10.010

Alexander, J. M., Gilstrap, L. C., Cox, S. M., McIntire, D. M., and Leveno, K. J. (1998). Clinical chorioamnionitis and the prognosis for very low birth weight infants. *Obstet. Gynecol.* 91, 725–729.

Altman, D. I., Powers, W. J., Perlman, J. M., Herscovitch, P., Volpe, S. L., and Volpe, J. J. (1988). Cerebral blood flow requirement for brain viability in newborn infants is lower than in adults. *Ann. Neurol.* 24, 218–226. doi: 10.1002/ana.410240208

Anblagan, D., Pataky, R., Evans, M. J., Telford, E. J., Serag, A., Sparrow, S., et al. (2016). Association between preterm brain injury and exposure to chorioamnionitis during fetal life. *Sci. Rep.* 6:37932. doi: 10.1038/srep37932

Anderson, P. J., De Luca, C. R., Hutchinson, E., Spencer-Smith, M. M., Roberts, G., and Doyle, L. W. (2011). Attention problems in a representative sample of extremely preterm/extremely low birth weight children. *Dev. Neuropsychol.* 36, 57–73. doi: 10.1080/87565641.2011.540538

Anderson, P. J., and Doyle, L. W. (2008). Cognitive and educational deficits in children born extremely preterm. *Semin. Perinatol.* 32, 51–58. doi: 10.1053/j.semperi.2007.12.009

Anderson, C. M., and Swanson, R. A. (2000). Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* 32, 1–14. doi: 10.1002/1098-1136(200010)32:1<1::AID-GLIA10>3.0.CO;2-W

Anderson, P. J., Treyvaud, K., Neil, J. J., Cheong, J. L. Y., Hunt, R. W., Thompson, D. K., et al. (2017). Associations of newborn brain magnetic resonance imaging with long-term neurodevelopmental impairments in very preterm children. *J. Pediatr.* 187, 58.e1–65.e1. doi: 10.1016/j.jpeds.2017.04.059

Andiman, S. E., Haynes, R. L., Trachtenberg, F. L., Billiards, S. S., Folkerth, R. D., Volpe, J. J., et al. (2010). The cerebral cortex overlying periventricular leukomalacia: analysis of pyramidal neurons. *Brain Pathol.* 20, 803–814. doi: 10.1111/j.1750-3639.2010.00380.x

Arranz, A. M., Hussein, A., Alix, J. J. P., Pérez-Cerdá, F., Allcock, N., Matute, C., et al. (2008). Functional glutamate transport in rodent optic nerve axons and glia. *Glia* 56, 1353–1367. doi: 10.1002/glia.20703

Assefa, B. T., Gebre, A. K., and Altaye, B. M. (2018). Reactive astrocytes as drug target in Alzheimer's disease. *Biomed. Res. Int.* 2018:10. doi: 10.1155/2018/4160247

Azzopardi, D. V., Strohm, B., Edwards, A. D., Dyet, L., Halliday, H. L., Juszczak, E., et al. (2009). Moderate hypothermia to treat perinatal asphyxial encephalopathy. *N. Engl. J. Med.* 361, 1349–1358. doi: 10.1056/NEJMoa0900854

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathol.* 134, 331–349. doi: 10.1007/s00401-017-1718-6

Back, S. A., Craig, A., Kayton, R. J., Luo, N. L., Meshul, C. K., Allcock, N., et al. (2007a). Hypoxia – ischemia preferentially triggers glutamate depletion

from oligodendroglia and axons in perinatal cerebral white matter. *J. Cereb. Blood Flow Metab.* 27, 334–347. doi: 10.1038/sj.jcbfm.9600344

Back, S. A., Gan, X., Li, Y., Rosenberg, P. A., and Volpe, J. J. (1998). Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. *J. Neurosci.* 18, 6241–6253. doi: 10.1523/JNEUROSCI.18-16-06241.1998

Back, S. A., Han, B. H., Luo, N. L., Chricton, C. A., Xanthoudakis, S., Tam, J., et al. (2002). Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. *J. Neurosci.* 22, 455–463. doi: 10.1523/JNEUROSCI.22-02-00455.2002

Back, S. A., Luo, N. L., Borenstein, N. S., Levine, J. M., Volpe, J. J., and Kinney, H. C. (2001). Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J. Neurosci.* 21, 1302–1312. doi: 10.1523/JNEUROSCI.21-04-01302.2001

Back, S. A., Luo, N. L., Mallinson, R. A., O'Malley, J. P., Wallen, L. D., Frei, B., et al. (2005). Selective vulnerability of preterm white matter to oxidative damage defined by F2-isoprostanes. *Ann. Neurol.* 58, 108–120. doi: 10.1002/ana.20530

Back, S. A., Riddle, A., and McClure, M. M. (2007b). Maturation-dependent vulnerability of perinatal white matter in premature birth. *Stroke* 38, 724–730. doi: 10.1161/01.STR.0000254729.27386.05

Back, S. A., and Rosenberg, P. A. (2014). Pathophysiology of glia in perinatal white matter injury. *Glia* 62, 1790–1815. doi: 10.1002/glia.22658

Ball, G., Boardman, J. P., Rueckert, D., Aljabar, P., Arichi, T., Merchant, N., et al. (2012). The effect of preterm birth on thalamic and cortical development. *Cereb. Cortex* 22, 1016–1024. doi: 10.1093/cercor/bhr176

Barnett, M. L., Tusor, N., Ball, G., Chew, A., Falconer, S., Aljabar, P., et al. (2018). Exploring the multiple-hit hypothesis of preterm white matter damage using diffusion MRI. *Neuroimage Clin.* 17, 596–606. doi: 10.1016/j.nicl.2017.11.017

Baud, O., Emilie, D., Pelletier, E., Lacaze-Masmonteil, T., Zupan, V., Fernandez, H., et al. (1999). Amniotic fluid concentrations of interleukin-1beta, interleukin-6 and TNF-alpha in chorioamnionitis before 32 weeks of gestation: histological associations and neonatal outcome. *Br. J. Obstet. Gynaecol.* 106, 72–77.

Baud, O., Greene, A. E., Li, J., Wang, H., Volpe, J. J., and Rosenberg, P. A. (2004). Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *J. Neurosci.* 24, 1531–1540. doi: 10.1523/JNEUROSCI.3989-03.2004

Bax, M., Goldstein, M., Rosenbaum, P., Leviton, A., Paneth, N., Dan, B., et al. (2005). Proposed definition and classification of cerebral palsy, April 2005. *Dev. Med. Child Neurol.* 47, 571–576. doi: 10.1017/S001216220500112X

Bax, M., Tydeman, C., and Flodmark, O. (2006). Clinical and mri correlates of cerebral palsy: the european cerebral palsy study. *JAMA* 296, 1602–1608. doi: 10.1001/jama.296.13.1602

Beaino, G., Khoshnood, B., Kaminski, M., Pierrat, V., Marret, S., Matis, J., et al. (2010). Predictors of cerebral palsy in very preterm infants: the EPIPAGE prospective population-based cohort study. *Dev. Med. Child Neurol.* 52, e119–e125. doi: 10.1111/j.1469-8749.2010.03612.x

Beart, P. M., and O'Shea, R. D. (2007). Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br. J. Pharmacol.* 150, 5–17. doi: 10.1038/sj.bjp.0706949

Benediktsson, A. M., Marrs, G. S., Tu, J. C., Worley, P. F., Rothstein, J. D., Bergles, D. E., et al. (2012). Neuronal activity regulates glutamate transporter dynamics in developing astrocytes. *Glia* 60, 175–188. doi: 10.1002/glia.21249

Benveniste, H., Drejer, J., Schousboe, A., and Diemer, N. H. (1984). Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* 43, 1369–1374. doi: 10.1111/j.1471-4159.1984.tb05396.x

Bergles, D. E., Tzingounis, A. V., and Jahr, C. E. (2002). Comparison of coupled and uncoupled currents during glutamate uptake by GLT-1 transporters. *J. Neurosci.* 22, 10153–10162. doi: 10.1523/JNEUROSCI.22-23-10153.2002

Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Rizzini, B. L., et al. (1998). Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* 391, 281–285. doi: 10.1038/34651

Bi, D., Chen, M., Zhang, X., Wang, H., Xia, L., Shang, Q., et al. (2014). The association between sex-related interleukin-6 gene polymorphisms and the risk for cerebral palsy. *J. Neuroinflammation* 11:100. doi: 10.1186/1742-2094-11-100

Biran, V., Verney, C., and Ferriero, D. M. (2012). Perinatal cerebellar injury in human and animal models. *Neurol. Res. Int.* 2012:858929. doi: 10.1155/2012/858929

Bliss, T. V., and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361, 31–39. doi: 10.1038/361031a0

Bodensteiner, J. B., and Johnsen, S. D. (2005). Cerebellar injury in the extremely premature infant: newly recognized but relatively common outcome. *J. Child Neurol.* 20, 139–142. doi: 10.1177/08830738050200021101

Boycott, H. E., Wilkinson, J. A., Boyle, J. P., Pearson, H. A., and Peers, C. (2008). Differential involvement of TNF alpha in hypoxic suppression of astrocyte glutamate transporters. *Glia* 56, 998–1004. doi: 10.1002/glia.20673

Bristol, L. A., and Rothstein, J. D. (1996). Glutamate transporter gene expression in amyotrophic lateral sclerosis motor cortex. *Ann. Neurol.* 39, 676–679. doi: 10.1002/ana.410390519

Bruce, A. J., Boling, W., Kindy, M. S., Peschon, J., Kraemer, P. J., Carpenter, M. K., et al. (1996). Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nat. Med.* 2, 788–794. doi: 10.1038/nm0796-788

Buser, J. R., Segovia, K. N., Dean, J. M., Nelson, K., Beardsley, D., Gong, X., et al. (2010). Timing of appearance of late oligodendrocyte progenitors coincides with enhanced susceptibility of preterm rabbit cerebral white matter to hypoxia-ischemia. *J. Cereb. Blood Flow Metab.* 30, 1053–1065. doi: 10.1038/jcbfm.2009.286

Carmignoto, G. (2000). Reciprocal communication systems between astrocytes and neurones. *Prog. Neurobiol.* 62, 561–581. doi: 10.1016/S0301-0082(00)00029-0

Castillo-Melendez, M., Chow, J. A., and Walker, D. W. (2004). Lipid peroxidation, caspase-3 immunoreactivity, and pyknosis in late-gestation fetal sheep brain after umbilical cord occlusion. *Pediatr. Res.* 55, 864–871. doi: 10.1203/01.PDR.0000115679.86566.C4

Chahboune, H., Ment, L. R., Stewart, W. B., Rothman, D. L., Vaccarino, F. M., Hyder, F., et al. (2009). Hypoxic injury during neonatal development in murine brain: correlation between in vivo DTI findings and behavioral assessment. *Cereb. Cortex* 19, 2891–2901. doi: 10.1093/cercor/bhp068

Chao, X. D., Fei, F., and Fei, Z. (2010). The role of excitatory amino acid transporters in cerebral ischemia. *Neurochem. Res.* 35, 1224–1230. doi: 10.1007/s11064-010-0178-3

Chen, W., Mahadomrongkul, V., Berger, U. V., Bassan, M., DeSilva, T., Tanaka, K., et al. (2004). The glutamate transporter GLT1a is expressed in excitatory axon terminals of mature hippocampal neurons. *J. Neurosci.* 24, 1136–1148. doi: 10.1523/JNEUROSCI.1586-03.2004

Cheung, N. S., Pascoe, C. J., Giardina, S. F., John, C. A., and Beart, P. M. (1998). Micromolar L-glutamate induces extensive apoptosis in an apoptotic-necrotic continuum of insult-dependent, excitotoxic injury in cultured cortical neurones. *Neuropharmacology* 37, 1419–1429. doi: 10.1016/S0028-3908(98)00123-3

Choi, D. W. (1992). Excitotoxic cell death. *J. Neurobiol.* 23, 1261–1276. doi: 10.1002/neu.480230915

Cimarosti, H., Jones, N. M., O'Shea, R. D., Pow, D. V., Salbego, C., and Beart, P. M. (2005). Hypoxic preconditioning in neonatal rat brain involves regulation of excitatory amino acid transporter 2 and estrogen receptor alpha. *Neurosci. Lett.* 385, 52–57. doi: 10.1016/j.neulet.2005.05.006

Clark, E. A. S., Weiner, S. J., Rouse, D. J., Mercer, B. M., Reddy, U. M., Iams, J. D., et al. (2018). Genetic variation, magnesium sulfate exposure, and adverse neurodevelopmental outcomes following preterm birth. *Am. J. Perinatol.* 35, 1012–1022. doi: 10.1055/s-0038-1635109

Constantinou, J. C., Adamson-Macedo, E. N., Mirmiran, M., and Fleisher, B. E. (2007). Movement, imaging and neurobehavioral assessment as predictors of cerebral palsy in preterm infants. *J. Perinatol.* 27, 225–229. doi: 10.1038/sj.jp.7211664

Costantine, M. M., Clark, E. A., Lai, Y., Rouse, D. J., Spong, C. Y., Mercer, B. M., et al. (2012). Association of polymorphisms in neuroprotection and oxidative stress genes and neurodevelopmental outcomes after preterm birth. *Obstet. Gynecol.* 120, 542–550. doi: 10.1097/AOG.0b013e318265f232

Counsell, S. J., Allsop, J. M., Harrison, M. C., Larkman, D. J., Kennea, N. L., Kapellou, O., et al. (2003). Diffusion-weighted imaging of the brain in preterm infants with focal and diffuse white matter abnormality. *Pediatrics* 112, 1–7. doi: 10.1542/peds.112.1.1

Counsell, S. J., and Boardman, J. P. (2005). Differential brain growth in the infant born preterm: current knowledge and future developments from brain imaging. *Semin. Fetal Neonatal Med.* 10, 403–410. doi: 10.1016/j.siny.2005.05.003

Craig, A., Ling Luo, N., Beardsley, D. J., Wingate-Pearse, N., Walker, D. W., Hohimer, A. R., et al. (2003). Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Exp. Neurol.* 181, 231–240. doi: 10.1016/S0014-4886(03)00032-3

Curtis, D. R., and Johnston, G. A. (1974). Amino acid transmitters in the mammalian central nervous system. *Ergeb. Physiol.* 69, 97–188.

Dallas, M., Boycott, H. E., Atkinson, L., Miller, A., Boyle, J. P., Pearson, H. A., et al. (2007). Hypoxia suppresses glutamate transport in astrocytes. *J. Neurosci.* 27, 3946–3955. doi: 10.1523/JNEUROSCI.5030-06.2007

Dammann, O. (2007). Persistent neuro-inflammation in cerebral palsy: a therapeutic window of opportunity? *Acta Paediatr.* 96, 6–7. doi: 10.1111/j.1651-2227.2007.00097.x

Dammann, O., Kuban, K. C., and Leviton, A. (2002). Perinatal infection, fetal inflammatory response, white matter damage, and cognitive limitations in children born preterm. *Ment. Retard. Dev. Disabil. Res. Rev.* 8, 46–50. doi: 10.1002/mrdd.10005

Dammann, O., and Leviton, A. (1998). Infection remote from the brain, neonatal white matter damage, and cerebral palsy in the preterm infant. *Semin. Pediatr. Neurol.* 5, 190–201. doi: 10.1016/S1071-9091(98)80034-X

Dammann, O., and Leviton, A. (2000). Role of the fetus in perinatal infection and neonatal brain damage. *Curr. Opin. Pediatr.* 12, 99–104. doi: 10.1097/00008480-200004000-00002

Dammann, O., and Leviton, A. (2004). Inflammatory brain damage in preterm newborns – dry numbers, wet lab, and causal inferences. *Early Hum. Dev.* 79, 1–15. doi: 10.1016/j.earlhumdev.2004.04.009

Danbolt, N. C. (2001). Glutamate uptake. *Prog. Neurobiol.* 65, 1–105. doi: 10.1016/S0301-0082(00)00067-8

Danbolt, N. C., Furness, D. N., and Zhou, Y. (2016). Neuronal vs glial glutamate uptake: resolving the conundrum. *Neurochem. Int.* 98, 29–45. doi: 10.1016/j.neuint.2016.05.009

Dang, Y. X., Shi, K. N., and Wang, X. M. (2017). Early changes in glutamate metabolism and perfusion in basal ganglia following hypoxia-ischemia in neonatal piglets: a multi-sequence 3.0T MR study. *Front. Physiol.* 8:237. doi: 10.3389/fphys.2017.00237

De Reuck, J. L. (1984). Cerebral angioarchitecture and perinatal brain lesions in premature and full-term infants. *Acta Neurol. Scand.* 70, 391–395. doi: 10.1111/j.1600-0404.1984.tb00843.x

de Vivo, L., Melone, M., Rothstein, J. D., and Conti, F. (2010). GLT-1 promoter activity in astrocytes and neurons of mouse hippocampus and somatic sensory cortex. *Front. Neuroanat.* 3:31. doi: 10.3389/neuro.05.031.2009

de Vries, L. S., Eken, P., and Dubowitz, L. M. S. (1992). The spectrum of leukomalacia using cranial ultrasound. *Behav. Brain Res.* 49, 1–6. doi: 10.1016/S0166-4328(05)80189-5

De Vries, L. S., Van Haastert, I. L., Rademaker, K. J., Koopman, C., and Groenendaal, F. (2004). Ultrasound abnormalities preceding cerebral palsy in high-risk preterm infants. *J. Pediatr.* 144, 815–820. doi: 10.1016/j.jpeds.2004.03.034

Dean, J. M., McClendon, E., Hansen, K., Azimi-Zonooz, A., Chen, K., Riddle, A., et al. (2013). Prenatal cerebral ischemia disrupts MRI-defined cortical microstructure through disturbances in neuronal arborization. *Sci. Transl. Med.* 5:168ra7. doi: 10.1126/scitranslmed.3004669

Dean, J. M., Moravec, M. D., Grafe, M., Abend, N., Ren, J., Gong, X., et al. (2011). Strain-specific differences in perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Dev. Neurosci.* 33, 251–260. doi: 10.1159/000327242

Delobel-Ayoub, M., Arnaud, C., White-Koning, M., Casper, C., Pierrat, V., Garel, M., et al. (2009). Behavioral problems and cognitive performance at 5 years of age after very preterm birth: the EPIPAGE study. *Pediatrics* 123, 1485–1492. doi: 10.1542/peds.2008-1216

Deng, W. (2010). Neurobiology of injury to the developing brain. *Nat. Rev. Neurol.* 6, 328–336. doi: 10.1038/nrneurol.2010.53

Deng, W., Rosenberg, P. A., Volpe, J. J., and Jensen, F. E. (2003). Calcium-permeable AMPA/kainate receptors mediate toxicity and preconditioning by oxygen-glucose deprivation in oligodendrocyte precursors. *Proc. Natl. Acad. Sci. USA* 100, 6801–6806. doi: 10.1073/pnas.1136624100

Descloux, C., Ginet, V., Rummel, C., Truttmann, A. C., and Puyal, J. (2018). Enhanced autophagy contributes to excitotoxic lesions in a rat model of preterm brain injury. *Cell Death Dis.* 9:853. doi: 10.1038/s41419-018-0916-z

Desilva, T. M., Billiards, S. S., Borenstein, N. S., Trachtenberg, F. L., Volpe, J. J., Kinney, H. C., et al. (2008). Glutamate transporter EAAT2 expression is up-regulated in reactive astrocytes in human periventricular leukomalacia. *J. Comp. Neurol.* 508, 238–248. doi: 10.1002/cne.21667

DeSilva, T. M., Borenstein, N. S., Volpe, J. J., Kinney, H. C., and Rosenberg, P. A. (2012). Expression of EAAT2 in neurons and protoplasmic astrocytes during human cortical development. *J. Comp. Neurol.* 520, 3912–3932. doi: 10.1002/cne.23130

DeSilva, T. M., Kabakov, A. Y., Goldhoff, P. E., Volpe, J. J., and Rosenberg, P. A. (2009). Regulation of glutamate transport in developing rat oligodendrocytes. *J. Neurosci.* 29, 7898–7908. doi: 10.1523/JNEUROSCI.6129-08.2009

Desilva, T. M., Kinney, H. C., Borenstein, N. S., Trachtenberg, F. L., Irwin, N., Volpe, J. J., et al. (2007). The glutamate transporter EAAT2 is transiently expressed in developing human cerebral white matter. *J. Comp. Neurol.* 501, 879–890. doi: 10.1002/cne.21289

Dienel, G. A., and Hertz, L. (2005). Astrocytic contributions to bioenergetics of cerebral ischemia. *Glia* 50, 362–388. doi: 10.1002/glia.20157

Doble, A. (1999). The role of excitotoxicity in neurodegenerative disease: implications for therapy. *Pharmacol. Ther.* 81, 163–221. doi: 10.1016/S0163-7258(98)00042-4

Dollner, H., Vatten, L., Halgunset, J., Rahimipoor, S., and Austgulen, R. (2002). Histologic chorioamnionitis and umbilical serum levels of pro-inflammatory cytokines and cytokine inhibitors. *BJOG* 109, 534–539. doi: 10.1111/j.1471-0528.2002.01028.x

Domercq, M., Etxebarria, E., Perez-Samartin, A., and Matute, C. (2005). Excitotoxic oligodendrocyte death and axonal damage induced by glutamate transporter inhibition. *Glia* 52, 36–46. doi: 10.1002/glia.20221

Domercq, M., Sánchez-Gómez, M. V., Areso, P., and Matute, C. (1999). Expression of glutamate transporters in rat optic nerve oligodendrocytes. *Eur. J. Neurosci.* 11, 2226–2236. doi: 10.1046/j.1460-9568.1999.00639.x

Domingues, A. M., Taylor, M., and Fern, R. (2010). Glia as transmitter sources and sensors in health and disease. *Neurochem. Int.* 57, 359–366. doi: 10.1016/j.neuint.2010.03.024

Dommergues, M. A., Patkai, J., Renauld, J. C., Evrard, P., and Gressens, P. (2000). Proinflammatory cytokines and interleukin-9 exacerbate excitotoxic lesions of the newborn murine neopallium. *Ann. Neurol.* 47, 54–63. doi: 10.1002/1531-8249(200001)47:1<54::AID-ANA10>3.0.CO;2-Y

Doyle, L. W., Crowther, C. A., Middleton, P., Marret, S., and Rouse, D. (2009). Magnesium sulphate for women at risk of preterm birth for neuroprotection of the fetus. *Cochrane Database Syst. Rev.* CD004661. doi: 10.1002/14651858.CD004661.pub3

Duerden, E. G., Taylor, M. J., and Miller, S. P. (2013). Brain development in infants born preterm: looking beyond injury. *Semin. Pediatr. Neurol.* 20, 65–74. doi: 10.1016/j.spen.2013.06.007

Duggan, P. J., Maalouf, E. F., Watts, T. L., Sullivan, M. H., Counsell, S. J., Allsop, J., et al. (2001). Intrauterine T-cell activation and increased proinflammatory cytokine concentrations in preterm infants with cerebral lesions. *Lancet* 358, 1699–1700. doi: 10.1016/S0140-6736(01)06723-X

Eklind, S., Mallard, C., Leverin, A. L., Gilland, E., Blomgren, K., Mattsby-Baltzer, I., et al. (2001). Bacterial endotoxin sensitizes the immature brain to hypoxic--ischaemic injury. *Eur. J. Neurosci.* 13, 1101–1106. doi: 10.1046/j.0953-816x.2001.01474.x

Ellison, V. J., Mocatta, T. J., Winterbourn, C. C., Darlow, B. A., Volpe, J. J., and Inder, T. E. (2005). The relationship of CSF and plasma cytokine levels to cerebral white matter injury in the premature newborn. *Pediatr. Res.* 57, 282–286. doi: 10.1203/01.PDR.0000148286.53572.95

Favrais, G., Schwendimann, L., Gressens, P., and Lelievre, V. (2007). Cyclooxygenase-2 mediates the sensitizing effects of systemic IL-1-beta on excitotoxic brain lesions in newborn mice. *Neurobiol. Dis.* 25, 496–505. doi: 10.1016/j.nbd.2006.10.012

Fern, R., and Moller, T. (2000). Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop. *J. Neurosci.* 20, 34–42. doi: 10.1523/JNEUROSCI.20-01-00034.2000

Fetters, L., and Huang, H. H. (2007). Motor development and sleep, play, and feeding positions in very-low-birthweight infants with and without white matter disease. *Dev. Med. Child Neurol.* 49, 807–813. doi: 10.1111/j.1469-8749.2007.00807.x

Fine, S. M., Angel, R. A., Perry, S. W., Epstein, L. G., Rothstein, J. D., Dewhurst, S., et al. (1996). Tumor necrosis factor alpha inhibits glutamate uptake by primary human astrocytes. Implications for pathogenesis of HIV-1 dementia. *J. Biol. Chem.* 271, 15303–15306. doi: 10.1074/jbc.271.26.15303

Fleiss, B., and Gressens, P. (2012). Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? *Lancet Neurol.* 11, 556–566. doi: 10.1016/S1474-4422(12)70058-3

Fogarty, M. J. (2018). Driven to decay: excitability and synaptic abnormalities in amyotrophic lateral sclerosis. *Brain Res. Bull.* 140, 318–333. doi: 10.1016/j.brainresbull.2018.05.023

Follett, P. L., Deng, W., Dai, W., Talos, D. M., Massillon, L. J., Rosenberg, P. A., et al. (2004). Glutamate receptor-mediated oligodendrocyte toxicity in periventricular leukomalacia: a protective role for topiramate. *J. Neurosci.* 24, 4412–4420. doi: 10.1523/JNEUROSCI.0477-04.2004

Follett, P. L., Rosenberg, P. A., Volpe, J. J., and Jensen, F. E. (2000). NBQX attenuates excitotoxic injury in developing white matter. *J. Neurosci.* 20, 9235–9241. doi: 10.1523/JNEUROSCI.20-24-09235.2000

Fontana, A. C. (2015). Current approaches to enhance glutamate transporter function and expression. *J. Neurochem.* 134, 982–1007. doi: 10.1111/jnc.13200

Fragoso, G., Martinez-Bermudez, A. K., Liu, H. N., Khorchid, A., Chemtob, S., Mushynski, W. E., et al. (2004). Developmental differences in HO-induced oligodendrocyte cell death: role of glutathione, mitogen-activated protein kinases and caspase 3. *J. Neurochem.* 90, 392–404. doi: 10.1111/j.1471-4159.2004.02488.x

Frizzo, J. K., Cardoso, M. P., de Assis, A. M., Perry, M. L., Volonte, C., and Frizzo, M. E. (2010). Effects of acute perinatal asphyxia in the rat hippocampus. *Cell. Mol. Neurobiol.* 30, 683–692. doi: 10.1007/s10571-009-9492-1

Fukamachi, S., Furuta, A., Ikeda, T., Ikenoue, T., Kaneoka, T., Rothstein, J. D., et al. (2001). Altered expressions of glutamate transporter subtypes in rat model of neonatal cerebral hypoxia-ischemia. *Brain Res. Dev. Brain Res.* 132, 131–139. doi: 10.1016/S0165-3806(01)00303-0

Furness, D. N., Dehnes, Y., Akhtar, A. Q., Rossi, D. J., Hamann, M., Grutle, N. J., et al. (2008). A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). *Neuroscience* 157, 80–94. doi: 10.1016/j.neuroscience.2008.08.043

Furuta, A., Rothstein, J. D., and Martin, L. J. (1997). Glutamate transporter protein subtypes are expressed differentially during rat CNS development. *J. Neurosci.* 17, 8363–8375. doi: 10.1523/JNEUROSCI.17-21-08363.1997

Gilles, F., Gressens, P., Dammann, O., and Leviton, A. (2018). Hypoxia–ischemia is not an antecedent of most preterm brain damage: the illusion of validity. *Dev. Med. Child Neurol.* 60, 120–125. doi: 10.1111/dmcn.13483

Gimenez, M., Junque, C., Narberhaus, A., Botet, F., Bargallo, N., and Mercader, J. M. (2006). Correlations of thalamic reductions with verbal fluency impairment in those born prematurely. *Neuroreport* 17, 463–466. doi: 10.1097/01.wnr.0000209008.93846.24

Glass, H. C., Costarino, A. T., Stayer, S. A., Brett, C., Cladis, F., and Davis, P. J. (2015). Outcomes for extremely premature infants. *Anesth. Analg.* 120, 1337–1351. doi: 10.1213/ANE.0000000000000705

Glass, H. C., Fujimoto, S., Ceppi-Cozzio, C., Bartha, A. I., Vigneron, D. B., Barkovich, A. J., et al. (2008). White-matter injury is associated with impaired gaze in premature infants. *Pediatr. Neurol.* 38, 10–15. doi: 10.1016/j.pediatrneurol.2007.08.019

Gleason, C. A., Hamm, C., and Jones, M. D. Jr. (1989). Cerebral blood flow, oxygenation, and carbohydrate metabolism in immature fetal sheep in utero. *Am. J. Phys.* 256, R1264–R1268. doi: 10.1152/ajpregu.1989.256.6.R1264

Gluckman, P. D., Wyatt, J. S., Azzopardi, D., Ballard, R., Edwards, A. D., Ferriero, D. M., et al. (2005). Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet* 365, 663–670. doi: 10.1016/S0140-6736(05)70932-6

Goodwani, S., Saternos, H., Alasmari, F., and Sari, Y. (2017). Metabotropic and ionotropic glutamate receptors as potential targets for the treatment of alcohol use disorder. *Neurosci. Biobehav. Rev.* 77, 14–31. doi: 10.1016/j.neubiorev.2017.02.024

Gopagondanahalli, K. R., Li, J., Fahey, M. C., Hunt, R. W., Jenkin, G., Miller, S. L., et al. (2016). Preterm hypoxic-ischemic encephalopathy. *Front. Pediatr.* 4:114. doi: 10.3389/fped.2016.00114

Gouix, E., Leveille, F., Nicole, O., Melon, C., Had-Aissouni, L., and Buisson, A. (2009). Reverse glial glutamate uptake triggers neuronal cell death through extrasynaptic NMDA receptor activation. *Mol. Cell. Neurosci.* 40, 463–473. doi: 10.1016/j.mcn.2009.01.002

Greisen, G. (1986). Cerebral blood flow in preterm infants during the first week of life. *Acta Paediatr. Scand.* 75, 43–51.

Gressens, P., Marret, S., and Evrard, P. (1996). Developmental spectrum of the excitotoxic cascade induced by ibotenate: a model of hypoxic insults in fetuses and neonates. *Neuropathol. Appl. Neurobiol.* 22, 498–502. doi: 10.1111/j.1365-2990.1996.tb01123.x

Grether, J. K., and Nelson, K. B. (1997). Maternal infection and cerebral palsy in infants of normal birth weight. *JAMA* 278, 207–211. doi: 10.1001/jama.1997.03550030047032

Grewer, C., Gameiro, A., Zhang, Z., Tao, Z., Braams, S., and Rauen, T. (2008). Glutamate forward and reverse transport: from molecular mechanism to transporter-mediated release after ischemia. *IUBMB Life* 60, 609–619. doi: 10.1002/iub.98

Grewer, C., and Rauen, T. (2005). Electrogenic glutamate transporters in the CNS: molecular mechanism, pre-steady-state kinetics, and their impact on synaptic signaling. *J. Membr. Biol.* 203, 1–20. doi: 10.1007/s00232-004-0731-6

Hagberg, H., Gilland, E., Diemer, N. H., and Andine, P. (1994). Hypoxia-ischemia in the neonatal rat brain: histopathology after post-treatment with NMDA and non-NMDA receptor antagonists. *Biol. Neonate* 66, 205–213.

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Hagberg, H., Peebles, D., and Mallard, C. (2002). Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults. *Ment. Retard. Dev. Disabil. Res. Rev.* 8, 30–38. doi: 10.1002/mrdd.10007

Hagberg, H., Thornberg, E., Blennow, M., Kjellmer, I., Lagercrantz, H., Thiringer, K., et al. (1993). Excitatory amino acids in the cerebrospinal fluid of asphyxiated infants: relationship to hypoxic-ischemic encephalopathy. *Acta Paediatr.* 82, 925–929. doi: 10.1111/j.1651-2227.1993.tb12601.x

Hansel, G., Ramos, D. B., Delgado, C. A., Souza, D. G., Almeida, R. F., Portela, L. V., et al. (2014). The potential therapeutic effect of guanosine after cortical focal ischemia in rats. *PLoS One* 9:e90693. doi: 10.1371/journal.pone.0090693

Hansel, G., Tonon, A. C., Guella, F. L., Pettenuzzo, L. F., Duarte, T., Duarte, M. M., et al. (2015). Guanosine protects against cortical focal ischemia. Involvement of inflammatory response. *Mol. Neurobiol.* 52, 1791–1803. doi: 10.1007/s12035-014-8978-0

Harding, R., Rawson, J. A., Griffiths, P. A., and Thorburn, G. D. (1984). The influence of acute hypoxia and sleep states on the electrical activity of the cerebellum in the sheep fetus. *Electroencephalogr. Clin. Neurophysiol.* 57, 166–173. doi: 10.1016/0013-4694(84)90175-5

Haugeto, O., Ullensvang, K., Levy, L. M., Chaudhry, F. A., Honore, T., Nielsen, M., et al. (1996). Brain glutamate transporter proteins form homomultimers. *J. Biol. Chem.* 271, 27715–27722. doi: 10.1074/jbc.271.44.27715

Heep, A., Behrendt, D., Nitsch, P., Fimmers, R., Bartmann, P., and Dembinski, J. (2003). Increased serum levels of interleukin 6 are associated with severe intraventricular haemorrhage in extremely premature infants. *Arch. Dis. Child. Fetal Neonatal Ed.* 88, F501–F504. doi: 10.1136/fn.88.6.F501

Himpens, E., Van den Broeck, C., Oostra, A., Calders, P., and Vanhaesebrouck, P. (2008). Prevalence, type, distribution, and severity of cerebral palsy in relation to gestational age: a meta-analytic review. *Dev. Med. Child Neurol.* 50, 334–340. doi: 10.1111/j.1469-8749.2008.02047.x

Hirvonen, M., Ojala, R., Korhonen, P., Haataja, P., Eriksson, K., Gissler, M., et al. (2014). Cerebral palsy among children born moderately and late preterm. *Pediatrics* 134, e1584–e1593. doi: 10.1542/peds.2014-0945

Huang, J., Zhang, L., Kang, B., Zhu, T., Li, Y., Zhao, F., et al. (2017). Association between perinatal hypoxic-ischemia and periventricular leukomalacia in preterm infants: a systematic review and meta-analysis. *PLoS One* 12:e0184993. doi: 10.1371/journal.pone.0189461

Hutton, L. C., Yan, E., Yawno, T., Castillo-Melendez, M., Hirst, J. J., and Walker, D. W. (2014). Injury of the developing cerebellum: a brief review of the effects of endotoxin and asphyxial challenges in the late gestation sheep fetus. *Cerebellum* 13, 777–786. doi: 10.1007/s12311-014-0602-3

Iida, K., Takashima, S., and Ueda, K. (1995). Immunohistochemical study of myelination and oligodendrocyte in infants with periventricular leukomalacia. *Pediatr. Neurol.* 13, 296–304. doi: 10.1016/0887-8994(95)00192-1

Ikeda, T., Mishima, K., Aoo, N., Egashira, N., Iwasaki, K., Fujiwara, M., et al. (2004). Combination treatment of neonatal rats with hypoxia-ischemia and

endotoxin induces long-lasting memory and learning impairment that is associated with extended cerebral damage. *Am. J. Obstet. Gynecol.* 191, 2132–2141. doi: 10.1016/j.ajog.2004.04.039

Inage, Y. W., Itoh, M., and Takashima, S. (2000). Correlation between cerebrovascular maturity and periventricular leukomalacia. *Pediatr. Neurol.* 22, 204–208. doi: 10.1016/S0887-8994(99)00153-8

Inage, Y. W., Itoh, M., Wada, K., and Takashima, S. (1998). Expression of two glutamate transporters, GLAST and EAAT4, in the human cerebellum: their correlation in development and neonatal hypoxic-ischemic damage. *J. Neuropathol. Exp. Neurol.* 57, 554–562. doi: 10.1097/00005072-199806000-00003

Inder, T. E., Anderson, N. J., Spencer, C., Wells, S., and Volpe, J. J. (2003). White matter injury in the premature infant: a comparison between serial cranial sonographic and MR findings at term. *AJNR Am. J. Neuroradiol.* 24, 805–809.

Inder, T. E., Warfield, S. K., Wang, H., Huppi, P. S., and Volpe, J. J. (2005). Abnormal cerebral structure is present at term in premature infants. *Pediatrics* 115, 286–294. doi: 10.1542/peds.2004-0326

Innocenti, G. M., and Berbel, P. (1991a). Analysis of an experimental cortical network: I. Architectonics of visual areas 17 and 18 after neonatal injections of ibotenic acid; similarities with human microgyria. *J. Neural Transplant. Plast.* 2, 1–28.

Innocenti, G. M., and Berbel, P. (1991b). Analysis of an experimental cortical network: II. Connections of visual areas 17 and 18 after neonatal injections of ibotenic acid. *J. Neural Transplant. Plast.* 2, 29–54.

Jabaudon, D., Shimamoto, K., Yasuda-Kamatani, Y., Scanziani, M., Gahwiler, B. H., and Gerber, U. (1999). Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. *Proc. Natl. Acad. Sci. USA* 96, 8733–8738.

Jacobs, S. E., Morley, C. J., Inder, T. E., Stewart, M. J., Smith, K. R., McNamara, P. J., et al. (2011). Whole-body hypothermia for term and near-term newborns with hypoxic-ischemic encephalopathy: a randomized controlled trial. *Arch. Pediatr. Adolesc. Med.* 165, 692–700. doi: 10.1001/archpediatrics.2011.43

Jantzie, L. L., Cheung, P. Y., Johnson, S. T., Bigam, D. L., and Todd, K. G. (2010). Cerebral amino acid profiles after hypoxia-reoxygenation and N-acetylcysteine treatment in the newborn piglet. *Neonatology* 97, 195–203. doi: 10.1159/000252972

Jantzie, L. L., Talos, D. M., Jackson, M. C., Park, H. K., Graham, D. A., Lechpammer, M., et al. (2015). Developmental expression of N-methyl-D-aspartate (NMDA) receptor subunits in human white and gray matter: potential mechanism of increased vulnerability in the immature brain. *Cereb. Cortex* 25, 482–495. doi: 10.1093/cercor/bht246

Johnsen, S. D., Bodensteiner, J. B., and Lotze, T. E. (2005). Frequency and nature of cerebellar injury in the extremely premature survivor with cerebral palsy. *J. Child Neurol.* 20, 60–64. doi: 10.1177/08830738050200011001

Johnston, M. V. (2005). Excitotoxicity in perinatal brain injury. *Brain Pathol.* 15, 234–240. doi: 10.1111/j.1750-3639.2005.tb00526.x

Kaindl, A. M., Favrais, G., and Gressens, P. (2009). Molecular mechanisms involved in injury to the preterm brain. *J. Child Neurol.* 24, 1112–1118. doi: 10.1177/0883073809337920

Kapitanovic Vidak, H., Catela Ivkovic, T., Jokic, M., Spaventi, R., and Kapitanovic, S. (2012). The association between proinflammatory cytokine polymorphisms and cerebral palsy in very preterm infants. *Cytokine* 58, 57–64. doi: 10.1016/j.cyto.2011.12.018

Karki, P., Smith, K., Johnson, J., Aschner, M., and Lee, E. Y. (2015). Genetic dys-regulation of astrocytic glutamate transporter EAAT2 and its implications in neurological disorders and manganese toxicity. *Neurochem. Res.* 40, 380–388. doi: 10.1007/s11064-014-1391-2

Kaukola, T., Herva, R., Perhomaa, M., Paakko, E., Kingsmore, S., Vainionpaa, L., et al. (2006). Population cohort associating chorioamnionitis, cord inflammatory cytokines and neurologic outcome in very preterm, extremely low birth weight infants. *Pediatr. Res.* 59, 478–483. doi: 10.1203/01.pdr.0000182596.66175.ee

Kaukola, T., Satyaraj, E., Patel, D. D., Tchernev, V. T., Grimwade, B. G., Kingsmore, S. F., et al. (2004). Cerebral palsy is characterized by protein mediators in cord serum. *Ann. Neurol.* 55, 186–194. doi: 10.1002/ana.10809

Kendall, G. S., Hristova, M., Horn, S., Dafou, D., Acosta-Saltos, A., Almolda, B., et al. (2011). TNF gene cluster deletion abolishes lipopolysaccharide-mediated sensitization of the neonatal brain to hypoxic ischemic insult. *Lab. Investig.* 91, 328–341. doi: 10.1038/labinvest.2010.192

Kesler, S. R., Reiss, A. L., Vohr, B., Watson, C., Schneider, K. C., Katz, K. H., et al. (2008). Brain volume reductions within multiple cognitive systems in male preterm children at age twelve. *J. Pediatr.* 152, 513–520, 520.e1. doi: 10.1016/j.jpeds.2007.08.009

Khandaker, G., Smithers-Sheedy, H., Islam, J., Alam, M., Jung, J., Novak, I., et al. (2015). Bangladesh cerebral palsy register (BCPR): a pilot study to develop a national cerebral palsy (CP) register with surveillance of children for CP. *BMC Neurol.* 15:173. doi: 10.1186/s12883-015-0427-9

Kim, R., Healey, K. L., Sepulveda-Orengo, M. T., and Reissner, K. J. (2018). Astroglial correlates of neuropsychiatric disease: from astrocytopathy to astrogliosis. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 87, 126–146. doi: 10.1016/j.pnpbp.2017.10.002

Kim, K., Lee, S.-G., Kegelman, T. P., Su, Z.-Z., Das, S. K., Dash, R., et al. (2011). Role of excitatory amino acid transporter-2 (EAAT2) and glutamate in neurodegeneration: opportunities for developing novel therapeutics. *J. Cell. Physiol.* 226, 2484–2493. doi: 10.1002/jcp.22609

Kiryk, A., Aida, T., Tanaka, K., Banerjee, P., Wilczynski, G. M., Meyza, K., et al. (2008). Behavioral characterization of GLT1 (+/−) mice as a model of mild glutamatergic hyperfunction. *Neurotox. Res.* 13, 19–30. doi: 10.1007/BF03033364

Korzeniewski, S. J., Romero, R., Cortez, J., Pappas, A., Schwartz, A. G., Kim, C. J., et al. (2014). A "multi-hit" model of neonatal white matter injury: cumulative contributions of chronic placental inflammation, acute fetal inflammation and postnatal inflammatory events. *J. Perinat. Med.* 42, 731–743. doi: 10.1515/jpm-2014-0250

Laptook, A. R. (2016). Birth asphyxia and hypoxic-ischemic brain injury in the preterm infant. *Clin. Perinatol.* 43, 529–545. doi: 10.1016/j.clp.2016.04.010

Larouche, A., Roy, M., Kadhim, H., Tsanaclis, A. M., Fortin, D., and Sebire, G. (2005). Neuronal injuries induced by perinatal hypoxic-ischemic insults are potentiated by prenatal exposure to lipopolysaccharide: animal model for perinatally acquired encephalopathy. *Dev. Neurosci.* 27, 134–142. doi: 10.1159/000085985

Lawrence, E. J., Froudist-Walsh, S., Neilan, R., Nam, K. W., Giampietro, V., McGuire, P., et al. (2014). Motor fMRI and cortical grey matter volume in adults born very preterm. *Dev. Cogn. Neurosci.* 10, 1–9. doi: 10.1016/j.dcn.2014.06.002

Lehnardt, S., Massillon, L., Follett, P., Jensen, F. E., Ratan, R., Rosenberg, P. A., et al. (2003). Activation of innate immunity in the CNS triggers neurodegeneration through a toll-like receptor 4-dependent pathway. *Proc. Natl. Acad. Sci. USA* 100, 8514–8519. doi: 10.1073/pnas.1432609100

Lehre, K. P., Levy, L. M., Ottersen, O. P., Storm-Mathisen, J., and Danbolt, N. C. (1995). Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *J. Neurosci.* 15, 1835–1853. doi: 10.1523/JNEUROSCI.15-03-01835.1995

Lepore, A. C., O'Donnell, J., Kim, A. S., Yang, E. J., Tuteja, A., Haidet-Phillips, A., et al. (2011). Reduction in expression of the astrocyte glutamate transporter, GLT1, worsens functional and histological outcomes following traumatic spinal cord injury. *Glia* 59, 1996–2005. doi: 10.1002/glia.21241

Levi, G., and Raiteri, M. (1993). Carrier-mediated release of neurotransmitters. *Trends Neurosci.* 16, 415–419.

Leviton, A., Fichorova, R. N., O'Shea, T. M., Kuban, K., Paneth, N., Dammann, O., et al. (2013). Two-hit model of brain damage in the very preterm newborn: small for gestational age and postnatal systemic inflammation. *Pediatr. Res.* 73, 362–370. doi: 10.1038/pr.2012.188

Leviton, A., and Paneth, N. (1990). White matter damage in preterm newborns – an epidemiologic perspective. *Early Hum. Dev.* 24, 1–22. doi: 10.1016/0378-3782(90)90002-Z

Leviton, A., Paneth, N., Reuss, M. L., Susser, M., Allred, E. N., Dammann, O., et al. (1999). Maternal infection, fetal inflammatory response, and brain damage in very low birth weight infants. Developmental epidemiology network investigators. *Pediatr. Res.* 46, 566–575. doi: 10.1203/00006450-199911000-00013

Levy, L. M., Lehre, K. P., Walaas, S. I., Storm-Mathisen, J., and Danbolt, N. C. (1995). Down-regulation of glial glutamate transporters after glutamatergic denervation in the rat brain. *Eur. J. Neurosci.* 7, 2036–2041. doi: 10.1111/j.1460-9568.1995.tb00626.x

Liao, S. L., and Chen, C. J. (2001). Differential effects of cytokines and redox potential on glutamate uptake in rat cortical glial cultures. *Neurosci. Lett.* 299, 113–116. doi: 10.1016/S0304-3940(01)01499-9

Limperopoulos, C., Bassan, H., Gauvreau, K., Robertson, R. L. Jr., Sullivan, N. R., Benson, C. B., et al. (2007). Does cerebellar injury in premature infants

contribute to the high prevalence of long-term cognitive, learning, and behavioral disability in survivors? *Pediatrics* 120, 584–593. doi: 10.1542/peds.2007-1041

Limperopoulos, C., Soul, J. S., Gauvreau, K., Huppi, P. S., Warfield, S. K., Bassan, H., et al. (2005a). Late gestation cerebellar growth is rapid and impeded by premature birth. *Pediatrics* 115, 688–695. doi: 10.1542/peds.2004-1169

Limperopoulos, C., Soul, J. S., Haidar, H., Huppi, P. S., Bassan, H., Warfield, S. K., et al. (2005b). Impaired trophic interactions between the cerebellum and the cerebrum among preterm infants. *Pediatrics* 116, 844–850. doi: 10.1542/peds.2004-2282

Lingam, I., and Robertson, N. J. (2018). Magnesium as a neuroprotective agent: a review of its use in the fetus, term infant with neonatal encephalopathy, and the adult stroke patient. *Dev. Neurosci.* 40, 1–12. doi: 10.1159/000484891

Lipton, S. A., and Rosenberg, P. A. (1994). Excitatory amino acids as a final common pathway for neurologic disorders. *N. Engl. J. Med.* 330, 613–622. doi: 10.1056/NEJM199403033300907

Litt, J., Taylor, H. G., Klein, N., and Hack, M. (2005). Learning disabilities in children with very low birthweight: prevalence, neuropsychological correlates, and educational interventions. *J. Learn. Disabil.* 38, 130–141. doi: 10.1177/00222194050380020301

Liu, L., Oza, S., Hogan, D., Chu, Y., Perin, J., Zhu, J., et al. (2016). Global, regional, and national causes of under-5 mortality in 2000-15: an updated systematic analysis with implications for the sustainable development goals. *Lancet* 388, 3027–3035. doi: 10.1016/S0140-6736(16)31593-8

Locatelli, A., Incerti, M., Paterlini, G., Doria, V., Consonni, S., Provero, C., et al. (2010). Antepartum and intrapartum risk factors for neonatal encephalopathy at term. *Am. J. Perinatol.* 27, 649–654. doi: 10.1055/s-0030-1249761

Loeliger, M., Watson, C. S., Reynolds, J. D., Penning, D. H., Harding, R., Bocking, A. D., et al. (2003). Extracellular glutamate levels and neuropathology in cerebral white matter following repeated umbilical cord occlusion in the near term fetal sheep. *Neuroscience* 116, 705–714. doi: 10.1016/S0306-4522(02)00756-X

Lou, H. C., Lassen, N. A., and Friis-Hansen, B. (1979). Impaired autoregulation of cerebral blood flow in the distressed newborn infant. *J. Pediatr.* 94, 118–121. doi: 10.1016/S0022-3476(79)80373-X

Lozovaya, N. A., Kopanitsa, M. V., Boychuk, Y. A., and Krishtal, O. A. (1999). Enhancement of glutamate release uncovers spillover-mediated transmission by N-methyl-D-aspartate receptors in the rat hippocampus. *Neuroscience* 91, 1321–1330. doi: 10.1016/S0306-4522(98)00638-1

Maalouf, E. F., Duggan, P. J., Counsell, S. J., Rutherford, M. A., Cowan, F., Azzopardi, D., et al. (2001). Comparison of findings on cranial ultrasound and magnetic resonance imaging in preterm infants. *Pediatrics* 107, 719–727. doi: 10.1542/peds.107.4.719

MacLennan, A. H., Thompson, S. C., and Gecz, J. (2015). Cerebral palsy: causes, pathways, and the role of genetic variants. *Am. J. Obstet. Gynecol.* 213, 779–788. doi: 10.1016/j.ajog.2015.05.034

Mallard, E. C., Rees, S., Stringer, M., Cock, M. L., and Harding, R. (1998). Effects of chronic placental insufficiency on brain development in fetal sheep. *Pediatr. Res.* 43, 262–270. doi: 10.1203/00006450-199802000-00018

Mangham, L. J., Petrou, S., Doyle, L. W., Draper, E. S., and Marlow, N. (2009). The cost of preterm birth throughout childhood in England and Wales. *Pediatrics* 123, e312–e327. doi: 10.1542/peds.2008-1827

Manning, S. M., Talos, D. M., Zhou, C., Selip, D. B., Park, H.-K., Park, C.-J., et al. (2008). NMDA receptor blockade with memantine attenuates white matter injury in a rat model of periventricular leukomalacia. *J. Neurosci.* 28, 6670–6678. doi: 10.1523/JNEUROSCI.1702-08.2008

Marret, S., Mukendi, R., Gadisseux, J. F., Gressens, P., and Evrard, P. (1995). Effect of ibotenate on brain development: an excitotoxic mouse model of microgyria and posthypoxic-like lesions. *J. Neuropathol. Exp. Neurol.* 54, 358–370. doi: 10.1097/00005072-199505000-00009

Martin, L. J., Brambrink, A., Koehler, R. C., and Traystman, R. J. (1997a). Primary sensory and forebrain motor systems in the newborn brain are preferentially damaged by hypoxia-ischemia. *J. Comp. Neurol.* 377, 262–285.

Martin, L. J., Brambrink, A. M., Lehmann, C., Portera-Cailliau, C., Koehler, R., Rothstein, J., et al. (1997b). Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum. *Ann. Neurol.* 42, 335–348.

McClendon, E., Chen, K., Gong, X., Sharifnia, E., Hagen, M., Cai, V., et al. (2014). Prenatal cerebral ischemia triggers dysmaturation of caudate projection neurons. *Ann. Neurol.* 75, 508–524. doi: 10.1002/ana.24100

McClure, M., Riddle, A., Manese, M., Luo, N. L., Rorvik, D. A., Kelly, K. A., et al. (2008). Cerebral blood flow heterogeneity in preterm sheep: lack of physiological support for vascular boundary zones in fetal cerebral white matter. *J. Cereb. Blood Flow Metab.* 28, 995–1008. doi: 10.1038/sj.jcbfm.9600597

McDonald, J. W., and Johnston, M. V. (1990). Pharmacology of N-methyl-D-aspartate-induced brain injury in an in vivo perinatal rat model. *Synapse* 6, 179–188. doi: 10.1002/syn.890060210

McDonald, J. W., Silverstein, F. S., and Johnston, M. V. (1988). Neurotoxicity of N-methyl-D-aspartate is markedly enhanced in developing rat central nervous system. *Brain Res.* 459, 200–203. doi: 10.1016/0006-8993(88)90306-X

McQuillen, P. S., Sheldon, R. A., Shatz, C. J., and Ferriero, D. M. (2003). Selective vulnerability of subplate neurons after early neonatal hypoxia-ischemia. *J. Neurosci.* 23, 3308–3315. doi: 10.1523/JNEUROSCI.23-08-03308.2003

Meldrum, B. S. (2000). Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J. Nutr.* 130, 1007s–1015s.

Melone, M., Bellesi, M., and Conti, F. (2009). Synaptic localization of GLT-1a in the rat somatic sensory cortex. *Glia* 57, 108–117. doi: 10.1002/glia.20744

Mercier, C. E., Dunn, M. S., Ferrelli, K. R., Howard, D. B., and Soll, R. F. (2010). Neurodevelopmental outcome of extremely low birth weight infants from the Vermont Oxford network: 1998-2003. *Neonatology* 97, 329–338. doi: 10.1159/000260136

Mercuri, E., He, J., Curati, W. L., Dubowitz, L. M., Cowan, F. M., and Bydder, G. M. (1997). Cerebellar infarction and atrophy in infants and children with a history of premature birth. *Pediatr. Radiol.* 27, 139–143. doi: 10.1007/s002470050085

Miller, S. P., Cozzio, C. C., Goldstein, R. B., Ferriero, D. M., Partridge, J. C., Vigneron, D. B., et al. (2003). Comparing the diagnosis of white matter injury in premature newborns with serial MR imaging and transfontanel ultrasonography findings. *AJNR Am. J. Neuroradiol.* 24, 1661–1669.

Mitani, A., and Tanaka, K. (2003). Functional changes of glial glutamate transporter GLT-1 during ischemia: an in vivo study in the hippocampal CA1 of normal mice and mutant mice lacking GLT-1. *J. Neurosci.* 23, 7176–7182. doi: 10.1523/JNEUROSCI.23-18-07176.2003

Miyawaki, T., Matsui, K., and Takashima, S. (1998). Developmental characteristics of vessel density in the human fetal and infant brains. *Early Hum. Dev.* 53, 65–72.

Moore, T., Hennessy, E. M., Myles, J., Johnson, S. J., Draper, E. S., Costeloe, K. L., et al. (2012). Neurological and developmental outcome in extremely preterm children born in England in 1995 and 2006: the EPICure studies. *BMJ* 345, 274–275. doi: 10.1136/bmj.e7961

Moretto, M. B., Arteni, N. S., Lavinsky, D., Netto, C. A., Rocha, J. B., Souza, D. O., et al. (2005). Hypoxic-ischemic insult decreases glutamate uptake by hippocampal slices from neonatal rats: prevention by guanosine. *Exp. Neurol.* 195, 400–406. doi: 10.1016/j.expneurol.2005.06.005

Moretto, M. B., Boff, B., Lavinsky, D., Netto, C. A., Rocha, J. B., Souza, D. O., et al. (2009). Importance of schedule of administration in the therapeutic efficacy of guanosine: early intervention after injury enhances glutamate uptake in model of hypoxia-ischemia. *J. Mol. Neurosci.* 38, 216–219. doi: 10.1007/s12031-008-9154-7

Munch, C., Zhu, B. G., Leven, A., Stamm, S., Einkorn, H., Schwalenstocker, B., et al. (2003). Differential regulation of 5′ splice variants of the glutamate transporter EAAT2 in an in vivo model of chemical hypoxia induced by 3-nitropropionic acid. *J. Neurosci. Res.* 71, 819–825. doi: 10.1002/jnr.10536

Murphy, D. J., Sellers, S., MacKenzie, I. Z., Yudkin, P. L., and Johnson, A. M. (1995). Case-control study of antenatal and intrapartum risk factors for cerebral palsy in very preterm singleton babies. *Lancet* 346, 1449–1454. doi: 10.1016/S0140-6736(95)92471-X

Newcomer, J. W., Farber, N. B., and Olney, J. W. (2000). NMDA receptor function, memory, and brain aging. *Dialogues Clin. Neurosci.* 2, 219–232.

Nicholls, D., and Attwell, D. (1990). The release and uptake of excitatory amino acids. *Trends Pharmacol. Sci.* 11, 462–468. doi: 10.1016/0165-6147(90)90129-V

Northington, F. J., Traystman, R. J., Koehler, R. C., and Martin, L. J. (1999). GLT1, glial glutamate transporter, is transiently expressed in neurons and develops astrocyte specificity only after midgestation in the ovine fetal brain.

J. Neurobiol. 39, 515–526. doi: 10.1002/(SICI)1097-4695(19990615)39:4<515::AID-NEU5>3.0.CO;2-U

Northington, F. J., Traystman, R. J., Koehler, R. C., Rothstein, J. D., and Martin, L. J. (1998). Regional and cellular expression of glial (GLT1) and neuronal (EAAC1) glutamate transporter proteins in ovine fetal brain. *Neuroscience* 85, 1183–1194. doi: 10.1016/S0306-4522(97)00673-8

Nosarti, C., Allin, M. P., Frangou, S., Rifkin, L., and Murray, R. M. (2005). Hyperactivity in adolescents born very preterm is associated with decreased caudate volume. *Biol. Psychiatry* 57, 661–666. doi: 10.1016/j.biopsych.2004.12.003

Nosarti, C., Giouroukou, E., Healy, E., Rifkin, L., Walshe, M., Reichenberg, A., et al. (2008). Grey and white matter distribution in very preterm adolescents mediates neurodevelopmental outcome. *Brain* 131, 205–217. doi: 10.1093/brain/awm282

Novak, I., Hines, M., Goldsmith, S., and Barclay, R. (2012). Clinical prognostic messages from a systematic review on cerebral palsy. *Pediatrics* 130, e1285–e1312. doi: 10.1542/peds.2012-0924

O'Callaghan, M. E., Maclennan, A. H., Gibson, C. S., McMichael, G. L., Haan, E. A., Broadbent, J. L., et al. (2013). Genetic and clinical contributions to cerebral palsy: a multi-variable analysis. *J. Paediatr. Child Health* 49, 575–581. doi: 10.1111/jpc.12279

O'Callaghan, M. E., Maclennan, A. H., Gibson, C. S., McMichael, G. L., Haan, E. A., Broadbent, J. L., et al. (2012). Fetal and maternal candidate single nucleotide polymorphism associations with cerebral palsy: a case-control study. *Pediatrics* 129, e414–e423. doi: 10.1542/peds.2011-0739

O'Callaghan, M. E., MacLennan, A. H., Haan, E. A., and Dekker, G. (2009). The genomic basis of cerebral palsy: a HuGE systematic literature review. *Hum. Genet.* 126, 149–172. doi: 10.1007/s00439-009-0638-5

O'Donovan, S. M., Sullivan, C. R., and McCullumsmith, R. E. (2017). The role of glutamate transporters in the pathophysiology of neuropsychiatric disorders. *NPJ Schizophr.* 3:32. doi: 10.1038/s41537-017-0037-1

Ofek-Shlomai, N., and Berger, I. (2014). Inflammatory injury to the neonatal brain – what can we do? *Front. Pediatr.* 2:30. doi: 10.3389/fped.2014.00030

Ohshima, M., Coq, J. O., Otani, K., Hattori, Y., Ogawa, Y., Sato, Y., et al. (2016). Mild intrauterine hypoperfusion reproduces neurodevelopmental disorders observed in prematurity. *Sci. Rep.* 6:39377. doi: 10.1038/srep39377

Oka, A., Belliveau, M. J., Rosenberg, P. A., and Volpe, J. J. (1993). Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. *J. Neurosci.* 13, 1441–1453. doi: 10.1523/JNEUROSCI.13-04-01441.1993

Olmos, G., and Llado, J. (2014). Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. *Mediat. Inflamm.* 2014:861231. doi: 10.1155/2014/861231

Olney, J. W. (1969). Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. *Science* 164, 719–721. doi: 10.1126/science.164.3880.719

O'Shea, T. M., Klinepeter, K. L., and Dillard, R. G. (1998). Prenatal events and the risk of cerebral palsy in very low birth weight infants. *Am. J. Epidemiol.* 147, 362–369. doi: 10.1093/oxfordjournals.aje.a009458

O'Shea, R. D., Lau, C. L., Farso, M. C., Diwakarla, S., Zagami, C. J., Svendsen, B. B., et al. (2006). Effects of lipopolysaccharide on glial phenotype and activity of glutamate transporters: evidence for delayed up-regulation and redistribution of GLT-1. *Neurochem. Int.* 48, 604–610. doi: 10.1016/j.neuint.2005.12.028

Oskoui, M., Coutinho, F., Dykeman, J., Jetté, N., and Pringsheim, T. (2013). An update on the prevalence of cerebral palsy: a systematic review and meta-analysis. *Dev. Med. Child Neurol.* 55, 509–519. doi: 10.1111/dmcn.12080

Otis, T. S., and Kavanaugh, M. P. (2000). Isolation of current components and partial reaction cycles in the glial glutamate transporter EAAT2. *J. Neurosci.* 20, 2749–2757.

Ottersen, O. P., Laake, J. H., Reichelt, W., Haug, F. M., and Torp, R. (1996). Ischemic disruption of glutamate homeostasis in brain: quantitative immunocytochemical analyses. *J. Chem. Neuroanat.* 12, 1–14. doi: 10.1016/S0891-0618(96)00178-0

Parker, J., Mitchell, A., Kalpakidou, A., Walshe, M., Jung, H. Y., Nosarti, C., et al. (2008). Cerebellar growth and behavioural & neuropsychological outcome in preterm adolescents. *Brain* 131, 1344–1351. doi: 10.1093/brain/awn062

Parkin, G. M., Udawela, M., Gibbons, A., and Dean, B. (2018). Glutamate transporters, EAAT1 and EAAT2, are potentially important in the pathophysiology and treatment of schizophrenia and affective disorders. *World J. Psychiatry* 8, 51–63. doi: 10.5498/wjp.v8.i2.51

Parsons, M. P., and Raymond, L. A. (2014). Extrasynaptic NMDA receptor involvement in central nervous system disorders. *Neuron* 82, 279–293. doi: 10.1016/j.neuron.2014.03.030

Persson, M., Brantefjord, M., Hansson, E., and Ronnback, L. (2005). Lipopolysaccharide increases microglial GLT-1 expression and glutamate uptake capacity in vitro by a mechanism dependent on TNF-alpha. *Glia* 51, 111–120. doi: 10.1002/glia.20191

Peterson, B. S., Anderson, A. W., Ehrenkranz, R., Staib, L. H., Tageldin, M., Colson, E., et al. (2003). Regional brain volumes and their later neurodevelopmental correlates in term and preterm infants. *Pediatrics* 111, 939–948. doi: 10.1542/peds.111.5.939

Peterson, B. S., Vohr, B., Staib, L. H., Cannistraci, C. J., Dolberg, A., Schneider, K. C., et al. (2000). Regional brain volume abnormalities and long-term cognitive outcome in preterm infants. *JAMA* 284, 1939–1947.

Petr, G. T., Sun, Y., Frederick, N. M., Zhou, Y., Dhamne, S. C., Hameed, M. Q., et al. (2015). Conditional deletion of the glutamate transporter GLT-1 reveals that astrocytic GLT-1 protects against fatal epilepsy while neuronal GLT-1 contributes significantly to glutamate uptake into synaptosomes. *J. Neurosci.* 35, 5187–5201. doi: 10.1523/JNEUROSCI.4255-14.2015

Platt, S. R. (2007). The role of glutamate in central nervous system health and disease – a review. *Vet. J.* 173, 278–286. doi: 10.1016/j.tvjl.2005.11.007

Pow, D. V., Naidoo, T., Lingwood, B. E., Healy, G. N., Williams, S. M., Sullivan, R. K., et al. (2004). Loss of glial glutamate transporters and induction of neuronal expression of GLT-1B in the hypoxic neonatal pig brain. *Brain Res. Dev. Brain Res.* 153, 1–11. doi: 10.1016/j.devbrainres.2004.06.019

Pryds, O. (1991). Control of cerebral circulation in the high-risk neonate. *Ann. Neurol.* 30, 321–329. doi: 10.1002/ana.410300302

Pryds, O., Andersen, G. E., and Friis-Hansen, B. (1990). Cerebral blood flow reactivity in spontaneously breathing, preterm infants shortly after birth. *Acta Paediatr. Scand.* 79, 391–396.

Rajatileka, S., Odd, D., Robinson, M. T., Spittle, A. C., Dwomoh, L., Williams, M., et al. (2017). Variants of the EAAT2 glutamate transporter gene promoter are associated with cerebral palsy in preterm infants. *Mol. Neurobiol.* 55, 2013–2024. doi: 10.1007/s12035-017-0462-1

Rao, V. L., Bowen, K. K., and Dempsey, R. J. (2001a). Transient focal cerebral ischemia down-regulates glutamate transporters GLT-1 and EAAC1 expression in rat brain. *Neurochem. Res.* 26, 497–502. doi: 10.1023/A:1010956711295

Rao, V. L., Dogan, A., Bowen, K. K., Todd, K. G., and Dempsey, R. J. (2001b). Antisense knockdown of the glial glutamate transporter GLT-1 exacerbates hippocampal neuronal damage following traumatic injury to rat brain. *Eur. J. Neurosci.* 13, 119–128. doi: 10.1111/j.1460-9568.2001.01367.x

Raymond, M., Li, P., Mangin, J. M., Huntsman, M., and Gallo, V. (2011). Chronic perinatal hypoxia reduces glutamate-aspartate transporter function in astrocytes through the Janus kinase/signal transducer and activator of transcription pathway. *J. Neurosci.* 31, 17864–17871. doi: 10.1523/JNEUROSCI.3179-11.2011

Rees, S., Mallard, C., Breen, S., Stringer, M., Cock, M., and Harding, R. (1998). Fetal brain injury following prolonged hypoxemia and placental insufficiency: a review. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 119, 653–660. doi: 10.1016/S1095-6433(98)01001-0

Rees, S., Stringer, M., Just, Y., Hooper, S. B., and Harding, R. (1997). The vulnerability of the fetal sheep brain to hypoxemia at mid-gestation. *Brain Res. Dev. Brain Res.* 103, 103–118. doi: 10.1016/S0165-3806(97)81787-7

Rehm, H. L. (2017). Evolving health care through personal genomics. *Nat. Rev. Genet.* 18, 259–267. doi: 10.1038/nrg.2016.162

Rezaie, P., and Dean, A. (2002). Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. *Neuropathology* 22, 106–132. doi: 10.1046/j.1440-1789.2002.00438.x

Riddle, A., Luo, N. L., Manese, M., Beardsley, D. J., Green, L., Rorvik, D. A., et al. (2006). Spatial heterogeneity in oligodendrocyte lineage maturation and not cerebral blood flow predicts fetal ovine periventricular white matter injury. *J. Neurosci.* 26, 3045–3055. doi: 10.1523/JNEUROSCI.5200-05.2006

Riikonen, R. S., Kero, P. O., and Simell, O. G. (1992). Excitatory amino acids in cerebrospinal fluid in neonatal asphyxia. *Pediatr. Neurol.* 8, 37–40. doi: 10.1016/0887-8994(92)90050-9

Rimaniol, A. C., Haik, S., Martin, M., Le Grand, R., Boussin, F. D., Dereuddre-Bosquet, N., et al. (2000). Na+−dependent high-affinity glutamate transport in macrophages. *J. Immunol.* 164, 5430–5438. doi: 10.4049/jimmunol.164.10.5430

Roberts, R. C., Roche, J. K., and McCullumsmith, R. E. (2014). Localization of excitatory amino acid transporters EAAT1 and EAAT2 in human postmortem

cortex: a light and electron microscopic study. *Neuroscience* 277, 522–540. doi: 10.1016/j.neuroscience.2014.07.019

Rocha-Ferreira, E., and Hristova, M. (2016). Plasticity in the neonatal brain following hypoxic-ischaemic injury. *Neural Plast.* 2016:4901014. doi: 10.1155/2016/4901014

Rosenbaum, P., Paneth, N., Leviton, A., Goldstein, M., Bax, M., Damiano, D., et al. (2007). A report: the definition and classification of cerebral palsy April 2006. *Dev. Med. Child Neurol. Suppl.* 109, 8–14. doi: 10.1111/j.1469-8749.2007.tb12610.x

Rothstein, J. D., Dykes-Hoberg, M., Pardo, C. A., Bristol, L. A., Jin, L., Kuncl, R. W., et al. (1996). Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16, 675–686. doi: 10.1016/S0896-6273(00)80086-0

Rothstein, J. D., Martin, L., Levey, A. I., Dykes-Hoberg, M., Jin, L., Wu, D., et al. (1994). Localization of neuronal and glial glutamate transporters. *Neuron* 13, 713–725. doi: 10.1016/0896-6273(94)90038-8

Sathyanesan, A., Kundu, S., Abbah, J., and Gallo, V. (2018). Neonatal brain injury causes cerebellar learning deficits and Purkinje cell dysfunction. *Nat. Commun.* 9:3235. doi: 10.1038/s41467-018-05656-w

Sattler, R., and Tymianski, M. (2001). Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol. Neurobiol.* 24, 107–129. doi: 10.1385/MN:24:1-3:107

Schlapbach, L. J., Aebischer, M., Adams, M., Natalucci, G., Bonhoeffer, J., Latzin, P., et al. (2011). Impact of sepsis on neurodevelopmental outcome in a Swiss National Cohort of extremely premature infants. *Pediatrics* 128, e348–e357. doi: 10.1542/peds.2010-3338

Segovia, K. N., McClure, M., Moravec, M., Luo, N. L., Wan, Y., Gong, X., et al. (2008). Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann. Neurol.* 63, 520–530. doi: 10.1002/ana.21359

Seki, Y., Feustel, P. J., Keller, R. W. Jr., Tranmer, B. I., and Kimelberg, H. K. (1999). Inhibition of ischemia-induced glutamate release in rat striatum by dihydrokinate and an anion channel blocker. *Stroke* 30, 433–440. doi: 10.1161/01.STR.30.2.433

Serdaroglu, G., Tekgul, H., Kitis, O., Serdaroglu, E., and Gökben, S. (2004). Correlative value of magnetic resonance imaging for neurodevelopmental outcome in periventricular leukomalacia. *Dev. Med. Child Neurol.* 46, 733–739. doi: 10.1111/j.1469-8749.2004.tb00992.x

Shah, D. K., Anderson, P. J., Carlin, J. B., Pavlovic, M., Howard, K., Thompson, D. K., et al. (2006). Reduction in cerebellar volumes in preterm infants: relationship to white matter injury and neurodevelopment at two years of age. *Pediatr. Res.* 60, 97–102. doi: 10.1203/01.pdr.0000220324.27597.f0

Shankaran, S., Langer, J. C., Kazzi, S. N., Laptook, A. R., and Walsh, M. (2006). Cumulative index of exposure to hypocarbia and hyperoxia as risk factors for periventricular leukomalacia in low birth weight infants. *Pediatrics* 118, 1654–1659. doi: 10.1542/peds.2005-2463

Shankaran, S., Laptook, A. R., Ehrenkranz, R. A., Tyson, J. E., McDonald, S. A., Donovan, E. F., et al. (2005). Whole-body hypothermia for neonates with hypoxic–ischemic encephalopathy. *N. Engl. J. Med.* 353, 1574–1584. doi: 10.1056/NEJMcps050929

Shibata, T., Watanabe, M., Tanaka, K., Wada, K., and Inoue, Y. (1996). Dynamic changes in expression of glutamate transporter mRNAs in developing brain. *Neuroreport* 7, 705–709. doi: 10.1097/00001756-199602290-00006

Simbruner, G., Mittal, R. A., Rohlmann, F., and Muche, R. (2010). Systemic hypothermia after neonatal encephalopathy: outcomes of neo.nEURO.Network RCT. *Pediatrics* 126, e771–e778. doi: 10.1542/peds.2009-2441

Soria-Pastor, S., Gimenez, M., Narberhaus, A., Falcon, C., Botet, F., Bargallo, N., et al. (2008). Patterns of cerebral white matter damage and cognitive impairment in adolescents born very preterm. *Int. J. Dev. Neurosci.* 26, 647–654. doi: 10.1016/j.ijdevneu.2008.08.001

Soria-Pastor, S., Padilla, N., Zubiaurre-Elorza, L., Ibarretxe-Bilbao, N., Botet, F., Costas-Moragas, C., et al. (2009). Decreased regional brain volume and cognitive impairment in preterm children at low risk. *Pediatrics* 124, e1161–e1170. doi: 10.1542/peds.2009-0244

Soul, J. S., Hammer, P. E., Tsuji, M., Saul, J. P., Bassan, H., Limperopoulos, C., et al. (2007). Fluctuating pressure-passivity is common in the cerebral circulation of sick premature infants. *Pediatr. Res.* 61, 467–473. doi: 10.1203/pdr.0b013e31803237f6

Spittle, A. J., Boyd, R. N., Inder, T. E., and Doyle, L. W. (2009). Predicting motor development in very preterm infants at 12 months' corrected age: the role of qualitative magnetic resonance imaging and general movements assessments. *Pediatrics* 123, 512–517. doi: 10.1542/peds.2008-0590

Spittle, A. J., Brown, N. C., Doyle, L. W., Boyd, R. N., Hunt, R. W., Bear, M., et al. (2008). Quality of general movements is related to white matter pathology in very preterm infants. *Pediatrics* 121, e1184–e1189. doi: 10.1542/peds.2007-1924

Spittle, A. J., Morgan, C., Olsen, J. E., Novak, I., and Cheong, J. L. Y. (2018). Early diagnosis and treatment of cerebral palsy in children with a history of preterm birth. *Clin. Perinatol.* 45, 409–420. doi: 10.1016/j.clp.2018.05.011

Srinivasan, L., Dutta, R., Counsell, S. J., Allsop, J. M., Boardman, J. P., Rutherford, M. A., et al. (2007). Quantification of deep gray matter in preterm infants at term-equivalent age using manual volumetry of 3-tesla magnetic resonance images. *Pediatrics* 119, 759–765. doi: 10.1542/peds.2006-2508

Stanley, F. J. (1992). Survival and cerebral palsy in low birthweight infants: implications for perinatal care. *Paediatr. Perinat. Epidemiol.* 6, 298–310. doi: 10.1111/j.1365-3016.1992.tb00769.x

Stanley, F., Blair, E., and Alberman, E. (2000). *Cerebral palsies: Epidemiology and causal pathways*. (London: MacKeith Press).

Stavsky, M., Mor, O., Mastrolia, S. A., Greenbaum, S., Than, N. G., and Erez, O. (2017). Cerebral palsy – trends in epidemiology and recent development in prenatal mechanisms of disease, treatment, and prevention. *Front. Pediatr.* 5:21. doi: 10.3389/fped.2017.00021

Stoll, B. J., Hansen, N., Fanaroff, A. A., Wright, L. L., Carlo, W. A., Ehrenkranz, R. A., et al. (2002). Late-onset sepsis in very low birth weight neonates: the experience of the NICHD neonatal research network. *Pediatrics* 110, 285–291. doi: 10.1542/peds.110.2.285

Su, Z. Z., Leszczyniecka, M., Kang, D. C., Sarkar, D., Chao, W., Volsky, D. J., et al. (2003). Insights into glutamate transport regulation in human astrocytes: cloning of the promoter for excitatory amino acid transporter 2 (EAAT2). *Proc. Natl. Acad. Sci. USA* 100, 1955–1960. doi: 10.1073/pnas.0136555100

Sutherland, M. L., Delaney, T. A., and Noebels, J. L. (1996). Glutamate transporter mRNA expression in proliferative zones of the developing and adult murine CNS. *J. Neurosci.* 16, 2191–2207. doi: 10.1523/JNEUROSCI.16-07-02191.1996

Szatkowski, M., Barbour, B., and Attwell, D. (1990). Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* 348, 443–446. doi: 10.1038/348443a0

Szymonowicz, W., Walker, A. M., Cussen, L., Cannata, J., and Yu, V. Y. (1988). Developmental changes in regional cerebral blood flow in fetal and newborn lambs. *Am. J. Phys.* 254, H52–H58. doi: 10.1152/ajpheart.1988.254.1.H52

Takahashi, K., Foster, J. B., and Lin, C.-L. G. (2015). Glutamate transporter EAAT2: regulation, function, and potential as a therapeutic target for neurological and psychiatric disease. *Cell. Mol. Life Sci.* 72, 3489–3506. doi: 10.1007/s00018-015-1937-8

Takasaki, C., Okada, R., Mitani, A., Fukaya, M., Yamasaki, M., Fujihara, Y., et al. (2008). Glutamate transporters regulate lesion-induced plasticity in the developing somatosensory cortex. *J. Neurosci.* 28, 4995–5006. doi: 10.1523/JNEUROSCI.0861-08.2008

Takashima, S., and Tanaka, K. (1978). Development of cerebrovascular architecture and its relationship to periventricular leukomalacia. *Arch. Neurol.* 35, 11–16. doi: 10.1001/archneur.1978.00500250015003

Takeuchi, H., Jin, S., Wang, J., Zhang, G., Kawanokuchi, J., Kuno, R., et al. (2006). Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. *J. Biol. Chem.* 281, 21362–21368. doi: 10.1074/jbc.M600504200

Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., et al. (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* 276, 1699–1702. doi: 10.1126/science.276.5319.1699

Thornton, C., Rousset, C. I., Kichev, A., Miyakuni, Y., Vontell, R., Baburamani, A. A., et al. (2012). Molecular mechanisms of neonatal brain injury. *Neurol. Res. Int.* 2012:506320. doi: 10.1155/2012/506320

Tilleux, S., and Hermans, E. (2007). Neuroinflammation and regulation of glial glutamate uptake in neurological disorders. *J. Neurosci. Res.* 85, 2059–2070. doi: 10.1002/jnr.21325

Torp, R., Lekieffre, D., Levy, L. M., Haug, F. M., Danbolt, N. C., Meldrum, B. S., et al. (1995). Reduced postischemic expression of a glial glutamate transporter, GLT1, in the rat hippocampus. *Exp. Brain Res.* 103, 51–58. doi: 10.1007/BF00241964

Tronnes, H., Wilcox, A. J., Lie, R. T., Markestad, T., and Moster, D. (2014). Risk of cerebral palsy in relation to pregnancy disorders and preterm birth: a national cohort study. *Dev. Med. Child Neurol.* 56, 779–785. doi: 10.1111/dmcn.12430

Tzingounis, A. V., and Wadiche, J. I. (2007). Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat. Rev. Neurosci.* 8, 935–947. doi: 10.1038/nrn2274

Ullensvang, K., Lehre, K. P., Storm-Mathisen, J., and Danbolt, N. C. (1997). Differential developmental expression of the two rat brain glutamate transporter proteins GLAST and GLT. *Eur. J. Neurosci.* 9, 1646–1655. doi: 10.1111/j.1460-9568.1997.tb01522.x

United Nations (2015). Millennium development goals report 2015. doi: 10.18356/98544aa9-en

van der Burg, J. W., Sen, S., Chomitz, V. R., Seidell, J. C., Leviton, A., and Dammann, O. (2016). The role of systemic inflammation linking maternal BMI to neurodevelopment in children. *Pediatr. Res.* 79, 3–12. doi: 10.1038/pr.2015.179

Van Steenwinckel, J., Schang, A. L., Sigaut, S., Chhor, V., Degos, V., Hagberg, H., et al. (2014). Brain damage of the preterm infant: new insights into the role of inflammation. *Biochem. Soc. Trans.* 42, 557–563. doi: 10.1042/BST20130284

van Tilborg, E., Achterberg, E. J. M., van Kammen, C. M., van der Toorn, A., Groenendaal, F., Dijkhuizen, R. M., et al. (2018). Combined fetal inflammation and postnatal hypoxia causes myelin deficits and autism-like behavior in a rat model of diffuse white matter injury. *Glia* 66, 78–93. doi: 10.1002/glia.23216

Vandenberg, R. J., and Ryan, R. M. (2013). Mechanisms of glutamate transport. *Physiol. Rev.* 93, 1621–1657. doi: 10.1152/physrev.00007.2013

Verkhratsky, A., Steardo, L., Parpura, V., and Montana, V. (2016). Translational potential of astrocytes in brain disorders. *Prog. Neurobiol.* 144, 188–205. doi: 10.1016/j.pneurobio.2015.09.003

Verma, U., Tejani, N., Klein, S., Reale, M. R., Beneck, D., Figueroa, R., et al. (1997). Obstetric antecedents of intraventricular hemorrhage and periventricular leukomalacia in the low-birth-weight neonate. *Am. J. Obstet. Gynecol.* 176, 275–281. doi: 10.1016/S0002-9378(97)70485-X

Volpe, J. J. (2008). *Neurology of the newborn*. (Philadelphia, PA: Saunders Elsevier).

Volpe, J. J. (2009a). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 8, 110–124. doi: 10.1016/S1474-4422(08)70294-1

Volpe, J. J. (2009b). Cerebellum of the premature infant: rapidly developing, vulnerable, clinically important. *J. Child Neurol.* 24, 1085–1104. doi: 10.1177/0883073809338067

Volpe, J. J. (2009c). The encephalopathy of prematurity--brain injury and impaired brain development inextricably intertwined. *Semin. Pediatr. Neurol.* 16, 167–178. doi: 10.1016/j.spen.2009.09.005

Volpe, J. J., Kinney, H. C., Jensen, F. E., and Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *Int. J. Dev. Neurosci.* 29, 423–440. doi: 10.1016/j.ijdevneu.2011.02.012

Wang, L. W., Chang, Y. C., Lin, C. Y., Hong, J. S., and Huang, C. C. (2010). Low-dose lipopolysaccharide selectively sensitizes hypoxic ischemia-induced white matter injury in the immature brain. *Pediatr. Res.* 68, 41–47. doi: 10.1203/PDR.0b013e3181df5f6b

Wang, X., Hagberg, H., Nie, C., Zhu, C., Ikeda, T., and Mallard, C. (2007). Dual role of intrauterine immune challenge on neonatal and adult brain vulnerability to hypoxia-ischemia. *J. Neuropathol. Exp. Neurol.* 66, 552–561. doi: 10.1097/01.jnen.0000263870.91811.6f

Wang, X., Stridh, L., Li, W., Dean, J., Elmgren, A., Gan, L., et al. (2009). Lipopolysaccharide sensitizes neonatal hypoxic-ischemic brain injury in a MyD88-dependent manner. *J. Immunol.* 183, 7471–7477. doi: 10.4049/jimmunol.0900762

Watkins, J. C., and Evans, R. H. (1981). Excitatory amino acid transmitters. *Annu. Rev. Pharmacol. Toxicol.* 21, 165–204. doi: 10.1146/annurev.pa.21.040181.001121

Wilke, S., Thomas, R., Allcock, N., and Fern, R. (2004). Mechanism of acute ischemic injury of oligodendroglia in early myelinating white matter: the importance of astrocyte injury and glutamate release. *J. Neuropathol. Exp. Neurol.* 63, 872–881.

Wilson-Costello, D., Friedman, H., Minich, N., Fanaroff, A. A., and Hack, M. (2005). Improved survival rates with increased neurodevelopmental disability for extremely low birth weight infants in the 1990s. *Pediatrics* 115, 997–1003. doi: 10.1542/peds.2004-0221

Wisnowski, J. L., Bluml, S., Paquette, L., Zelinski, E., Nelson, M. D. Jr., Painter, M. J., et al. (2013). Altered glutamatergic metabolism associated with punctate white matter lesions in preterm infants. *PLoS One* 8:e56880. doi: 10.1371/journal.pone.0056880

World Health Organization (2012) in *Born too soon: The global action report on preterm birth*. eds. C. P. Howson, M. V. Kinney, and J. E. Lawn (Geneva: World Health Organization).

World Health Organization (2016). *Global Health estimates 2015: Disease burden by cause, age, sex, by country and by region, 2000–2015*. (Geneva: World Health Organization).

Wu, Y. W. (2002). Systematic review of chorioamnionitis and cerebral palsy. *Ment. Retard. Dev. Disabil. Res. Rev.* 8, 25–29. doi: 10.1002/mrdd.10003

Wu, Y. W., and Colford, J. M. Jr. (2000). Chorioamnionitis as a risk factor for cerebral palsy: a meta-analysis. *JAMA* 284, 1417–1424.

Wu, D., Zou, Y. F., Xu, X. Y., Feng, X. L., Yang, L., Zhang, G. C., et al. (2011). The association of genetic polymorphisms with cerebral palsy: a meta-analysis. *Dev. Med. Child Neurol.* 53, 217–225. doi: 10.1111/j.1469-8749.2010.03884.x

Yamada, K., Watanabe, M., Shibata, T., Nagashima, M., Tanaka, K., and Inoue, Y. (1998). Glutamate transporter GLT-1 is transiently localized on growing axons of the mouse spinal cord before establishing astrocytic expression. *J. Neurosci.* 18, 5706–5713. doi: 10.1523/JNEUROSCI.18-15-05706.1998

Yanni, D., Korzeniewski, S. J., Allred, E. N., Fichorova, R. N., O'Shea, T. M., Kuban, K., et al. (2017). Both antenatal and postnatal inflammation contribute information about the risk of brain damage in extremely preterm newborns. *Pediatr. Res.* 82, 691–696. doi: 10.1038/pr.2017.128

Ying, W. (1997). Deleterious network: a testable pathogenetic concept of Alzheimer's disease. *Gerontology* 43, 242–253.

Yoon, B. H., Jun, J. K., Romero, R., Park, K. H., Gomez, R., Choi, J. H., et al. (1997). Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. *Am. J. Obstet. Gynecol.* 177, 19–26. doi: 10.1016/S0002-9378(97)70432-0

Yoon, B. H., Romero, R., Park, J. S., Kim, C. J., Kim, S. H., Choi, J. H., et al. (2000). Fetal exposure to an intra-amniotic inflammation and the development of cerebral palsy at the age of three years. *Am. J. Obstet. Gynecol.* 182, 675–681. doi: 10.1067/mob.2000.104207

Yoon, B. H., Romero, R., Yang, S. H., Jun, J. K., Kim, I. O., Choi, J. H., et al. (1996). Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. *Am. J. Obstet. Gynecol.* 174, 1433–1440. doi: 10.1016/S0002-9378(96)70585-9

Zamanian, J. L., Xu, L., Foo, L. C., Nouri, N., Zhou, L., Giffard, R. G., et al. (2012). Genomic analysis of reactive astrogliosis. *J. Neurosci.* 32, 6391–6410. doi: 10.1523/JNEUROSCI.6221-11.2012

Zhang, Y., Tan, F., Xu, P., and Qu, S. (2016). Recent advance in the relationship between excitatory amino acid transporters and Parkinson's disease. *Neural Plast.* 2016:8. doi: 10.1155/2016/8941327

Zhou, Y., and Danbolt, N. C. (2013). GABA and glutamate transporters in brain. *Front. Endocrinol.* 4:165. doi: 10.3389/fendo.2013.00165

Zhou, Y., Waanders, L. F., Holmseth, S., Guo, C., Berger, U. V., Li, Y., et al. (2014). Proteome analysis and conditional deletion of the EAAT2 glutamate transporter provide evidence against a role of EAAT2 in pancreatic insulin secretion in mice. *J. Biol. Chem.* 289, 1329–1344. doi: 10.1074/jbc.M113.529065

Zhou, X.-w., Wang, X., Yang, Y., Luo, J.-w., Dong, H., Liu, Y.-h., et al. (2016). Biomarkers related with seizure risk in glioma patients: a systematic review. *Clin. Neurol. Neurosurg.* 151, 113–119. doi: 10.1016/j.clineuro.2016.10.001

Zhu, M. Y., Milligan, N., Keating, S., Windrim, R., Keunen, J., Thakur, V., et al. (2016). The hemodynamics of late-onset intrauterine growth restriction by MRI. *Am. J. Obstet. Gynecol.* 214, 367.e1–367.e17. doi: 10.1016/j.ajog.2015.10.004

Zonouzi, M., Scafidi, J., Li, P., McEllin, B., Edwards, J., Dupree, J. L., et al. (2015). GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury. *Nat. Neurosci.* 18, 674–682. doi: 10.1038/nn.3990

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Pregnolato, Chakkarapani, Isles and Luyt. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 25 April 2019
doi: 10.3389/fphys.2019.00463

Iron Metabolism and Brain Development in Premature Infants

*Yafeng Wang[1,2,3], Yanan Wu[2], Tao Li[1,2,3], Xiaoyang Wang[2,4] and Changlian Zhu[2,3]**

[1]Department of Neonatology (NICU), Children's Hospital Affiliated Zhengzhou University, Zhengzhou, China, [2]Henan Key Laboratory of Child Brain Injury, Institute of Neuroscience and Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3]Department of Clinical Neuroscience, Center for Brain Repair and Rehabilitation, Institute of Neuroscience and Physiology, University of Gothenburg, Gothenburg, Sweden, [4]Department of Physiology, Sahlgrenska Academy, Institute of Neuroscience and Physiology, University of Gothenburg, Gothenburg, Sweden

Iron is important for a remarkable array of essential functions during brain development, and it needs to be provided in adequate amounts, especially to preterm infants. In this review article, we provide an overview of iron metabolism and homeostasis at the cellular level, as well as its regulation at the mRNA translation level, and we emphasize the importance of iron for brain development in fetal and early life in preterm infants. We also review the risk factors for disrupted iron metabolism that lead to high risk of developing iron deficiency and subsequent adverse effects on neurodevelopment in preterm infants. At the other extreme, iron overload, which is usually caused by excess iron supplementation in iron-replete preterm infants, might negatively impact brain development or even induce brain injury. Maintaining the balance of iron during the fetal and neonatal periods is important, and thus iron status should be monitored routinely and evaluated thoroughly during the neonatal period or before discharge of preterm infants so that iron supplementation can be individualized.

Keywords: brain development, brain injury, iron metabolism, iron homeostasis, preterm infants

OPEN ACCESS

Edited by:
Mary Tolcos,
RMIT University, Australia

Reviewed by:
Rebecca Maree Dyson,
University of Otago, New Zealand
Max Berry,
University of Otago, New Zealand

***Correspondence:**
Changlian Zhu
changlian.zhu@neuro.gu.se

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *30 September 2018*
Accepted: *04 April 2019*
Published: *25 April 2019*

Citation:
Wang Y, Wu Y, Li T, Wang X and Zhu C (2019) Iron Metabolism and Brain Development in Premature Infants. Front. Physiol. 10:463. doi: 10.3389/fphys.2019.00463

INTRODUCTION

Iron is a transition metal with the ability to transport oxygen and transfer electrons, and it acts as a catalyst in the active sites of oxidases, oxygenases, and certain antioxidants. All cells require iron due to iron's role in important physiological processes such as oxidative phosphorylation and energy metabolism. For example, the cytochromes and succinate dehydrogenase that play critical roles in the tricarboxylic acid cycle are iron-containing proteins (Bartnikas, 2012). The huge demand for iron in the late fetal and early postnatal period is for hemoglobin (Hb) synthesis; however, iron's function should not be undervalued during the development of all other organ systems. As with all nutrients, there is a greater requirement for iron during rapid growth and development.

Premature infants are at high risk of iron deficiency (ID) due to inadequate iron storage caused by the factors of preterm birth, early onset of postnatal erythropoiesis, and rapid growth after birth (Choudhury et al., 2015; Pettei et al., 2016). The lack of a gold standard to describe iron status clinically for healthy preterm infants is still a weakness. Previous studies showed lower iron storage in premature neonates compared with full term neonates, and the smaller premature neonates are at birth, the more susceptible they are to ID due to their

proportionately smaller iron storage at birth (Haga, 1980; Schiza et al., 2007; Takala et al., 2010). Thus, most preterm infants need to be supplied with a certain dose of iron for the prevention of anemia of prematurity, and with iron supplementation and reasonable breast feeding, the situation of ID gradually improves with age (Takala et al., 2010; Schneider and Garcia-Rodenas, 2017). Cumulative evidence suggests that iron imbalance—both ID and iron overload—has negative consequences on infant development (Hare et al., 2015; Cusick et al., 2018). In this review article, we summarize the iron metabolism status in early life and its relation with brain development, and we focus especially on impact of iron dysregulation in preterm infants.

IRON METABOLISM AND REGULATION

Iron balance is strictly regulated by preventing both ID and iron overload. This homeostasis is achieved through iron storage, erythrocyte iron reutilization, and iron absorption (Finch and Huebers, 1982). Therefore, when the iron level of the body is inadequate, absorption is maximized, and when the iron level is adequate, iron absorption is restricted (Gkouvatsos et al., 2012). When iron is in overabundance, excess iron is kept in enterocytes as ferritin and in the liver, spleen, and bone marrow as hemosiderin (Saito, 2014). The ferroportin-mediated release of free iron ions into the plasma is essential for iron absorption, iron recycling, and overall iron homeostasis (Nemeth et al., 2004). Iron flux is controlled by hepcidin in the organs expressing ferroportin, and its expression is regulated by iron, hypoxia, inflammation, and other factors (Rivera et al., 2005; Chung et al., 2007; Vecchi et al., 2009). Conversely, ID, anemia, and hypoxia all inhibit hepcidin mRNA transcription (Vela, 2018), which results in unrestricted duodenal iron absorption and iron release from macrophages.

Under normal physiological conditions, protein-bound iron is the iron transport and storage form because it does not induce free radical reactions. However, protein-bound iron could be released from its binding proteins following perinatal asphyxia and/or postnatal hypoxia, and this is the common risk factor associated with brain injury in preterm infants (Albertsson et al., 2014; Laptook, 2016; Pillers, 2017). The blood pH decreases after asphyxia, causing transferrin to release iron and induce free radical production and iron accumulation, which could be seen in injured neurons and white matter (Rathnasamy et al., 2011; Beppu et al., 2014). These free radicals cause more iron to be released after mobilization from ferritin, and the reperfusion and reoxygenation after hypoxia could produce a great quantity of nitric oxide in the neonatal brain, causing the release of even more iron from its binding protein (Niatsetskaya et al., 2012). These mechanisms activate a cascade of iron release and free radical production that lead to extensive cellular oxidative stress and cell death (Shouman et al., 2008). After perinatal asphyxia and/or postnatal hypoxia, as an end product of lipid peroxidation, the serum levels of malondialdehyde are elevated in newborns (El Bana et al., 2016). The level of lipid peroxides and the severity of cell damage can be decreased by the iron chelator deferoxamine (Rathnasamy et al., 2011). It has also been suggested that iron-mediated ferroptosis might play an important role in preterm infants after perinatal asphyxia and/or postnatal hypoxia-induced brain injury (Wu et al., 2019).

IRON METABOLISM AND BRAIN DEVELOPMENT

Processes and pathways involved in central nervous system (CNS) iron homeostasis at the cellular level are shown in the left part of **Figure 1**. After iron enters the brain across the blood–brain barrier and the choroid plexus, the iron is processed by endocytosis (Simpson et al., 2015). Particularly, as a highly specific form of membrane-bound ceruloplasmin, it controls and regulates the activity of ferroportin, which is a ferroxidase enzyme that normally functions as the main copper-carrying protein in the blood and that is also expressed by the adjacent endfeet of astrocytes (McCarthy and Kosman, 2014). Generally, this interaction and regulation between astrocytes and brain capillary epithelial cell hephaestin is enabled by a negative feedback loop (McCarthy and Kosman, 2015). Under physiological conditions, high levels of transferrin receptors (TfRs) are expressed in neurons which could obtain the greater part of their iron from transferrin (Morris et al., 2018). The initial neuronal uptake of transferrin-bound iron (TBI) is achieved by the formation of TfR1 and incoming TBI complex and then is internalized by way of clathrin-mediated endocytosis (Liu et al., 2017). Microglia internalizes TBI by way of TfRs commonly, and by dicarboxylic acid receptor as well as possibly also the lactoferrin receptor. Neurons and other glial cells also obtain non-TBI from upregulated divalent metal transporter-1 (DMT-19) under inflammatory conditions (Morris et al., 2018). At the cellular level (**Figure 1**, right part), iron metabolism is controlled post-transcriptionally by the IRE (iron responsive element)/IRP (iron regulatory protein) system (Hentze et al., 2010; Wang and Pantopoulos, 2011; Zhou and Tan, 2017). IRP1 and IRP2 are two iron regulatory proteins that bind to IREs in order to regulate the translation or stability of these IRE-containing mRNAs. These mRNAs encode crucial iron metabolic proteins, such as δ-aminolevulinate synthase 2 (ALAS2), H- and L-ferritin, DMT-1, TfR1, ferroportin, hypoxia inducible factor-2α (HIF-2α), and others (Sanchez et al., 2007; Sebastiani and Pantopoulos, 2011; Lane et al., 2015; Yoshinaga et al., 2017). IRPs are activated by ID and other stimuli to bind to cognate IREs, which stabilizes TfR1 and DMT-1 mRNAs and inhibits specific translation of H- and L-ferritin, ferroportin, ALAS2, and HIF-2α mRNAs (Sebastiani and Pantopoulos, 2011).

During the first year of age, the brain experiences an extraordinary transformation from a relatively original into a complex organ. During this period, essential neurodevelopmental processes include synaptogenesis, the organization of neurotransmitter systems, and the onset of myelination, especially within the hippocampus, visual system, and auditory system (**Figure 2**, top part; Thompson and Nelson, 2001; Georgieff and Innis, 2005), and iron impacts on these developmental processes at multiple levels. Iron is a key nutrient that contributes to fetal and neonatal brain development is associated with critical

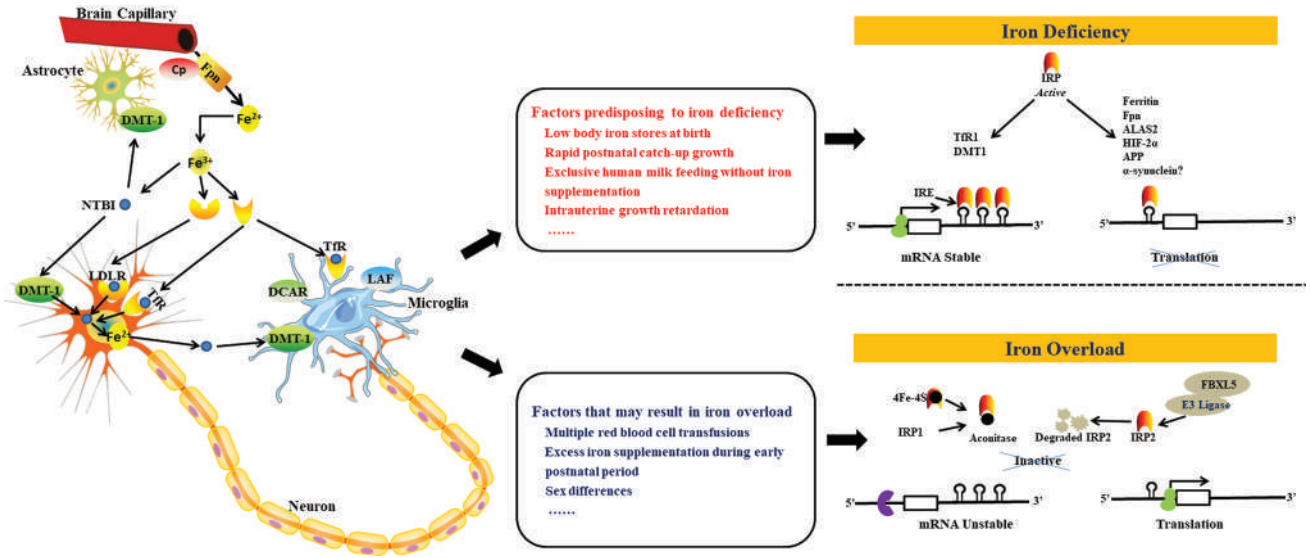


FIGURE 1 | Schematic depiction of processes and pathways involved in iron homeostasis and regulation in the brain. Iron can enter the brain through the blood–brain barrier and the choroid plexus (1). Transport of iron across the blood–brain barrier is mediated by the TfR-DMT-1-Fpn pathway in a similar manner to cells in the periphery. Fe^{2+} released from the basolateral surface of brain capillary endothelial cells by Fpn is rapidly oxidized to Fe^{3+} by Cp, secreted into the interstitium through the astrocyte endfeet, and then captured by transferrin that is expressed by cells of the choroid plexus. Iron can also enter the brain through astrocytes (2). A significant amount of Fe^{3+} ions in the CNS circulate attached to low molecular mass molecules secreted by astrocytes such as ascorbate, citrate, or ATP. The CNS also contains a significantly greater amount of NTBI than the periphery. Neurons express high levels of TfRs and acquire the bulk of their iron from transferrin under physiological conditions. Astrocytes, on the other hand, express DMT-1 and internalize Fe^{2+} ions in the form of NTBI. Microglia internalize TBI *via* TfRs as expected, but also utilize the dicarboxylic acid receptor and probably also the lactoferrin receptor. Neurons and other glial cells also acquire NTBI from upregulated DMT-1 under inflammatory conditions (*left part*). Some factors might disrupt this iron balance resulting in iron deficiency (*middle top*) or iron overload (*middle bottom*). The IRP-IRE system regulates iron uptake and storage by modulating the expression of mRNAs coding for iron uptake, storage, and export proteins. When CNS iron levels are low (*right top*), IRP binds to the 3′ IREs of target mRNAs (e.g. TfR1 and DMT1) thus stabilizing the transcript in order to enable translation and the subsequent increase in iron uptake. Concomitant binding to the 5′ IREs of target mRNAs (ferritin, Fpn, ALAS2, HIF-2α, APP, and, possibly, a-synuclein) prevents binding of the 43S preinitiation complex, thus inhibiting translation and reducing iron storage and efflux. In the presence of excess iron in the CNS (*right bottom*), IRP1 incorporates ISCs in order to acquire aconitase activity, while IRP2 is degraded. IRPs thus lose their affinity for IREs, resulting in the degradation of mRNAs with 3′ IRE sequences that code for iron uptake proteins and in the translation of mRNAs with 5′ IREs that code for iron storage and efflux proteins. Figure adapted and get permission from references (Singh et al., 2014; Morris et al., 2018). DMT-1, divalent metal transporter-1; Fpn, ferroportin; Cp, caeruloplasmin; CNS, central nervous system; NTBI, non-transferrin-bound iron; TfR, transferrin receptor; TBI, transferrin-bound iron; LDLR, low density lipoprotein receptor; DCDR, dicarboxylic acid receptor; LAF, lactoferrin; ALAS2, δ-aminolevulinate synthase 2; APP, amyloid precursor protein; HIF-2α, hypoxia-inducible factor-2α; ISC, iron–sulfur cluster; IREs, iron-responsive elements; IRP, iron regulatory protein.

cellular processes in the immature brain, including the maintenance of neural cell energy status, myelination, and monoamine neurotransmitter homeostasis (Bianco et al., 2008; Todorich et al., 2009; Cheli et al., 2018). The oligodendrocytes are related to myelin production (Sun et al., 2019), and there is an extremely complicated relationship between iron acquisition and myelin production. As a co-factor for cholesterol and lipid biosynthesis, iron directly participates in myelin production and is indirectly involved in oxidative metabolism (which is more likely to occur in oligodendrocytes than in other cells of the brain) (Todorich et al., 2009; Stephenson et al., 2014; Xu et al., 2014).

The neonatal brain is in a highly metabolic state, consuming approximately 60% of the total body oxygen consumption, while the adult brain only consumes about 20% of the total body oxygen consumption (Erecinska and Silver, 1989). Studies in rodents have shown that the high rate of metabolism is iron dependent. After feeding with purified diet of different levels of iron in young rats, cytochrome c and muscle myoglobin show similar degree impact as hemoglobin (Dallman, 1986). During the period of differentiation in neuronal and glial cells, a great amount of metabolic energy is needed for the process of migration, myelination, the establishment of synaptic contacts, and the extension of neuritic processes, especially in rapidly developing brain areas (Simons and Trajkovic, 2006; Li et al., 2011; Zhang et al., 2017a). Iron is mainly located in oligodendrocytes and microglia and is involved in numerous metabolic activities such as myelination, oligodendrocyte maturation, and microglial activation (Zhang et al., 2006; Bishop et al., 2011). Specific iron-requiring enzymes that contribute to maintaining a high level of metabolic activity, including the cytochrome oxidase system, glucose-6-phosphate dehydrogenase, dioxygenase, NADH dehydrogenase, and succinic dehydrogenase, which are all increased in oligodendrocytes compared with other kinds of cells in the brain (Todorich et al., 2009).

Because brain continues to develop during infancy and childhood, diet might have an influence on cognitive ability and behavior (Bryan et al., 2004; Huo et al., 2012; Prado and Dewey, 2014).

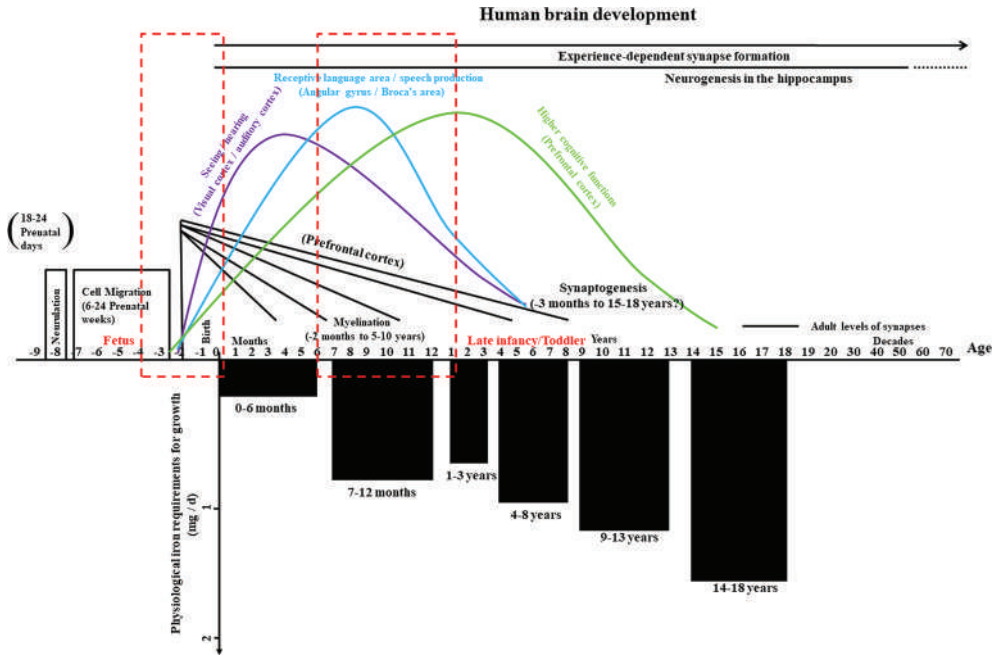


FIGURE 2 | Overview of human brain development and physiological iron requirement for growth in infancy and childhood. The upper part of this graph illustrates the important prenatal events – such as the formation of the neural tube (neurulation) and cell migration, critical aspects (seeing/hearing, receptive language area/speech production, and cognitive functions) of synapse formation and myelination beyond year three, and the formation of synapses based on experience – as well as neurogenesis in a key region of the hippocampus throughout much of life. Periods with high risks for alterations in iron metabolism during early human brain development are highlighted with dashed red boxes. The lower part of the graph shows the physiological iron requirements for growth in different stages during infancy and childhood. Chart adapted and appropriated permission have been obtained from references (Thompson and Nelson, 2001; Georgieff and Innis, 2005; Hider and Kong, 2013).

Iron is one of the most important micronutrients, and meeting its requirement is likely to have an advantageous impact on cognitive development in children (Sachdev et al., 2005). Imbalanced iron status has negative effects on psychological function due to altered activity of iron-containing enzymes in the brain (Benton, 2008; Scott et al., 2018). The neurodevelopmental effects of iron in preterm infants include its influences on the speed of neural processing and on general cognition. For example, early iron supplementation in preterm infants leads to a tendency toward beneficial impact on neurocognitive development at 5.3 years of age; however, as the original study was not designed to assess impacts on neurocognitive development, the power of the study was inadequate to investigate small but possible clinically related improvements, and further research in larger cases to prove this tendency are needed (Steinmacher et al., 2007).

IMPACT OF IRON IMBALANCE ON BRAIN DEVELOPMENT IN PRETERM INFANTS

Consequences of Iron Deficiency on Brain Function in Preterm Infants

Studies in animal models showed that compromised iron status leads to significant loss of cytochrome c oxidase activity in selected brain structures, especially in the hippocampus and prefrontal area (Carlson et al., 2007; Bastian et al., 2016). In preterm infants, ID has been found to lead to poor physical growth, decreased immunity, and temperature instability (Ohls et al., 2004; Ekiz et al., 2005; Aly et al., 2018). The impacts of ID are extensive and involve numerous organ systems; for example, skeletal muscle dysfunction and altered cardiac contractility might be caused by tissue ID (Stugiewicz et al., 2016; Hoes et al., 2018), but the main concern of early ID is its impact on brain development.

Preterm infants with the lowest quartile cord ferritin concentrations (<76 μg/L) have slower central nerve conduction velocities as measured by auditory brainstem-evoked response (Amin et al., 2010). A clinical trial investigating anemia (Hb ≤ 10 g/dl) and low iron stores (serum ferritin ≤76 μg/L) in preterm infants showed an increased number of abnormal neurologic reflexes (such as glabella reflex, Babinski reflex, plantar grasp, palmar grasp, passive movement of the arms, and passive movement of the legs) at 37 weeks gestational age compared with nonanemic, iron-replete infants (Armony-Sivan et al., 2004). Steinmacher et al. (2007) examined the effects of iron supplementation on neurocognitive development in low birth weight (LBW) premature newborns and found that iron supplementation during the early period (<61 days of age) had a tendency to ameliorate neurocognitive development compared with late supplementation (≥61 days of age). Interestingly, full-term infants with neonatal ID are more likely to be at risk of cognitive deficits, but motor

deficits such as fidgety movements (the movements could occur continuously in awake infants except during fussing and crying, and mainly refer to the small amplitude, moderate speed, and variable acceleration that occur in the neck, trunk, and limbs in all directions) seem to predominate in preterm infants (Bruggink et al., 2008).

Recent studies have demonstrated an correlation between ID/iron-deficiency anemia (IDA) and poor neuronal/cognitive consequences in newborns that lasts beyond the period of ID and might affect motor development, recognition memory, social-emotional behavior, and maturation of the CNS (Shafir et al., 2008; Luo et al., 2015; Scott et al., 2018; Otero et al., 2019; Wenger et al., 2019). Studies in term infants and animal models have shown great impacts of perinatal ID on brain development both acutely during the period of deficiency and long-term after iron levels have been restored. These effects include impaired learning and memory, poorer auditory recognition memory, and less cooperative, confident, and persistent personality (Lozoff and Georgieff, 2006; Lozoff et al., 2014; Geng et al., 2015).

More evidence comes from experimental studies that have shown that ID in the time period corresponding to human preterm infants has impacts on cognitive and behavioral function. Perinatal ID (from gestational day 2 until postnatal day 10) in a rat model reduced neuronal metabolic activity and negatively affected memory processing in selected regions of the neonatal brain (Siddappa et al., 2003). Other studies using rodents reported the impact of decreased levels of brain iron on various dopaminergic functions and dopamine-mediated behaviors by measuring brain iron and dopamine transporters and dopamine receptor density, especially when ID occurs in the first 3 weeks after birth (Beard et al., 2003). In addition, Unger et al. (2012) showed that ID in early life (including the gestational period and up to 8 days after birth) leads to acute and persistent changes in regional monoamine concentrations and significant abnormal motor performance in rats. A study using iron-deficient newborn rat pups found that replenishment of iron starting at postnatal day 4 could rectify the influence of ID on both iron levels and monoamine function in a variety of brain regions (Beard et al., 2007). Iron supplementation after the development of hypomyelination, however, is capable of correcting the motor and cognitive abnormalities due to the early ID. These specific deficits could be found in the striatal dopamine system (Youdim, 2008) demonstrating adverse development of the basal ganglia system, which plays critical roles in the initiation and control of movement, as well as in the hippocampus and cortex that are crucial for the functions of memory and cognition (Leisman et al., 2014; Qiu et al., 2016). A study in a rat perinatal ID model found that disrupting dendritic growth in the hippocampus has negative impacts on synaptogenesis (Jorgenson et al., 2003) and that ID also increases the risk of the developing brain even in response to mild hypoxia-ischemia (Rao et al., 2007). A correlation study in weanling rats showed an interesting association between iron, anxiety-like behavior, and dopaminergic system. What is more, nose pokes and rates of habituation were related to prefrontal cortical iron levels, whereas spontaneous activities, which had higher correlation with iron concentrations and the density of dopamine receptors in the ventral midbrain (Han and Kim, 2015). Another study also showed that iron levels in the brain are one of critical factors for anxiety-like behaviors (Breton et al., 2015). Thus, it is clear that ID can be detrimental to brain development and can increase the risk of poor neurodevelopment both in premature newborns and in neonatal animal models. ID might interfere with neurotransmitter metabolism and myelination (Estrada et al., 2014; Deoni et al., 2018), which affects the cognitive and behavioral function of the brain. Moreover, iron plays an important role in the synthesis of hemoglobin and myoglobin, and ID affects the transport and storage of oxygen and energy expenditure, which is adverse to the function of the brain (Wenger et al., 2019). In addition, some of the preclinical works we mentioned above were conducted only in males or females, or they used equal male or female pups in different groups, which indicate that there is no impact of sex differences on iron status in these preclinical studies.

Consequences of Iron Overload on Brain Development in Preterm Infants

Recent postmortem studies showed that premature neonates who receive multiple blood transfusions often exhibit iron excess (Park and Kim, 2015; Trevino-Baez et al., 2017). Free iron might be released from senescent red blood cells by transfusion hemolysis, and low circulating levels of transferrin and other iron-binding proteins in premature neonates might increase circulation of non-protein-bound iron. It has been shown that there is a tendency for iron excess because of the lacking of the ability of down-regulating iron absorption in neonatal animal models (Leong et al., 2003), and excess iron supplementation in infants might result in higher risk of impaired growth, infection, and disturbed metabolism of other minerals (Domellof, 2007; Stark et al., 2013).

Excess free iron is common in the pathogenesis of intraventricular hemorrhage (IVH) and has been shown to have adverse effects on the brain (Wu et al., 2019). IVH is particularly common in preterm neonates and carries with it high morbidity and mortality (Christian et al., 2016; Song et al., 2016). In the IVH rat model, injection of lysed red blood cells into the ventricles resulted in upregulation of periventricular heme oxygenase-1 (HO-1), while iron injection led to ependymal cell injury with mitochondrial swelling and loss of cilia (Gao et al., 2014). In addition, overexpression of HO-1 is involved in increased activated microglia, which produce more reactive oxygen species (ROS) after hemorrhagic brain injury (Zhang et al., 2017b). Another study with neonatal rats showed that Hb injection led to iron overload in the subventricular zone, which is a site of neuronal stem and progenitor cell proliferation (Strahle et al., 2014). IVH has also been shown to induce substantial damage to the bordering hippocampus in an iron-dependent fashion (Chen et al., 2011), which is likely to be mediated by iron-activated c-Jun N-terminal kinase apoptotic pathways (Garton et al., 2016a,b). In IVH, blood can disperse within the ventricular system, and free iron can accumulate in the ependymal and subependymal

regions as indicated by elevated ferritin and iron deposition in these cells (Garton et al., 2016a). Excess free iron released in the red blood cells can also increase the risk of oxidative injury due to hydroxyl radical generation (Lu and Black, 2016; Wu et al., 2019).

Studies have also reported negative effects of iron overload on cognitive development in experimental animal models of preterm infants (Schroder et al., 2013). Kaur et al. (2007) administered iron-fortified formula dose of iron to newborn mice and showed reduced dopamine levels in striatum, neurodegeneration in midbrain and enhanced vulnerability to toxic injury.

Oxidative stress mediated by excessive free iron under conditions of poor antioxidant capacity has been presumed to initiate the progressive loss of brain function in several diseases through the generation of ROS in preterm infants. The possible mechanism behind the negative impact of iron overload is still unknown but might be associated with the pro-oxidative impacts of iron overload or probably an association between iron and other nutrients involved in growth. Iron overload might augment brain oxidative stress status and decrease brain serotonin and dopamine by reacting with hydrogen peroxide and superoxide anions and by producing hydroxyl radicals and ROS as a result of brain cell injury (Elseweidy and Abd El-Baky, 2008; Yu et al., 2011). In these related oxidative stress pathologies, brain cell damage through lipid and protein peroxidation is caused by free iron, which is released from iron stores. Increased levels of lipid and protein peroxidation have been reported in hypoxic neonates, and the more severe the hypoxia the greater the intra-erythrocyte free iron release, ROS production, and oxidative damage (Lu et al., 2015; Sun et al., 2017).

RISK FACTORS FOR IRON IMBALANCE IN PRETERM INFANTS

Risk Factors for Iron Deficiency in Preterm Infants

Shorter Gestation Period

Many factors alone or combined contribute to negative iron balance in preterm infants, which is seen in 25–80% of preterm infants at some point during infancy (Vucic et al., 2013; Ferri et al., 2014). Different from term neonates, in whom the condition typically occurs during the second half of infancy, premature newborns are at risk for developing ID throughout infancy (MacQueen et al., 2017). In normal pregnancy, more than 80% of the iron in the body accumulates during the third trimester of gestation (Widdowson and Spray, 1951), whereas total body iron and hemoglobin content as well as serum and storage iron levels are much lower in premature infants (Siddappa et al., 2007), and premature infants are commonly born with much less than half of term infant's total body iron at birth. After birth, many preterm infants undergo severe and rapid reduction in hemoglobin (anemia of prematurity) and iron storage due to rapid growth, reduced erythropoiesis, and blood loss due to repeated phlebotomy (Jeon and Sin, 2013). Follow-up studies of premature neonates have indicated that ID can occur within 2 months of discharge from the neonatal intensive care unit (NICU) (Domellof and Georgieff, 2015) because preterm infants begin life in the NICU where a great amount of things can further perturb iron balance. In addition, a clinical study revealed that IDA of prematurity has a significant positive correlation with elevated zinc protoporphyrin/heme ratios (Bjorklund et al., 2017).

Timing of Umbilical Cord Clamping at Birth

The amount of blood that is transfused from the placenta to the neonates is very important for the total body iron level. Clinical studies have demonstrated that delayed cord clamping is helpful for establishing iron stores and preventing ID at 3–6 months of age in newborns with normal birth weight (Andersson et al., 2011, 2014; Kc et al., 2017). Delayed cord clamping might be even more crucial in LBW preterm neonates, and Mercer et al. (2006) concluded that delayed cord clamping of premature neonates is involved in decreased demand for blood transfusion, reduced incidence of IVH, and reduced incidence of late-onset sepsis.

Maternal Factors

Moderate maternal ID does not affect the iron endowment of their infants, but severe maternal ID does (Lonnerdal et al., 2015), and infants with ID are often born with low iron endowment, indicating the demand for sufficient iron stores at birth. The level of maternal iron status only accounts for about 6% of neonatal iron storage variability at birth, and it is not clear for other reasons that caused highly variation of birth endowment, but prematurity, LBW, intrauterine growth retardation, maternal smoking, and diabetes during pregnancy are likely to be significant factors (Siddappa et al., 2007; Lonnerdal et al., 2015). Neonates born to women with IDA during pregnancy mostly have serum iron concentrations and hematocrits at the same level as neonates born to iron-adequate women, but lower serum ferritin levels are likely to occur in newborns to iron-deficient mothers, indicating lower iron store levels (Shao et al., 2012). However, fetal iron exposure affects early infant growth but does not significantly improve iron status or absorption, and prenatal iron supplementation does not influence iron status of infants at 2 or 5 months of age (Finkelstein et al., 2013), which might indicate that maternal iron status only partly contributes to ID in preterm infants. In addition, the utilization of some drugs during pregnancy could have an impact on neonatal iron metabolism. For example, corticosteroid is used in some pregnant women with certain diseases even though it has a risk of neurodevelopmental impairment in newborns; however, it might increase the iron level (Naigamwalla et al., 2012; Boghossian et al., 2016).

Faster Growth

Tissue iron stores are consumed quickly in premature infants demonstrating rapid growth. The inadequate iron stores in these infants can be used up quickly during the first 6–8 weeks

after birth, coinciding with the onset of erythropoiesis and rapid catch-up growth (Rao and Georgieff, 2009). The minimum level of hematocrit/Hb ratio is lower and occurs earlier in the majority of preterm infants (gestational age 28–34 weeks) than in those born at a gestational age of 35–42 weeks, while this situation is even worse for premature infants (gestational age 23–28 weeks) due to early net fluid shifts with extravascular fluid moving into the vascular space, leading to dilution and a decrease in the hematocrit/Hb ratio (Jopling et al., 2009). From the age of 20–30 weeks, the average Hb level in premature newborns is lower than in full-term infants at the beginning of this age range, but this difference changes over the next 10 weeks. Interestingly, iron status at birth has no effect on the postnatal growth rate. Ferritin concentrations are initially lower in preterm infants, but these concentrations become similar between preterm infants and term infants over the course of the first year of life (Takala et al., 2010). With the increases in blood volume and Hb mass, the high rate of postnatal catch-up growth needs extra iron supplementation (Rao and Georgieff, 2009).

Feeding Mode

The iron concentration in human breast milk is about 0.35 mg/L (Bjorklund et al., 2012). Although iron absorption rate of human breast milk is better than neonate formula, it is obtained only 0.07 mg/kg per day for iron delivery from exclusive breast milk feeding. Although this iron can be well utilized, there is still a potential risk of developing IDA for neonates who are breastfed for more than 4–6 months without receiving iron-fortified complementary foods or iron supplements (Lonnerdal et al., 2015). Most premature neonates are not solely breastfed longer than 3 months and thus are dependent on iron in preterm and post-discharge formula. Low iron formulas containing less than 5 mg/L of iron do not satisfy the iron demands of the growing premature neonate (Baker et al., 2010). Newborns with poor iron status might require more iron; however, it is not clear if higher levels of iron fortification formula will lead to increased iron levels in infants who fed with formula up to 6 months old (Domellof et al., 2001). Interestingly, a recent study reported that preterm neonates have adequate iron storage at birth and at 2 months old and that they are not likely to require iron supplementation until at least 2 months of age (Saha et al., 2016). As mentioned above, many preterm infant characteristics and/or maternal factors have negative effects on iron status (**Figure 1**, middle upper). Although ID is more common in preterm neonates, other factors that cause iron overload should be considered.

Risk Factors for Iron Overload in Preterm Infants

Although IDA has been considered to be an issue in growing premature neonates, the impact of iron overload has not been thoroughly investigated. As an invasive test, liver biopsy is the gold standard for diagnosing iron excess, while serum ferritin level, which is a helpful biochemical assay, is usually utilized as a surrogate indicator to assess and guide treatment of iron excess in older children (Fleming and Ponka, 2012).

Medicinal Erythrocyte Transfusion

This is the main factor resulting in iron overload in preterm infants. Premature neonates that receive more erythrocyte transfusions not only could replace phlebotomy losses but also maintain certain level of Hb concentrations. Physicians use erythrocyte transfusions as a frequent intervention when treating preterm infants with very low birth weight (birth weight <1,500 g) (Trevino-Baez et al., 2017). This poses several risks, including iron overload (Park and Kim, 2015), because excess iron is not able to be eliminated by physiological pathways, even though the iron released after degradation of the transfused red blood cells increases body iron storage. Indeed, serum ferritin levels increase significantly with the first month after birth in premature neonates who receive multiple red blood cell transfusions, and there is a greater risk of iron excess in exposed preterm neonates in comparison with nonexposed infants (Herzlich et al., 2016).

Inappropriate Infant Formulas

The majority of neonate formulas contain 4–12 mg of iron/L, which is 10–60 times more compared with the concentration of iron in human breast milk. It might be debated whether neonate formula should contain such an excess of iron during the period of the first 6 months, which has no beneficial effect, so as to fit perceived iron demands at 6–12 months of life (Lonnerdal et al., 2015). A clinical study demonstrated that newborns with lower Hb (<106 g/L) benefit from neonate formula with a higher concentration of iron and indicated superior developmental outcomes at 10 years of age compared with those infants who were fed with formula with less iron concentration from 6 to 12 months of life (Lozoff et al., 2012). However, the infants with an initial Hb higher than 128 g/L had worse scores (especially for spatial memory and visual-motor integration) when formula with higher levels of iron was given.

Sex

Significant sex differences in iron overload have been observed during infancy. Molloy et al. studied 60 growing, stable premature neonates who had increased iron indices and found significantly greater increases in male infants (Molloy et al., 2009). Ziegler et al. also reported differences in iron levels between males and females and found sex differences in mean corpuscular volume (Ziegler et al., 2014).

In general, iatrogenic factors are responsible for excess iron accumulation in premature neonates, but other risk factors such as medical iron supplementation and infant formula containing a higher level of iron should not be ignored (**Figure 1**, middle bottom).

INDICATORS FOR IRON STATUS IN CLINICAL PRACTICE

Screening the iron status of mothers, neonates, and children is necessary to avoid long-term adverse health impacts for mothers and children, especially neurodevelopment abnormalities in the child caused by ID. In view of the current lack of

sufficient evidence, the standard for describing iron status clinically for healthy preterm infants is still unknown. Even though the iron level marker in amniotic fluid might be a potential indicator during pregnancy, some factors such as the expression of fetal oxidative stress factors might also significantly affect this trend Gazzolo et al. (2005). As summarized above, iron imbalance including both deficiency and overload has severe impacts on brain development, and thus it seems essential to establish the association between potential indicators such as non-TBI and neurological outcomes in infants.

Hb and ferritin are used as indicators of iron status in infants. Because most physiological changes in iron status and erythrocyte morphology occur during early development, age-specific cutoffs indicators of iron status should be utilized for preterm neonates with LBW (**Table 1**). In these clinical indicators for iron status, we could see that the thresholds appear to decrease slightly with advancing postnatal age, with the exception of the 2-month value (for example, Hb). For preterm infants with LBW, multiple factors can result in ID and iron overload even if the infants are without pathological disease, and so these clinical indicators are a little lower due to the different iron status in infants who choose to enroll in these studies (Domellof et al., 2002; Siddappa et al., 2007).

However, because ID is highly prevalent throughout the world, the indicators for detecting iron status are initially focused on identifying whether ID occurs. The present clinical indicators and proposed tests for monitoring ID include hematologic and non-hematologic measurements (**Table 2**). These indicators are changing gradually before individuals become more iron-deficient. However, to protect the developing brain, the two main points in this process are worthy to be noted. As summarized by Georgieff (2017), first, none of the markers directly index iron levels in brain tissue. In addition, it is unclear whether the brain is lacking iron in this process from sufficient to anemia unless it occurs prior to obvious anemia. Brain iron status detected by direct imaging would be expensive, as well as not currently possible due to the low sensitivity of MRI technology, which is not able to find low iron levels, although it can reveal iron excess (Langkammer et al., 2010). Neurobehavioral tests are attractive candidates as bio-indicators of brain iron status because it reflects iron-specific brain functions (Georgieff, 2017). However, an imbalance in other nutrients such as copper, zinc, and iodine can also lead to similar abnormal neurobehavior (Hagmeyer et al., 2014; Ganaie et al., 2015; Petro et al., 2016; Iglesias et al., 2018). Thus, none of the proper neurobehavioral tests can be used as a direct iron-specific indicator for indexing brain functions. However, some neurobehavioral tests such as the hesitancy and anxiety-like behavior tests might reflect iron status by affecting dopamine receptor/transporter status in animal models (Beard et al., 2002).

TABLE 1 | Clinical indicators for iron imbalance in LBW preterm infants at different ages.

	Newborn	2 months	4 months	6–24 months
ID: SF (μg/L)	<35	<40	<20	<10–12
IDA: Hb (g/L)	<135	<90	<105	<105
Iron overload: SF (μg/L)	>300	>300	>250	>200

ID, iron deficiency; LBW, low birth weight; SF, serum ferritin; IDA, iron deficiency anemia; Hb, hemoglobin. Data adapted from Domellof et al. (2002) and Siddappa et al. (2007).

TABLE 2 | Clinical indicators for monitoring iron deficiency and iron deficiency anemia.

Condition	Physiology	Current test	Proposed test
Mild ID	Mobilized available iron	↓Serum iron, ↓SF	↓Hepcidin, ↓CHr, Perl's staining (–)
Moderate ID	Increased iron delivery	↑TIBC, ↓TSAT, ↑sTfR	↓Hepcidin, ↓CHr, Perl's staining (–)
Moderate to severe ID	Altered RBC morphology	↓MCV, ↑ZPP	↓Hepcidin, ↓CHr, Perl's staining (–)
IDA	Impaired RBC production	↓Hb	↓↓Hepcidin, ↓CHr, Perl's staining (–)

ID, iron deficiency; IDA, iron deficiency anemia; MCV, mean corpuscular volume; RBC, red blood cell; SF, serum ferritin; sTfR, soluble transferrin receptor; TIBC, total iron binding capacity; TSAT, transferrin saturation; ZPP, zinc protoporphyrin; Hb, hemoglobin; CHr, reticulocyte hemoglobin concentration; Perl's staining, Perl's staining of bone marrow for iron. ↑, increased; ↓, reduced; (–), negative. Data adapted from Baker et al. (2010), Wilson and Sloan (2015), and Georgieff (2017).

CURRENT RECOMMENDATIONS FOR IRON SUPPLEMENTATION IN PRETERM INFANTS

The physiological iron requirements for growth vary in different stages during infancy and childhood (Hider and Kong, 2013; **Figure 2**, bottom part). It is assumed that the iron absorption rate can be up to 50% from human breast milk and that it is about 10% from neonate formula and iron-fortified complementary foods (Saarinen et al., 1977), but some factors, as summarized in this article, probably result in iron imbalance. Thus, proper iron supplementation is crucial, especially for preterm infants who are at high risk of iron imbalance. The recommendation for iron supplementation in preterm infants from the American Academy of Pediatrics is that breastfed premature infant requires to be supplemented with 2 mg/kg of iron per day from 1 to 12 months old. Premature neonates will get about 1.8–2.2 mg/kg/day of iron by feeding a standard neonate formula (14.6 mg/L of iron) or a standard full-term neonate formula (12.0 mg/L of iron; Baker et al., 2010).

Despite the use of iron-containing formulas, some preterm infants develop ID during the first year of life. Thus, some formula-fed premature neonates might require an extra iron supplement; nevertheless, there is insufficient evidence to confirm this as a common recommendation at this time. In clinical practice, premature neonates who receive multiple blood transfusions are exceptions, so they might not require any iron supplementation (Baker et al., 2010).

Another recommendation for iron supplementation in preterm infants from the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) concluded that iron

supplementation of preterm infants with slight LBW at a dose of 1–2 mg/kg/day up to 6 months has few adverse effects and decreases the risk for later adverse cognitive and behavioral performance (Lozoff et al., 2006; Domellof et al., 2014). According to the requirement of ESPGHAN enteral nutrition guidelines for premature neonates, newborn with birth weight <2,000 g should be supplemented with 2–3 mg/kg of iron (Agostoni et al., 2010). Because iron stores are usually used up at about 6 months old, iron-rich complementary foods are recommended. Even if iron-fortified follow-on formulas should be supplemented, determining the optimal level of iron in follow-on formulas still lacks sufficient evidence. When the infants grow up to 6 months old, it is necessary to give them iron-rich food. Before 12 months of age, unmodified cow's milk is not suggested to infants as the main milk drink (Agostoni et al., 2010).

REMARKS

Preterm infants are at high risk of iron imbalance, and ID and iron overload are important nutritional issues in preterm infants. The potential risk for neurodevelopmental abnormalities caused by ID requires regular screening and preventive measures. It is also beneficial and safe for preterm infants to be given iron supplementation. On the other side, iron overload is another significant concern in preterm infants; however, the management of premature infants who have excess iron has not been well investigated. Because iron levels in premature neonates vary greatly, we should monitor its status carefully during neonatal and post-discharge periods. The gestational age-specific clinical indicators for evaluating iron status and the neurobehavioral examinations reflecting iron-specific brain function are necessary to be developed. In the previous iron status and iron supplementation studies, most of them were conducted before the period of increased survival of high-risk premature neonates. Thus, randomized and well-controlled trials are required to establish iron supplement guidelines for these preterm infants.

AUTHOR CONTRIBUTIONS

CZ devised the review. YWa, YWu, and TL reviewed the literature and wrote the manuscript drafts. XW and CZ substantially contributed to the literature review and the writing of this manuscript.

FUNDING

This work was financed by the National Key Research and Development Program of China (2018YFC1004604), National Nature Science Foundation of China (31761133015, U1704281), the Swedish Research Council (2015-02845, 2013-2475, 2015-06276), Swedish Governmental grants to scientists working in health care (ALFGBG-717791, ALFGBG-429801), the Henan Provincial Science and Technology Department (171100310200), and the Brain Foundation (FO2018-0090).

REFERENCES

Agostoni, C., Buonocore, G., Carnielli, V. P., De Curtis, M., Darmaun, D., Decsi, T., et al. (2010). Enteral nutrient supply for preterm infants: commentary from the European society of paediatric gastroenterology, hepatology and nutrition committee on nutrition. *J. Pediatr. Gastroenterol. Nutr.* 50, 85–91. doi: 10.1097/MPG.0b013e3181adaee0

Albertsson, A. M., Bi, D., Duan, L., Zhang, X., Leavenworth, J. W., Qiao, L., et al. (2014). The immune response after hypoxia-ischemia in a mouse model of preterm brain injury. *J. Neuroinflammation* 11:153. doi: 10.1186/s12974-014-0153-z

Aly, S. S., Fayed, H. M., Ismail, A. M., and Abdel Hakeem, G. L. (2018). Assessment of peripheral blood lymphocyte subsets in children with iron deficiency anemia. *BMC Pediatr.* 18:49. doi: 10.1186/s12887-018-0990-5

Amin, S. B., Orlando, M., Eddins, A., MacDonald, M., Monczynski, C., and Wang, H. (2010). In utero iron status and auditory neural maturation in premature infants as evaluated by auditory brainstem response. *J. Pediatr.* 156, 377–381. doi: 10.1016/j.jpeds.2009.09.049

Andersson, O., Domellof, M., Andersson, D., and Hellstrom-Westas, L. (2014). Effect of delayed vs early umbilical cord clamping on iron status and neurodevelopment at age 12 months: a randomized clinical trial. *JAMA Pediatr.* 168, 547–554. doi: 10.1001/jamapediatrics.2013.4639

Andersson, O., Hellstrom-Westas, L., Andersson, D., and Domellof, M. (2011). Effect of delayed versus early umbilical cord clamping on neonatal outcomes and iron status at 4 months: a randomised controlled trial. *BMJ* 343:d7157. doi: 10.1136/bmj.d7157

Armony-Sivan, R., Eidelman, A. I., Lanir, A., Sredni, D., and Yehuda, S. (2004). Iron status and neurobehavioral development of premature infants. *J. Perinatol.* 24, 757–762. doi: 10.1038/sj.jp.7211178

Baker, R. D., Greer, F. R., and Committee on Nutrition American Academy of Pediatrics. (2010). Diagnosis and prevention of iron deficiency and iron-deficiency anemia in infants and young children (0–3 years of age). *Pediatrics* 126, 1040–1050. doi: 10.1542/peds.2010-2576

Bartnikas, T. B. (2012). Known and potential roles of transferrin in iron biology. *Biometals* 25, 677–686. doi: 10.1007/s10534-012-9520-3

Bastian, T. W., von Hohenberg, W. C., Mickelson, D. J., Lanier, L. M., and Georgieff, M. K. (2016). Iron deficiency impairs developing hippocampal neuron gene expression, energy metabolism, and dendrite complexity. *Dev. Neurosci.* 38, 264–276. doi: 10.1159/000448514

Beard, J. L., Erikson, K. M., and Jones, B. C. (2002). Neurobehavioral analysis of developmental iron deficiency in rats. *Behav. Brain Res.* 134, 517–524. doi: 10.1016/S0166-4328(02)00092-X

Beard, J., Erikson, K. M., and Jones, B. C. (2003). Neonatal iron deficiency results in irreversible changes in dopamine function in rats. *J. Nutr.* 133, 1174–1179. doi: 10.1093/jn/133.4.1174

Beard, J. L., Unger, E. L., Bianco, L. E., Paul, T., Rundle, S. E., and Jones, B. C. (2007). Early postnatal iron repletion overcomes lasting effects of gestational iron deficiency in rats. *J. Nutr.* 137, 1176–1182. doi: 10.1093/jn/137.5.1176

Benton, D. (2008). Micronutrient status, cognition and behavioral problems in childhood. *Eur. J. Nutr.* 47(Suppl. 3), 38–50. doi: 10.1007/s00394-008-3004-9

Beppu, K., Sasaki, T., Tanaka, K. F., Yamanaka, A., Fukazawa, Y., Shigemoto, R., et al. (2014). Optogenetic countering of glial acidosis suppresses glial glutamate release and ischemic brain damage. *Neuron* 81, 314–320. doi: 10.1016/j.neuron.2013.11.011

Bianco, L. E., Wiesinger, J., Earley, C. J., Jones, B. C., and Beard, J. L. (2008). Iron deficiency alters dopamine uptake and response to L-DOPA injection in Sprague-Dawley rats. *J. Neurochem.* 106, 205–215. doi: 10.1111/j.1471-4159.2008.05358.x

Bishop, G. M., Dang, T. N., Dringen, R., and Robinson, S. R. (2011). Accumulation of non-transferrin-bound iron by neurons, astrocytes, and microglia. *Neurotox. Res.* 19, 443–451. doi: 10.1007/s12640-010-9195-x

Bjorklund, G., Aaseth, J., Skalny, A. V., Suliburska, J., Skalnaya, M. G., Nikonorov, A. A., et al. (2017). Interactions of iron with manganese, zinc, chromium, and selenium as related to prophylaxis and treatment of iron deficiency. *J. Trace Elem. Med. Biol.* 41, 41–53. doi: 10.1016/j.jtemb.2017.02.005

Bjorklund, K. L., Vahter, M., Palm, B., Grander, M., Lignell, S., and Berglund, M. (2012). Metals and trace element concentrations in breast milk of first time healthy mothers: a biological monitoring study. *Environ. Health* 11:92. doi: 10.1186/1476-069X-11-92

Boghossian, N. S., McDonald, S. A., Bell, E. F., Carlo, W. A., Brumbaugh, J. E., Stoll, B. J., et al. (2016). Association of antenatal corticosteroids with mortality, morbidity, and neurodevelopmental outcomes in extremely preterm multiple gestation infants. *JAMA Pediatr.* 170, 593–601. doi: 10.1001/jamapediatrics.2016.0104

Breton, A. B., Fox, J. A., Brownson, M. P., and McEchron, M. D. (2015). Postnatal nutritional iron deficiency impairs dopaminergic-mediated synaptic plasticity in the CA1 area of the hippocampus. *Nutr. Neurosci.* 18, 241–247. doi: 10.1179/1476830514Y.0000000121

Bruggink, J. L., Einspieler, C., Butcher, P. R., Van Braeckel, K. N., Prechtl, H. F., and Bos, A. F. (2008). The quality of the early motor repertoire in preterm infants predicts minor neurologic dysfunction at school age. *J. Pediatr.* 153, 32–39. doi: 10.1016/j.jpeds.2007.12.047

Bryan, J., Osendarp, S., Hughes, D., Calvaresi, E., Baghurst, K., and van Klinken, J. W. (2004). Nutrients for cognitive development in school-aged children. *Nutr. Rev.* 62, 295–306. doi: 10.1111/j.1753-4887.2004.tb00055.x

Carlson, E. S., Stead, J. D., Neal, C. R., Petryk, A., and Georgieff, M. K. (2007). Perinatal iron deficiency results in altered developmental expression of genes mediating energy metabolism and neuronal morphogenesis in hippocampus. *Hippocampus* 17, 679–691. doi: 10.1002/hipo.20307

Cheli, V. T., Gonzalez Santiago, D. A., Marziali, L. N., Zamora, N. N., Guitart, M. E., Spreuer, V., et al. (2018). The divalent metal transporter 1 (DMT1) is required for iron uptake and normal development of oligodendrocyte progenitor cells. *J. Neurosci.* 38, 9142–9159. doi: 10.1523/JNEUROSCI.1447-18.2018

Chen, Z., Gao, C., Hua, Y., Keep, R. F., Muraszko, K., and Xi, G. (2011). Role of iron in brain injury after intraventricular hemorrhage. *Stroke* 42, 465–470. doi: 10.1161/STROKEAHA.110.602755

Choudhury, V., Amin, S. B., Agarwal, A., Srivastava, L. M., Soni, A., and Saluja, S. (2015). Latent iron deficiency at birth influences auditory neural maturation in late preterm and term infants. *Am. J. Clin. Nutr.* 102, 1030–1034. doi: 10.3945/ajcn.115.113084

Christian, E. A., Jin, D. L., Attenello, F., Wen, T., Cen, S., Mack, W. J., et al. (2016). Trends in hospitalization of preterm infants with intraventricular hemorrhage and hydrocephalus in the United States, 2000–2010. *J. Neurosurg. Pediatr.* 17, 260–269. doi: 10.3171/2015.7.PEDS15140

Chung, B., Matak, P., McKie, A. T., and Sharp, P. (2007). Leptin increases the expression of the iron regulatory hormone hepcidin in HuH7 human hepatoma cells. *J. Nutr.* 137, 2366–2370. doi: 10.1093/jn/137.11.2366

Cusick, S. E., Georgieff, M. K., and Rao, R. (2018). Approaches for reducing the risk of early-life iron deficiency-induced brain dysfunction in children. *Nutrients* 10, pii:E227. doi: 10.3390/nu10020227

Dallman, P. R. (1986). Biochemical basis for the manifestations of iron deficiency. *Annu. Rev. Nutr.* 6, 13–40. doi: 10.1146/annurev.nu.06.070186.000305

Deoni, S., Dean, D. 3rd, Joelson, S., O'Regan, J., and Schneider, N. (2018). Early nutrition influences developmental myelination and cognition in infants and young children. *NeuroImage* 178, 649–659. doi: 10.1016/j.neuroimage.2017.12.056

Domellof, M. (2007). Iron requirements, absorption and metabolism in infancy and childhood. *Curr. Opin. Clin. Nutr. Metab. Care* 10, 329–335. doi: 10.1097/MCO.0b013e3280523aaf

Domellof, M., Braegger, C., Campoy, C., Colomb, V., Decsi, T., Fewtrell, M., et al. (2014). Iron requirements of infants and toddlers. *J. Pediatr. Gastroenterol. Nutr.* 58, 119–129. doi: 10.1097/MPG.0000000000000206

Domellof, M., Cohen, R. J., Dewey, K. G., Hernell, O., Rivera, L. L., and Lonnerdal, B. (2001). Iron supplementation of breast-fed Honduran and Swedish infants from 4 to 9 months of age. *J. Pediatr.* 138, 679–687. doi: 10.1067/mpd.2001.112895

Domellof, M., Dewey, K. G., Lonnerdal, B., Cohen, R. J., and Hernell, O. (2002). The diagnostic criteria for iron deficiency in infants should be reevaluated. *J. Nutr.* 132, 3680–3686. doi: 10.1093/jn/132.12.3680

Domellof, M., and Georgieff, M. K. (2015). Postdischarge iron requirements of the preterm infant. *J. Pediatr.* 167(Suppl. 4), S31–S35. doi: 10.1016/j.jpeds.2015.07.018

Ekiz, C., Agaoglu, L., Karakas, Z., Gurel, N., and Yalcin, I. (2005). The effect of iron deficiency anemia on the function of the immune system. *Hematol. J.* 5, 579–583. doi: 10.1038/sj.thj.6200574

El Bana, S. M., Maher, S. E., Gaber, A. F., and Aly, S. S. (2016). Serum and urinary malondialdehyde (MDA), uric acid, and protein as markers of perinatal asphyxia. *Electron. Physician* 8, 2614–2619. doi: 10.19082/2614

Elseweidy, M. M., and Abd El-Baky, A. E. (2008). Effect of dietary iron overload in rat brain: oxidative stress, neurotransmitter level and serum metal ion in relation to neurodegenerative disorders. *Indian J. Exp. Biol.* 46, 855–858.

Erecinska, M., and Silver, I. A. (1989). ATP and brain function. *J. Cereb. Blood Flow Metab.* 9, 2–19. doi: 10.1038/jcbfm.1989.2

Estrada, J. A., Contreras, I., Pliego-Rivero, F. B., and Otero, G. A. (2014). Molecular mechanisms of cognitive impairment in iron deficiency: alterations in brain-derived neurotrophic factor and insulin-like growth factor expression and function in the central nervous system. *Nutr. Neurosci.* 17, 193–206. doi: 10.1179/1476830513Y.0000000084

Ferri, C., Procianoy, R. S., and Silveira, R. C. (2014). Prevalence and risk factors for iron-deficiency anemia in very-low-birth-weight preterm infants at 1 year of corrected age. *J. Trop. Pediatr.* 60, 53–60. doi: 10.1093/tropej/fmt077

Finch, C. A., and Huebers, H. (1982). Perspectives in iron metabolism. *N. Engl. J. Med.* 306, 1520–1528. doi: 10.1056/NEJM198206243062504

Finkelstein, J. L., O'Brien, K. O., Abrams, S. A., and Zavaleta, N. (2013). Infant iron status affects iron absorption in Peruvian breastfed infants at 2 and 5 mo of age. *Am. J. Clin. Nutr.* 98, 1475–1484. doi: 10.3945/ajcn.112.056945

Fleming, R. E., and Ponka, P. (2012). Iron overload in human disease. *N. Engl. J. Med.* 366, 348–359. doi: 10.1056/NEJMra1004967

Ganaie, M. A., Charoo, B. A., Sofi, R. A., Ahmed, A., and Bhat, J. I. (2015). Maternal overt hypothyroidism and neurobehavioral outcome of neonates: a cohort study from an iodine-deficient area of Northern India. *Indian Pediatr.* 52, 864–866. doi: 10.1007/s13312-015-0733-8

Gao, C., Du, H., Hua, Y., Keep, R. F., Strahle, J., and Xi, G. (2014). Role of red blood cell lysis and iron in hydrocephalus after intraventricular hemorrhage. *J. Cereb. Blood Flow Metab.* 34, 1070–1075. doi: 10.1038/jcbfm.2014.56

Garton, T. P., He, Y., Garton, H. J., Keep, R. F., Xi, G., and Strahle, J. M. (2016b). Hemoglobin-induced neuronal degeneration in the hippocampus after neonatal intraventricular hemorrhage. *Brain Res.* 1635, 86–94. doi: 10.1016/j.brainres.2015.12.060

Garton, T., Keep, R. F., Hua, Y., and Xi, G. (2016a). Brain iron overload following intracranial haemorrhage. *Stroke Vasc. Neurol.* 1, 172–184. doi: 10.1136/svn-2016-000042

Gazzolo, D., Perrone, S., Paffetti, P., Longini, M., Vezzosi, P., Bruschettini, M., et al. (2005). Non protein bound iron concentrations in amniotic fluid. *Clin. Biochem.* 38, 674–677. doi: 10.1016/j.clinbiochem.2005.03.010

Geng, F., Mai, X., Zhan, J., Xu, L., Zhao, Z., Georgieff, M., et al. (2015). Impact of fetal-neonatal iron deficiency on recognition memory at 2 months of age. *J. Pediatr.* 167, 1226–1232. doi: 10.1016/j.jpeds.2015.08.035

Georgieff, M. K. (2017). Iron assessment to protect the developing brain. *Am. J. Clin. Nutr.* 106(Suppl. 6), 1588S–1593S. doi: 10.3945/ajcn.117.155846

Georgieff, M. K., and Innis, S. M. (2005). Controversial nutrients that potentially affect preterm neurodevelopment: essential fatty acids and iron. *Pediatr. Res.* 57, 99R–103R. doi: 10.1203/01.PDR.0000160542.69840.0F

Gkouvatsos, K., Papanikolaou, G., and Pantopoulos, K. (2012). Regulation of iron transport and the role of transferrin. *Biochim. Biophys. Acta* 1820, 188–202. doi: 10.1016/j.bbagen.2011.10.013

Haga, P. (1980). Plasma ferritin concentrations in preterm infants in cord blood and during the early anaemia of prematurity. *Acta Paediatr. Scand.* 69, 637–641. doi: 10.1111/j.1651-2227.1980.tb07335.x

Hagmeyer, S., Haderspeck, J. C., and Grabrucker, A. M. (2014). Behavioral impairments in animal models for zinc deficiency. *Front. Behav. Neurosci.* 8:443. doi: 10.3389/fnbeh.2014.00443

Han, M., and Kim, J. (2015). Effect of dietary iron loading on recognition memory in growing rats. *PLoS One* 10:e0120609. doi: 10.1371/journal.pone.0120609

Hare, D. J., Arora, M., Jenkins, N. L., Finkelstein, D. I., Doble, P. A., and Bush, A. I. (2015). Is early-life iron exposure critical in neurodegeneration? *Nat. Rev. Neurol.* 11, 536–544. doi: 10.1038/nrneurol.2015.100

Hentze, M. W., Muckenthaler, M. U., Galy, B., and Camaschella, C. (2010). Two to tango: regulation of mammalian iron metabolism. *Cell* 142, 24–38. doi: 10.1016/j.cell.2010.06.028

Herzlich, J., Litmanovitz, I., Regev, R., Bauer, S., Sirota, G., Steiner, Z., et al. (2016). Iron homeostasis after blood transfusion in stable preterm infants - an observational study. *J. Perinat. Med.* 44, 919–923. doi: 10.1515/jpm-2015-0361

Hider, R. C., and Kong, X. (2013). Iron: effect of overload and deficiency. *Met. Ions Life Sci.* 13, 229–294. doi: 10.1007/978-94-007-7500-8_8

Hoes, M. F., Grote Beverborg, N., Kijlstra, J. D., Kuipers, J., Swinkels, D. W., Giepmans, B. N. G., et al. (2018). Iron deficiency impairs contractility of human cardiomyocytes through decreased mitochondrial function. *Eur. J. Heart Fail.* 20, 910–919. doi: 10.1002/ejhf.1154

Huo, K., Sun, Y., Li, H., Du, X., Wang, X., Karlsson, N., et al. (2012). Lithium reduced neural progenitor apoptosis in the hippocampus and ameliorated functional deficits after irradiation to the immature mouse brain. *Mol. Cell. Neurosci.* 51, 32–42. doi: 10.1016/j.mcn.2012.07.002

Iglesias, L., Canals, J., and Arija, V. (2018). Effects of prenatal iron status on child neurodevelopment and behavior: a systematic review. *Crit. Rev. Food Sci. Nutr.* 58, 1604–1614. doi: 10.1080/10408398.2016.1274285

Jeon, G. W., and Sin, J. B. (2013). Risk factors of transfusion in anemia of very low birth weight infants. *Yonsei Med. J.* 54, 366–373. doi: 10.3349/ymj.2013.54.2.366

Jopling, J., Henry, E., Wiedmeier, S. E., and Christensen, R. D. (2009). Reference ranges for hematocrit and blood hemoglobin concentration during the neonatal period: data from a multihospital health care system. *Pediatrics* 123, e333–e337. doi: 10.1542/peds.2008-2654

Jorgenson, L. A., Wobken, J. D., and Georgieff, M. K. (2003). Perinatal iron deficiency alters apical dendritic growth in hippocampal CA1 pyramidal neurons. *Dev. Neurosci.* 25, 412–420. doi: 10.1159/000075667

Kaur, D., Peng, J., Chinta, S. J., Rajagopalan, S., Di Monte, D. A., Cherny, R. A., et al. (2007). Increased murine neonatal iron intake results in Parkinson-like neurodegeneration with age. *Neurobiol. Aging* 28, 907–913. doi: 10.1016/j.neurobiolaging.2006.04.003

Kc, A., Rana, N., Malqvist, M., Jarawka Ranneberg, L., Subedi, K., and Andersson, O. (2017). Effects of delayed umbilical cord clamping vs early clamping on anemia in infants at 8 and 12 months: a randomized clinical trial. *JAMA Pediatr.* 171, 264–270. doi: 10.1001/jamapediatrics.2016.3971

Lane, D. J., Merlot, A. M., Huang, M. L., Bae, D. H., Jansson, P. J., Sahni, S., et al. (2015). Cellular iron uptake, trafficking and metabolism: key molecules and mechanisms and their roles in disease. *Biochim. Biophys. Acta* 1853, 1130–1144. doi: 10.1016/j.bbamcr.2015.01.021

Langkammer, C., Krebs, N., Goessler, W., Scheurer, E., Ebner, F., Yen, K., et al. (2010). Quantitative MR imaging of brain iron: a postmortem validation study. *Radiology* 257, 455–462. doi: 10.1148/radiol.10100495

Laptook, A. R. (2016). Birth asphyxia and hypoxic-ischemic brain injury in the preterm infant. *Clin. Perinatol.* 43, 529–545. doi: 10.1016/j.clp.2016.04.010

Leisman, G., Braun-Benjamin, O., and Melillo, R. (2014). Cognitive-motor interactions of the basal ganglia in development. *Front. Syst. Neurosci.* 8:16. doi: 10.3389/fnsys.2014.00016

Leong, W. I., Bowlus, C. L., Tallkvist, J., and Lonnerdal, B. (2003). Iron supplementation during infancy--effects on expression of iron transporters, iron absorption, and iron utilization in rat pups. *Am. J. Clin. Nutr.* 78, 1203–1211. doi: 10.1093/ajcn/78.6.1203

Li, H., Li, Q., Du, X., Sun, Y., Wang, X., Kroemer, G., et al. (2011). Lithium-mediated long-term neuroprotection in neonatal rat hypoxia-ischemia is associated with antiinflammatory effects and enhanced proliferation and survival of neural stem/progenitor cells. *J. Cereb. Blood Flow Metab.* 31, 2106–2115. doi: 10.1038/jcbfm.2011.75

Liu, Z., Shen, H. C., Lian, T. H., Mao, L., Tang, S. X., Sun, L., et al. (2017). Iron deposition in substantia nigra: abnormal iron metabolism, neuroinflammatory mechanism and clinical relevance. *Sci. Rep.* 7:14973. doi: 10.1038/s41598-017-14721-1

Lonnerdal, B., Georgieff, M. K., and Hernell, O. (2015). Developmental physiology of iron absorption, homeostasis, and metabolism in the healthy term infant. *J. Pediatr.* 167(Suppl. 4), S8–S14. doi: 10.1016/j.jpeds.2015.07.014

Lozoff, B., Beard, J., Connor, J., Barbara, F., Georgieff, M., and Schallert, T. (2006). Long-lasting neural and behavioral effects of iron deficiency in infancy. *Nutr. Rev.* 64(5 Pt 2), S34–S43; discussion S72-S91. doi: 10.1111/j.1753-4887.2006.tb00243.x

Lozoff, B., Castillo, M., Clark, K. M., and Smith, J. B. (2012). Iron-fortified vs low-iron infant formula: developmental outcome at 10 years. *Arch. Pediatr. Adolesc. Med.* 166, 208–215. doi: 10.1001/archpediatrics.2011.197

Lozoff, B., Castillo, M., Clark, K. M., Smith, J. B., and Sturza, J. (2014). Iron supplementation in infancy contributes to more adaptive behavior at 10 years of age. *J. Nutr.* 144, 838–845. doi: 10.3945/jn.113.182048

Lozoff, B., and Georgieff, M. K. (2006). Iron deficiency and brain development. *Semin. Pediatr. Neurol.* 13, 158–165. doi: 10.1016/j.spen.2006.08.004

Lu, Q., and Black, S. M. (2016). Iron metabolism, oxidative stress, and neonatal brain injury. *Neural Regen. Res.* 11, 725–726. doi: 10.4103/1673-5374.182691

Lu, Q., Harris, V. A., Rafikov, R., Sun, X., Kumar, S., and Black, S. M. (2015). Nitric oxide induces hypoxia ischemic injury in the neonatal brain via the disruption of neuronal iron metabolism. *Redox Biol.* 6, 112–121. doi: 10.1016/j.redox.2015.06.007

Luo, R., Shi, Y., Zhou, H., Yue, A., Zhang, L., Sylvia, S., et al. (2015). Micronutrient deficiencies and developmental delays among infants: evidence from a cross-sectional survey in rural China. *BMJ Open* 5:e008400. doi: 10.1136/bmjopen-2015-008400

MacQueen, B. C., Christensen, R. D., Ward, D. M., Bennett, S. T., O'Brien, E. A., Sheffield, M. J., et al. (2017). The iron status at birth of neonates with risk factors for developing iron deficiency: a pilot study. *J. Perinatol.* 37, 436–440. doi: 10.1038/jp.2016.234

McCarthy, R. C., and Kosman, D. J. (2014). Glial cell ceruloplasmin and hepcidin differentially regulate iron efflux from brain microvascular endothelial cells. *PLoS One* 9:e89003. doi: 10.1371/journal.pone.0089003

McCarthy, R. C., and Kosman, D. J. (2015). Iron transport across the blood-brain barrier: development, neurovascular regulation and cerebral amyloid angiopathy. *Cell. Mol. Life Sci.* 72, 709–727. doi: 10.1007/s00018-014-1771-4

Mercer, J. S., Vohr, B. R., McGrath, M. M., Padbury, J. F., Wallach, M., and Oh, W. (2006). Delayed cord clamping in very preterm infants reduces the incidence of intraventricular hemorrhage and late-onset sepsis: a randomized, controlled trial. *Pediatrics* 117, 1235–1242. doi: 10.1542/peds.2005-1706

Molloy, E. J., El-Khuffash, A., Bieda, A., Jelinek, M. M., and Baley, J. (2009). Elevated iron indices in preterm infants: association with male gender. *Am. J. Perinatol.* 26, 7–11. doi: 10.1055/s-0028-1090588

Morris, G., Berk, M., Carvalho, A. F., Maes, M., Walker, A. J., and Puri, B. K. (2018). Why should neuroscientists worry about iron? The emerging role of ferroptosis in the pathophysiology of neuroprogressive diseases. *Behav. Brain Res.* 341, 154–175. doi: 10.1016/j.bbr.2017.12.036

Naigamwalla, D. Z., Webb, J. A., and Giger, U. (2012). Iron deficiency anemia. *Can. Vet. J.* 53, 250–256.

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., et al. (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 306, 2090–2093. doi: 10.1126/science.1104742

Niatsetskaya, Z. V., Charlagorla, P., Matsukevich, D. A., Sosunov, S. A., Mayurasakorn, K., Ratner, V. I., et al. (2012). Mild hypoxemia during initial reperfusion alleviates the severity of secondary energy failure and protects brain in neonatal mice with hypoxic-ischemic injury. *J. Cereb. Blood Flow Metab.* 32, 232–241. doi: 10.1038/jcbfm.2011.164

Ohls, R. K., Ehrenkranz, R. A., Das, A., Dusick, A. M., Yolton, K., Romano, E., et al. (2004). Neurodevelopmental outcome and growth at 18 to 22 months' corrected age in extremely low birth weight infants treated with early erythropoietin and iron. *Pediatrics* 114, 1287–1291. doi: 10.1542/peds.2003-1129-L

Otero, G. A., Fernandez, T., Pliego-Rivero, F. B., and Mendieta, G. G. (2019). Iron therapy substantially restores qEEG maturational lag among iron-deficient anemic infants. *Nutr. Neurosci.* 22, 363–372. doi: 10.1080/1028415X.2017.1391529

Park, S. H., and Kim, H. M. (2015). The iron status of very low birth weight infants receiving multiple erythrocyte transfusions during hospitalization in the neonatal intensive care unit. *Pediatr. Gastroenterol. Hepatol. Nutr.* 18, 100–107. doi: 10.5223/pghn.2015.18.2.100

Petro, A., Sexton, H. G., Miranda, C., Rastogi, A., Freedman, J. H., and Levin, E. D. (2016). Persisting neurobehavioral effects of developmental copper exposure in wildtype and metallothionein 1 and 2 knockout mice. *BMC Pharmacol. Toxicol.* 17:55. doi: 10.1186/s40360-016-0096-3

Pettei, M. J., Committee, A. N. S. C. N., Weinstein, T., and Eden, A. (2016). Screening for iron deficiency. *Pediatrics* 137, pii:e20160714A. doi: 10.1542/peds.2016-0714A

Pillers, D. M. (2017). Cerebral palsy and asphyxia in 32–35 week preterm infants. *J. Perinatol.* 37, 899–900. doi: 10.1038/jp.2017.78

Prado, E. L., and Dewey, K. G. (2014). Nutrition and brain development in early life. *Nutr. Rev.* 72, 267–284. doi: 10.1111/nure.12102

Qiu, L., Zhu, C., Bodogan, T., Gomez-Galan, M., Zhang, Y., Zhou, K., et al. (2016). Acute and long-term effects of brief sevoflurane anesthesia during the early postnatal period in rats. *Toxicol. Sci.* 149, 121–133. doi: 10.1093/toxsci/kfv219

Rao, R., and Georgieff, M. K. (2009). Iron therapy for preterm infants. *Clin. Perinatol.* 36, 27–42. doi: 10.1016/j.clp.2008.09.013

Rao, R., Tkac, I., Townsend, E. L., Ennis, K., Gruetter, R., and Georgieff, M. K. (2007). Perinatal iron deficiency predisposes the developing rat hippocampus to greater injury from mild to moderate hypoxia-ischemia. *J. Cereb. Blood Flow Metab.* 27, 729–740. doi: 10.1038/sj.jcbfm.9600376

Rathnasamy, G., Ling, E. A., and Kaur, C. (2011). Iron and iron regulatory proteins in amoeboid microglial cells are linked to oligodendrocyte death in hypoxic neonatal rat periventricular white matter through production of proinflammatory cytokines and reactive oxygen/nitrogen species. *J. Neurosci.* 31, 17982–17995. doi: 10.1523/JNEUROSCI.2250-11.2011

Rivera, S., Nemeth, E., Gabayan, V., Lopez, M. A., Farshidi, D., and Ganz, T. (2005). Synthetic hepcidin causes rapid dose-dependent hypoferremia and is concentrated in ferroportin-containing organs. *Blood* 106, 2196–2199. doi: 10.1182/blood-2005-04-1766

Saarinen, U. M., Siimes, M. A., and Dallman, P. R. (1977). Iron absorption in infants: high bioavailability of breast milk iron as indicated by the extrinsic tag method of iron absorption and by the concentration of serum ferritin. *J. Pediatr.* 91, 36–39. doi: 10.1016/S0022-3476(77)80439-3

Sachdev, H., Gera, T., and Nestel, P. (2005). Effect of iron supplementation on mental and motor development in children: systematic review of randomised controlled trials. *Public Health Nutr.* 8, 117–132. doi: 10.1079/PHN2004677

Saha, B., Jeeva Sankar, M., Gupta, S., Agarwal, R., Gupta, N., Deorari, A., et al. (2016). Iron stores in term and late preterm small for gestational age and appropriate for gestational age neonates at birth and in early infancy. *Indian J. Pediatr.* 83, 622–627. doi: 10.1007/s12098-015-1960-7

Saito, H. (2014). Metabolism of iron stores. *Nagoya J. Med. Sci.* 76, 235–254.

Sanchez, M., Galy, B., Muckenthaler, M. U., and Hentze, M. W. (2007). Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency. *Nat. Struct. Mol. Biol.* 14, 420–426. doi: 10.1038/nsmb1222

Schiza, V., Giapros, V., Pantou, K., Theocharis, P., Challa, A., and Andronikou, S. (2007). Serum transferrin receptor, ferritin, and reticulocyte maturity indices during the first year of life in 'large' preterm infants. *Eur. J. Haematol.* 79, 439–446. doi: 10.1111/j.1600-0609.2007.00931.x

Schneider, N., and Garcia-Rodenas, C. L. (2017). Early Nutritional interventions for brain and cognitive development in preterm infants: a review of the literature. *Nutrients* 9, pii:E187. doi: 10.3390/nu9030187

Schroder, N., Figueiredo, L. S., and de Lima, M. N. (2013). Role of brain iron accumulation in cognitive dysfunction: evidence from animal models and human studies. *J. Alzheimers Dis.* 34, 797–812. doi: 10.3233/JAD-121996

Scott, S. P., Murray-Kolb, L. E., Wenger, M. J., Udipi, S. A., Ghugre, P. S., Boy, E., et al. (2018). Cognitive performance in indian school-going adolescents is positively affected by consumption of iron-biofortified pearl millet: a 6-month randomized controlled efficacy trial. *J. Nutr.* 148, 1462–1471. doi: 10.1093/jn/nxy113

Sebastiani, G., and Pantopoulos, K. (2011). Disorders associated with systemic or local iron overload: from pathophysiology to clinical practice. *Metallomics* 3, 971–986. doi: 10.1039/c1mt00082a

Shafir, T., Angulo-Barroso, R., Jing, Y., Angelilli, M. L., Jacobson, S. W., and Lozoff, B. (2008). Iron deficiency and infant motor development. *Early Hum. Dev.* 84, 479–485. doi: 10.1016/j.earlhumdev.2007.12.009

Shao, J., Lou, J., Rao, R., Georgieff, M. K., Kaciroti, N., Felt, B. T., et al. (2012). Maternal serum ferritin concentration is positively associated with newborn iron stores in women with low ferritin status in late pregnancy. *J. Nutr.* 142, 2004–2009. doi: 10.3945/jn.112.162362

Shouman, B. O., Mesbah, A., and Aly, H. (2008). Iron metabolism and lipid peroxidation products in infants with hypoxic ischemic encephalopathy. *J. Perinatol.* 28, 487–491. doi: 10.1038/jp.2008.22

Siddappa, A. M., Rao, R., Long, J. D., Widness, J. A., and Georgieff, M. K. (2007). The assessment of newborn iron stores at birth: a review of the literature and standards for ferritin concentrations. *Neonatology* 92, 73–82. doi: 10.1159/000100805

Siddappa, A. J., Rao, R. B., Wobken, J. D., Casperson, K., Leibold, E. A., Connor, J. R., et al. (2003). Iron deficiency alters iron regulatory protein and iron transport protein expression in the perinatal rat brain. *Pediatr. Res.* 53, 800–807. doi: 10.1203/01.PDR.0000058922.67035.D5

Simons, M., and Trajkovic, K. (2006). Neuron-glia communication in the control of oligodendrocyte function and myelin biogenesis. *J. Cell Sci.* 119, 4381–4389. doi: 10.1242/jcs.03242

Simpson, I. A., Ponnuru, P., Klinger, M. E., Myers, R. L., Devraj, K., Coe, C. L., et al. (2015). A novel model for brain iron uptake: introducing the concept of regulation. *J. Cereb. Blood Flow Metab.* 35, 48–57. doi: 10.1038/jcbfm.2014.168

Singh, N., Haldar, S., Tripathi, A. K., Horback, K., Wong, J., Sharma, D., et al. (2014). Brain iron homeostasis: from molecular mechanisms to clinical significance and therapeutic opportunities. *Antioxid. Redox Signal.* 20, 1324–1363. doi: 10.1089/ars.2012.4931

Song, J., Sun, H., Xu, F., Kang, W., Gao, L., Guo, J., et al. (2016). Recombinant human erythropoietin improves neurological outcomes in very preterm infants. *Ann. Neurol.* 80, 24–34. doi: 10.1002/ana.24677

Stark, M. J., Keir, A. K., and Andersen, C. C. (2013). Does non-transferrin bound iron contribute to transfusion related immune-modulation in preterms? *Arch. Dis. Child. Fetal Neonatal Ed.* 98, F424–F429. doi: 10.1136/archdischild-2012-303353

Steinmacher, J., Pohlandt, F., Bode, H., Sander, S., Kron, M., and Franz, A. R. (2007). Randomized trial of early versus late enteral iron supplementation in infants with a birth weight of less than 1301 grams: neurocognitive development at 5.3 years' corrected age. *Pediatrics* 120, 538–546. doi: 10.1542/peds.2007-0495

Stephenson, E., Nathoo, N., Mahjoub, Y., Dunn, J. F., and Yong, V. W. (2014). Iron in multiple sclerosis: roles in neurodegeneration and repair. *Nat. Rev. Neurol.* 10, 459–468. doi: 10.1038/nrneurol.2014.118

Strahle, J. M., Garton, T., Bazzi, A. A., Kilaru, H., Garton, H. J., Maher, C. O., et al. (2014). Role of hemoglobin and iron in hydrocephalus after neonatal intraventricular hemorrhage. *Neurosurgery* 75, 696–705; discussion 706. doi: 10.1227/NEU.0000000000000524

Stugiewicz, M., Tkaczyszyn, M., Kasztura, M., Banasiak, W., Ponikowski, P., and Jankowska, E. A. (2016). The influence of iron deficiency on the functioning of skeletal muscles: experimental evidence and clinical implications. *Eur. J. Heart Fail.* 18, 762–773. doi: 10.1002/ejhf.467

Sun, Y., Li, T., Xie, C., Xu, Y., Zhou, K., Rodriguez, J., et al. (2017). Haploinsufficiency in the mitochondrial protein CHCHD4 reduces brain injury in a mouse model of neonatal hypoxia-ischemia. *Cell Death Dis.* 8:e2781. doi: 10.1038/cddis.2017.196

Sun, L., Xia, L., Wang, M., Zhu, D., Wang, Y., Bi, D., et al. (2019). Variants of the OLIG2 gene are associated with cerebral palsy in chinese han infants with hypoxic-ischemic encephalopathy. *Neuromolecular Med.* 21, 75–84. doi: 10.1007/s12017-018-8510-1

Takala, T. I., Makela, E., Suominen, P., Matomaki, J., Lapinleimu, H., Lehtonen, L., et al. (2010). Blood cell and iron status analytes of preterm and full-term infants from 20 weeks onwards during the first year of life. *Clin. Chem. Lab. Med.* 48, 1295–1301. doi: 10.1515/CCLM.2010.242

Thompson, R. A., and Nelson, C. A. (2001). Developmental science and the media. Early brain development. *Am. Psychol.* 56, 5–15. doi: 10.1037/0003-066X.56.1.5

Todorich, B., Pasquini, J. M., Garcia, C. I., Paez, P. M., and Connor, J. R. (2009). Oligodendrocytes and myelination: the role of iron. *Glia* 57, 467–478. doi: 10.1002/glia.20784

Trevino-Baez, J. D., Briones-Lara, E., Alamillo-Velazquez, J., and Martinez-Moreno, M. I. (2017). Multiple red blood cell transfusions and iron overload in very low birthweight infants. *Vox Sang.* 112, 453–458. doi: 10.1111/vox.12528

Unger, E. L., Hurst, A. R., Georgieff, M. K., Schallert, T., Rao, R., Connor, J. R., et al. (2012). Behavior and monoamine deficits in prenatal and perinatal iron deficiency are not corrected by early postnatal moderate-iron or high-iron diets in rats. *J. Nutr.* 142, 2040–2049. doi: 10.3945/jn.112.162198

Vecchi, C., Montosi, G., Zhang, K., Lamberti, I., Duncan, S. A., Kaufman, R. J., et al. (2009). ER stress controls iron metabolism through induction of hepcidin. *Science* 325, 877–880. doi: 10.1126/science.1176639

Vela, D. (2018). Hepcidin, an emerging and important player in brain iron homeostasis. *J. Transl. Med.* 16:25. doi: 10.1186/s12967-018-1399-5

Vucic, V., Berti, C., Vollhardt, C., Fekete, K., Cetin, I., Koletzko, B., et al. (2013). Effect of iron intervention on growth during gestation, infancy, childhood, and adolescence: a systematic review with meta-analysis. *Nutr. Rev.* 71, 386–401. doi: 10.1111/nure.12037

Wang, J., and Pantopoulos, K. (2011). Regulation of cellular iron metabolism. *Biochem. J.* 434, 365–381. doi: 10.1042/BJ20101825

Wenger, M. J., DellaValle, D. M., Murray-Kolb, L. E., and Haas, J. D. (2019). Effect of iron deficiency on simultaneous measures of behavior, brain activity,

and energy expenditure in the performance of a cognitive task. *Nutr. Neurosci.* 22, 196–206. doi: 10.1080/1028415X.2017.1360559

Widdowson, E. M., and Spray, C. M. (1951). Chemical development in utero. *Arch. Dis. Child.* 26, 205–214. doi: 10.1136/adc.26.127.205

Wilson, K., and Sloan, J. M. (2015). Iron-deficiency anemia. *N. Engl. J. Med.* 373:485. doi: 10.1056/NEJMc1507104

Wu, Y., Song, J., Wang, Y., Wang, X., Culmsee, C., and Zhu, C. (2019). The Potential role of ferroptosis in neonatal brain injury. *Front. Neurosci.* 13:115. doi: 10.3389/fnins.2019.00115

Xu, Y., Wang, H., Sun, Y., Shang, Q., Chen, M., Li, T., et al. (2014). The association of apolipoprotein E gene polymorphisms with cerebral palsy in Chinese infants. *Mol. Gen. Genomics.* 289, 411–416. doi: 10.1007/s00438-014-0818-4

Yoshinaga, M., Nakatsuka, Y., Vandenbon, A., Ori, D., Uehata, T., Tsujimura, T., et al. (2017). Regnase-1 maintains iron homeostasis via the degradation of transferrin receptor 1 and prolyl-hydroxylase-domain-containing protein 3 mRNAs. *Cell Rep.* 19, 1614–1630. doi: 10.1016/j.celrep.2017.05.009

Youdim, M. B. (2008). Brain iron deficiency and excess; cognitive impairment and neurodegeneration with involvement of striatum and hippocampus. *Neurotox. Res.* 14, 45–56. doi: 10.1007/BF03033574

Yu, S., Feng, Y., Shen, Z., and Li, M. (2011). Diet supplementation with iron augments brain oxidative stress status in a rat model of psychological stress. *Nutrition* 27, 1048–1052. doi: 10.1016/j.nut.2010.11.007

Zhang, X., Rocha-Ferreira, E., Li, T., Vontell, R., Jabin, D., Hua, S., et al. (2017a). γδT cells but not alphabetaT cells contribute to sepsis-induced white matter injury and motor abnormalities in mice. *J. Neuroinflammation* 14:255. doi: 10.1186/s12974-017-1029-9

Zhang, Z., Song, Y., Zhang, Z., Li, D., Zhu, H., Liang, R., et al. (2017b). Distinct role of heme oxygenase-1 in early- and late-stage intracerebral hemorrhage in 12-month-old mice. *J. Cereb. Blood Flow Metab.* 37, 25–38. doi: 10.1177/0271678X16655814

Zhang, X., Surguladze, N., Slagle-Webb, B., Cozzi, A., and Connor, J. R. (2006). Cellular iron status influences the functional relationship between microglia and oligodendrocytes. *Glia* 54, 795–804. doi: 10.1002/glia.20416

Zhou, Z. D., and Tan, E. K. (2017). Iron regulatory protein (IRP)-iron responsive element (IRE) signaling pathway in human neurodegenerative diseases. *Mol. Neurodegener.* 12:75. doi: 10.1186/s13024-017-0218-4

Ziegler, E. E., Nelson, S. E., and Jeter, J. M. (2014). Iron stores of breastfed infants during the first year of life. *Nutrients* 6, 2023–2034. doi: 10.3390/nu6052023

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Wang, Wu, Li, Wang and Zhu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 09 May 2019
doi: 10.3389/fphys.2019.00540

The Potential of Stem Cell Therapy to Repair White Matter Injury in Preterm Infants: Lessons Learned From Experimental Models

*Josine E. G. Vaes[1,2], Marit A. Vink[1], Caroline G. M. de Theije[1], Freek E. Hoebeek[1], Manon J. N. L. Benders[2] and Cora H. A. Nijboer[1]**

[1] NIDOD Laboratory, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands, [2] Department of Neonatology, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Emily Camm,
University of Cambridge,
United Kingdom
Gavin John Clowry,
Newcastle University, United Kingdom

***Correspondence:**
Cora H. A. Nijboer
c.nijboer@umcutrecht.nl

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: 24 January 2019
Accepted: 17 April 2019
Published: 09 May 2019

Citation:
Vaes JEG, Vink MA, de Theije CGM, Hoebeek FE, Benders MJNL and Nijboer CHA (2019) The Potential of Stem Cell Therapy to Repair White Matter Injury in Preterm Infants: Lessons Learned From Experimental Models. Front. Physiol. 10:540. doi: 10.3389/fphys.2019.00540

Diffuse white matter injury (dWMI) is a major cause of morbidity in the extremely preterm born infant leading to life-long neurological impairments, including deficits in cognitive, motor, sensory, psychological, and behavioral functioning. At present, no treatment options are clinically available to combat dWMI and therefore exploration of novel strategies is urgently needed. In recent years, the pathophysiology underlying dWMI has slowly started to be unraveled, pointing towards the disturbed maturation of oligodendrocytes (OLs) as a key mechanism. Immature OL precursor cells in the developing brain are believed to be highly sensitive to perinatal inflammation and cerebral oxygen fluctuations, leading to impaired OL differentiation and eventually myelination failure. OL lineage development under normal and pathological circumstances and the process of (re)myelination have been studied extensively over the years, often in the context of other adult and pediatric white matter pathologies such as stroke and multiple sclerosis (MS). Various studies have proposed stem cell-based therapeutic strategies to boost white matter regeneration as a potential strategy against a wide range of neurological diseases. In this review we will discuss experimental studies focusing on mesenchymal stem cell (MSC) therapy to reduce white matter injury (WMI) in multiple adult and neonatal neurological diseases. What lessons have been learned from these previous studies and how can we translate this knowledge to application of MSCs for the injured white matter in the preterm infant? A perspective on the current state of stem cell therapy will be given and we will discuss different important considerations of MSCs including cellular sources, timing of treatment and administration routes. Furthermore, we reflect on optimization strategies that could potentially reinforce stem cell therapy, including preconditioning and genetic engineering of stem cells or using cell-free stem cell products, to optimize cell-based strategy for vulnerable preterm infants in the near future.

Keywords: preterm birth, white matter injury, mesenchymal stem cells, brain development, white matter pathology, cell therapies, regeneration, myelin loss

INTRODUCTION

Preterm birth is defined as birth before 37 weeks of gestation, and is relatively common with global prevalence rates ranging between 8 and 10%. Some of these children are born before 28 weeks of pregnancy (∼5% of all preterm births worldwide), and are labeled extremely preterm (Blencowe et al., 2012). White matter injury (WMI) is the most prevalent form of brain injury in the (extremely) preterm neonate and can lead to life-long neurological deficits (Back and Miller, 2014). While mortality rates following (extreme) preterm birth are steadily declining, the incidence of neurological sequelae remains high throughout the preterm population (Deng, 2010). It is estimated that about 25–50% of surviving extreme preterm infants encounter long-term neurological impairments, ranging from perceptual disabilities, impaired cognitive functioning, and behavioral problems, to an increased risk of psychiatric disorders (Larroque et al., 2008; Moster et al., 2008; Johnson et al., 2009; MacKay et al., 2010; Linsell et al., 2018). A smaller percentage of this population (5–10%) is believed to suffer from major motor problems, such as cerebral palsy (Larroque et al., 2008; Johnson et al., 2009).

Preterm WMI is thought to be the result of myelination failure during white matter development in the third trimester (Back et al., 2001; Khwaja and Volpe, 2008). The formation of myelin sheaths is essential for rapid, saltatory conduction of action potentials throughout the central nervous system (CNS), ensuring optimal brain connectivity, as well as protection of axonal integrity (Freeman and Rowitch, 2013). Even though the white matter is undeniably affected in preterm brain injury, evidence supporting brain injury in the preterm infant as a complex constellation of multiple neurodevelopmental disturbances, called "encephalopathy of prematurity," has increased over the years (Volpe, 2009). These disturbances were shown to primarily involve the white matter, accompanied by (secondary) neuronal/axonal deficits affecting multiple brain regions, such as thalamus, basal ganglia, cerebral cortex, cerebellum, and brain stem (Volpe, 2009). Interestingly, recent studies have shed light on the development of another important cell type emerging in the third trimester, the (cortical) interneuron. Preterm birth was shown to affect both interneuron neurogenesis and migration, leading to disturbed interneuron distribution in the cortex in both a rabbit model of preterm WMI and post-mortem human tissue (Panda et al., 2018; Tibrewal et al., 2018). However, due to the irrefutable and fundamental role of impaired white matter development in preterm brain injury, this review focuses specifically on the protective and/or regenerative potential of treatments on the *white matter* of the brain.

The nomenclature in preterm WMI is one that can be hard to decipher. Before going into detail on the pathophysiology underlying preterm WMI it is important to clear up these terms to avoid confusion. Attempts to provide a consistent nomenclature have been made by combining neuroimaging findings with neuropathological correlates (Volpe, 2017). "Preterm WMI" is a collective name for a range of pathologies of the white matter in the developing brain. Based on neuropathological studies subdivisions into periventricular leukomalacia (PVL) and diffuse white matter injury (dWMI) can be made. PVL can be subdivided based on severity of necrosis and cyst formation. Punctate white matter lesions, sometimes recognized as a separate entity, are believed to result from small necrotic lesions and can be categorized in the PVL spectrum (Volpe et al., 2011; Back, 2017; Lee, 2017; Volpe, 2017; Zaghloul and Ahmed, 2017). dWMI is characterized by diffuse, subtle alterations in the white matter microenvironment without focal necrosis. Currently, preterm dWMI is the most prevalent form of WMI observed in preterm infants; it is believed that 80% of affected preterm neonates suffer from this type of WMI, leading to global hypomyelination (Back and Miller, 2014; Back, 2017). For this reason, we mainly focus on dWMI in this review.

Despite being a cause of serious neurological morbidity, treatment options for dWMI in preterm infants are still lacking. Although preterm dWMI differs from other (adult) CNS disorders in etiology and symptoms, the majority of these other conditions are (in part) caused by damage to the white matter and/or insufficient (re)myelination, resulting in abnormal brain functioning. Therefore, research already performed from these other areas of white matter pathology could aid in the identification and optimization of potent treatment strategies to combat preterm dWMI. Here we will discuss the potency of stem cell-based treatments for dWMI, by reviewing a wide range of *in vitro* and *in vivo* studies in multiple adult and pediatric white matter diseases.

PRETERM WHITE MATTER INJURY: PATHOPHYSIOLOGY

Preterm infants are born at a very crucial period of cerebral white matter development, since myelination starts only around 32 weeks of gestation (Back et al., 2001; Knuesel et al., 2014). Prior to this gestational age, the myelin-forming cells of the brain, i.e., oligodendrocytes (OLs), undergo highly regulated and strictly timed developmental changes in order to transform into mature OLs capable of myelin production. OLs typically develop via a 4-stage program: (1) neural stem cells (NSCs) originating from different endogenous stem cell niches of the brain [for example the lateral subventricular zone (SVZ)] develop into, (2) OL precursor cells (OPCs), which migrate to designated brain regions. There, the OPC population will expand through proliferation and subsequently differentiates into, (3) immature pre-myelinating OLs (pre-OLs) that progress to the final stage of 4) mature myelinating OLs (Back et al., 2001; Emery, 2010; Volpe et al., 2011; van Tilborg et al., 2018). OPCs remain present in the brain throughout adulthood and are crucial for myelin maintenance and remyelination of axons after damage. Any disturbance in local OPC pools by differentiation, migration or cell death will be rapidly restored via multiple pathways that regulate OPC proliferation, ensuring a homeostatic number of OPCs (Bradl and Lassmann, 2010; van Tilborg et al., 2016). OL lineage maturation and migration in the developing brain has been described in detail in multiple excellent studies

(Kessaris et al., 2006; Jakovcevski et al., 2009; Mitew et al., 2014; van Tilborg et al., 2018).

The majority of OL lineage cells present in the brain of infants born between 24 and 32 weeks are OPCs and pre-OLs (Back et al., 2001, 2007; Volpe et al., 2011). These immature cell types have been reported to be very sensitive to preterm birth-related insults, while mature OLs are more resilient to damage (van Tilborg et al., 2016; Bennet et al., 2018). Accumulating evidence has identified inflammation and hypoxia, both insults unequivocally linked to preterm birth, as two main pathways involved in disruption of OL lineage development (Khwaja and Volpe, 2008; Volpe, 2009; Deng, 2010). These two detrimental types of insults are believed to work synergistically, as the incidence of WMI has shown to be higher in children exposed to multiple insults (Rezaie and Dean, 2002; Zhao et al., 2013). A pro-inflammatory state of the brain can result as a consequence of antenatal sequelae like maternal inflammation and/or intra-amniotic infections (often a trigger of preterm birth), or due to postnatal infections such as neonatal sepsis (Back and Miller, 2014; van Tilborg et al., 2016; Bennet et al., 2018). Irrespective of timing, inflammation is thought to contribute to WMI through systemic cytokine release and the activation of microglia (i.e., microgliosis), the innate immune cells of the brain. As a consequence of microgliosis, toxic compounds such as free radicals, glutamate and pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-17, interferon-γ (IFN-γ), and IL-1β, are secreted in the brain leading to pre-OL injury (Favrais et al., 2011; Hagberg et al., 2012; van Tilborg et al., 2016). In addition to inflammation being an important hit for WMI, preterm birth is also linked to disrupted cerebral oxygen levels in the perinatal period as preterm infants often need mechanical ventilation because of an underdeveloped respiratory system (Brown and DiBlasi, 2011). Furthermore, preterm infants have an underdeveloped cardiovascular system with disturbed autoregulation of cerebral blood flow (Fyfe et al., 2014). Taken together, these events can ultimately lead to low blood pressure, hypocapnia, leading to cerebral vasoconstriction, and brain hypoperfusion (Viscardi et al., 2004; Fyfe et al., 2014). Apart from the risk of hypoxia, oxygen disturbances in preterm infants are possibly also caused by periods of hyperoxia following excessive ventilation, all the while optimal oxygen saturation levels in preterm infants are still under debate (Lakshminrusimha et al., 2014; Stoll et al., 2015). Whereas mature OLs largely tolerate hypoxic insults, pre-OLs are very vulnerable to an imbalanced oxygen supply. In addition, disruptions of the oxygen supply leads to oxidative stress through various pathways, ultimately causing production and accumulation of reactive oxygen species within developing OLs (van Tilborg et al., 2016). For example, activation of nitric oxide synthase causes pre-OL injury by nitric oxide production (Gluckman et al., 1992; Khwaja and Volpe, 2008; Lee, 2017; Singh et al., 2018). Further, OPCs are extremely sensitive to oxidative damage, as they lack particular anti-oxidant enzymes (Perrone et al., 2015). Moreover, hypoxia has been shown to further fuel activation of the immune system, including activation of microglia thereby augmenting the release of pro-inflammatory cytokines (Singh et al., 2018).

Even though myelination failure is evident in preterm dWMI, the exact pathophysiology of the OL lineage has yet to be determined. On the one hand it is hypothesized and supported by human post-mortem and experimental animal studies that an initial wave of pre-OL cell death is compensated by an inadequate regenerative response from the large reservoir of early OL progenitors, leading to a secondary OL maturational arrest in these newly formed cells (Robinson et al., 2006; Segovia et al., 2008; Back, 2015). On the other hand, several human post-mortem studies failed to show any evidence of OL lineage cell death in dWMI, which suggests that arrested maturation of the large pool of pre-existing OPCs and pre-OLs underlies dysregulated myelination observed in preterm dWMI (Billiards et al., 2008; Buser et al., 2012; Verney et al., 2012).

Regardless of the exact nature of myelination failure in preterm dWMI, ultimately, the lack of proper myelination during brain development will negatively influence axonal processes leading to impaired connectivity and causing life-long neurodevelopmental deficits (van Tilborg et al., 2016).

STEM CELL THERAPY IN ADULT AND PEDIATRIC WHITE MATTER PATHOLOGIES

Although dWMI can cause long term neurological impairments, there are currently no treatment options available to reduce myelination deficits in the developing brain. A prospective therapy for preterm WMI would preferably be multifaceted, and thus act on multiple pathophysiological processes contributing to preterm WMI. Displaying both anti-inflammatory properties as well as providing trophic support, mesenchymal stem cells (MSCs) have been proposed as a potent therapeutic tool in numerous neuropathologies, including white matter diseases (Kassis et al., 2008; Liang et al., 2014). MSCs are believed to exert their regenerative abilities by adaptation of their secretome *in situ*, favoring endogenous repair of brain injury through paracrine signaling (van Velthoven et al., 2010a; Kassis et al., 2011; Liang et al., 2014; Paton et al., 2017). MSCs can be harvested from a wide range of tissues, including bone marrow, adipose tissue, and the umbilical cord (both from the Wharton's jelly and from cord blood) (Kobolak et al., 2016; Volkman and Offen, 2017). Moreover, MSC administration has a low risk of triggering the recipient's immune system, due to low expression of major histocompatibility complex (MHC) class I receptors, lack of MHC class II receptors and lack of co-stimulatory proteins (e.g., CD40, CD80, and CD86) on the MSC's plasma membrane (De Miguel et al., 2012; Jacobs et al., 2013). While MSC therapy could be an attractive therapeutic option, evidence supporting the regenerative effect of MSCs in dWMI is still scarce. Research on MSCs used in *in vitro* models or in other brain pathologies could contribute to more insight into development of an effective cell-based therapy for the vulnerable white matter of the preterm brain. Therefore we start by discussing data obtained in studies using MSC therapy in basic *in vitro* models of OL development

and in *in vivo* models of adult and neonatal conditions with pronounced WMI.

Mesenchymal Stem Cells in *in vitro* Models of OL Development

Evidence of a potential direct effect of MSCs on OL development in *in vitro* models of WMI is scarce. A few studies report a supportive role of the MSC secretome in OL differentiation. Zhang et al. (2016) studied the direct effect of rat ectodermal MSCs (derived from the neural crest) in both a non-contact (transwell) and cell-cell contact co-culture with OPCs. Interestingly, both non-contact and direct contact MSC co-cultures significantly improved the number of myelin basic protein (MBP), a structural component of mature myelin exclusively expressed by mature myelinating OLs, positive (mature) OLs and the length of the OL processes (important for sufficient axonal wrapping) compared to OPCs cultured without the presence of MSCs, indicating at least partially an important role of the MSCs' secretome. However, the most pronounced increase in mature OL numbers and process outgrowth was found in the direct contact co-culture, indicating an additional positive effect through direct cell-cell contact or near-proximity of the MSCs. A study adopting a similar setup, but using rat NSCs instead of OPCs, reported comparable results. Direct co-culture of NSCs and human Wharton's jelly derived MSCs (WJ-MSCs) led to a greater increase in the expression of MBP and the immature OL marker GalC, compared to exposure of NSCs to only the MSCs' secretome via either non-contact WJ-MSC-NSC co-cultures or by using WJ-MSC conditioned medium (Oppliger et al., 2017). Direct MSC contact is believed to lead to superior OL maturation and process outgrowth through the presence of gap junctions and extra-cellular matrix (ECM) proteins, such as laminin, produced by MSCs (Zhang et al., 2016; Oppliger et al., 2017). Multiple other studies have shown that MSCs are capable of mitochondrial transfer through microvesicles, gap junctions or nanotubes to cells with impaired mitochondrial function following oxidative stress, a detrimental hit to developing OLs (Liang et al., 2014; Lin et al., 2015; Mahrouf-Yorgov et al., 2017; Paliwal et al., 2018). Even though studies demonstrating mitochondrial transfer between MSCs and damaged OLs are currently lacking, this mechanism could contribute to the observed superior effect of cell proximity. The additive effect of cell proximity is not supported in all available *in vitro* studies. For instance, Rivera et al. (2006) demonstrated comparable effects of either direct cell-cell contact of MSCs or only using MSC-conditioned medium (CM) in the promotion of oligodendrogenesis in a rat NSC culture.

Thus, based on these findings it seems that MSCs and their secretome play a supportive role in OL maturation, even though a close proximity of the two cell types might be of importance for the most optimal effect. The final location of stem cells in the brain and their proximity to target cells could be further elucidated by studying the biodistribution, migration and cellular niches of transplanted MSCs in *in vivo* models of preterm dWMI for instance by using fluorescent labeled stem cells, bioluminescence or tracing of xenogenic transplants. It is, however, relevant to note that in none of the above described *in vitro* studies OPCs or NSCs were challenged, meaning that the effect of MSCs on oligodendrogenesis was studied under non-injured circumstances. What the effects of MSCs could be on maturation when OPCs are challenged with inflammatory or injury-mimicking stimuli is yet to be studied. Moreover, most *in vitro* studies focus on the effect of MSC on NSCs and endogenous regeneration of myelination through formation of new OL progenitors in the stem cell niche of the SVZ. However, the primary therapeutic target in dWMI might be the maturation-arrested pre-OLs residing in the injured white matter, so additional research needs to be done to study the potential beneficial effects of MSCs in *in vitro* models mimicking maturational arrest of OL progenitors.

Mesenchymal Stem Cells in Adult White Matter Disease

To assess the potency of MSCs in preterm WMI, this section focuses on adult pathologies in which the *white matter* of the brain is affected. The potency of MSCs in other adult brain diseases in which primarily the gray matter is affected can be found elsewhere (Laroni et al., 2013; Volkman and Offen, 2017).

Stroke

The effectiveness of MSC therapy to repair the brain following stroke, a pathological condition in which disturbances in cerebral blood flow lead to permanent neurological impairments, has been studied extensively over the past years. A recent meta-analysis by Sarmah et al. (2018) reported significant improvement of neurological deficits following ischemic stroke compared to controls in all included animal studies. Even though the majority of these studies mainly address the effects of MSC therapy on regeneration of the gray matter, the white matter is also damaged in cerebrovascular disease but fewer studies have specifically focused on the effects of MSC treatment on the damaged white matter after stroke (Jiang et al., 2006; Gutierrez-Fernandez et al., 2013b; Mifsud et al., 2014; Hayakawa and Lo, 2016). Yu et al. (2018) showed a significant increase in MBP levels, the key protein in myelin sheaths, after intraventricular rat bone marrow-derived MSC (BM-MSC) administration following transient middle cerebral artery occlusion (MCAO) in rats. The increase in MBP expression was hypothesized to be the result of the reported rise in proliferating OPCs following BM-MSC treatment. Moreover, fractional anisotropy (FA) values, a measure of WM integrity determined by MRI-DTI, were lower in animals that did not receive cell therapy. In addition, Gutierrez-Fernandez et al. (2013a) showed an incline in Olig2 protein levels, an OL marker that marks all developmental stages, after intravenous rat BM-MSC and rat adipose tissue-derived MSC (AD-MSC) treatment in a rat model of focal ischemia induced by permanent MCAO (Gutierrez-Fernandez et al., 2013a). Furthermore, in a rat model of subcortical stroke, intravenous rat AD-MSC administration increased the number of OL progenitors in the area of stroke and the number of mature OLs in the penumbra, leading to an increase in myelin formation and hence restoration of white matter integrity (Otero-Ortega et al., 2015). Thus, these *in vivo* studies show potent

reduction in myelination deficits following MSC therapy after stroke, which could be related to the observed increase in OL progenitor proliferation. Moreover, they also underline the proposed transient *paracrine* effects of MSCs as the surviving number of engrafted or differentiated transplanted cells is very small in models of stroke/MCAO (Dulamea, 2015; Cunningham et al., 2018; Sarmah et al., 2018).

Even though brain ischemia is responsible for the greater percentage of all strokes, the pathological term "stroke" does not only entail ischemic cerebrovascular accidents. *Hemorrhagic* stroke, including intracerebral hemorrhage (ICH), accounts for about 10–20% of strokes. However, studies investigating the potential of MSC treatment in experimental models of hemorrhagic stroke are less prevalent. A recent review by Bedini et al. (2018) did report enhanced functional recovery and reduction in lesion size in animal models of ICH as a result of MSC treatment. In a rat model of striatal ICH, intraventricular injection of human WJ-MSCs, led to a decrease in myelination deficits as shown by luxol fast blue staining (which stains phospholipids in myelin) and upregulation of MBP protein levels confirmed by Western Blot, suggestive of an increase in remyelination (Liu et al., 2010). Intranasal rat BM-MSC therapy in a rat model of subarachnoid hemorrhage (SAH), in which brain injury is evoked by presence of blood in the subarachnoid space and subsequent cerebral ischemia, was recently shown to reduce white matter loss, demonstrated by a rise in MBP expression (Nijboer et al., 2018).

The encouraging results in preclinical studies have prompted various clinical trials to assess the safety, feasibility and efficacy of MSC treatment in stroke patients. A meta-analysis analyzing a large number of the clinical studies in Asia confirmed the safety and efficacy of MSC therapy in ischemic stroke (Xue et al., 2018). Neurological deficits were significantly reduced, while no serious adverse events were reported. Interestingly, outcome parameters did not differ significantly between patient groups treated in the acute phase or chronic phase of ischemic stroke, indicating a wide treatment window using MSCs. Other sub-analyses for optimal dosage, cell origin and administration methods were not conclusive (Xue et al., 2018). Toyoshima et al. (2017), who reviewed a different subset of clinical studies, reported similar findings while stressing the need for additional research to determine optimal timing, route of administration and dosages.

Multiple Sclerosis

Multiple sclerosis (MS), a disorder in which a dysregulated autoimmune response is believed to result in transient and eventually chronic demyelination of the CNS, is one of the leading causes of neurological deficits in young adults (Compston and Coles, 2002; Uccelli et al., 2007). The exact pathophysiological mechanism of disease onset and progression is beyond the scope of this review and multiple excellent reviews on this subject can be found elsewhere (Ciccarelli et al., 2014; Garg and Smith, 2015; Correale et al., 2017; Thompson et al., 2018). Due to the persistent and uncontrolled T-cell, B-cell and microglial activation, a prospective therapy for MS should both attenuate the autoimmune attack, and promote remyelination/axonal regeneration (Uccelli et al., 2007). Over the years, animal studies have explored the potential of MSC therapy in MS and the results have been summarized in detail in many other reviews (Jadasz et al., 2012; Morando et al., 2012; Rivera and Aigner, 2012; Cohen, 2013; Laroni et al., 2013; Gharibi et al., 2015; Xiao et al., 2015; Genc et al., 2018). These studies frequently use animal models of either toxin-induced (i.e., cuprizone) demyelination or an experimental autoimmune encephalitic (EAE) model, mimicking inflammation-induced demyelination by active immunization with myelin- or OL associated antigens (Jadasz et al., 2012). Genetic models, like *Shiverer* mice, in which an autosomal recessive mutation leads to CNS hypomyelination, are also used to study MS. One of these studies, by Cristofanilli et al. (2011), used an unconventional approach by co-transplanting mouse BM-MSCs with allogenic OPCs intracranially to boost remyelination in demyelinated *Shiverer* mutants. They hypothesized that BM-MSCs would display immunosuppressive properties, boosting allogenic OPC engraftment. The co-transplantation resulted in an increase in myelination surrounding the injection site and was due to both reduction in inflammation and a boost of OPC engraftment, migration and differentiation. While BM-MSC therapy alone was shown to dampen the inflammatory response, a direct comparison between BM-MSC and BM-MSC plus OPC therapy on other outcome parameters was not made. Therefore it is unclear if the regenerative effect was the result of combination therapy, or could also be achieved by BM-MSC therapy alone. However, this study highlights the potent anti-inflammatory effect that MSCs can have in the injured white matter. Even though these preclinical studies all report improvement of histological and behavioral outcomes after MSC therapy (either applied intravenously, intracerebrally, or intraperitoneally) in models of relapse-remitting or chronic MS, the mechanism of action of MSCs in these models is unclear. Many of these studies report reduction in demyelination as a result of modulation of the immune system, reducing peripheral T-cell and B-cell influx or activation (Zappia et al., 2005; Zhang et al., 2005; Kassis et al., 2008; Bai et al., 2009; Liu et al., 2009; Cristofanilli et al., 2011; Liu et al., 2013). In contrast, the regenerative role of MSCs following white matter damage seems less pronounced in models of MS. However, some of the EAE studies do report an increase in endogenous oligodendrogenesis following MSC therapy, as a result of the MSCs' secretome (Zhang et al., 2005; Kassis et al., 2008; Bai et al., 2009; Jaramillo-Merchan et al., 2013; Liu et al., 2013). Nessler et al. (2013) used a mouse model of cuprizone-induced CNS demyelination, to assess the potency of MSCs to repair myelination deficits without interference of immune system activation. Interestingly, neither intranasal nor intravenous application of human or mouse BM-MSCs were shown to beneficially affect myelination. Another study in the same model performed by Cruz-Martinez et al. (2016) showed opposite results: intraventricular injection with mouse BM-MSCs led to increased OL progenitor proliferation in the SVZ and myelin regeneration at the lesion site. The contrast in outcome of these studies could be related to differences in administration routes of MSCs, as Nessler et al. (2013) concluded that the lack of myelin regeneration following MSC therapy was related to the intact blood brain barrier (BBB), limiting MSC migration toward

the lesion site following intravenous or intranasal administration. Cruz-Martinez et al. (2016) chose a direct approach by injecting the MSCs in the lateral ventricles of the brain. In conclusion, data from these studies indicate that neuroinflammation with a strong chemotactic signal and damaged BBB facilitates MSC migration and could be the key for MSC-based therapies to effectively remyelinate the damaged white matter.

Taken together, available data indicate that MSCs have beneficial effects in animal models of MS through either their anti-inflammatory properties or regenerative properties. Over the years, multiple small studies exploring safety and feasibility of MSCs therapy in MS patients have been published. A recent clinical review by Scolding et al. (2017) provides a clear overview of the outcome of these studies. In general, MSC therapy was warranted to be safe, with very little reported adverse events, including a study ruling out neoplasia formation (von Bahr et al., 2012; Scolding et al., 2017). Currently, several phase II trials using MSCs for MS are underway (SIAMMS-II; NCT01932593, ACTiMuS; NCT01815632, and MESEMS; NCT01854957).

In conclusion, in adult experimental models for stroke and MS it has been shown that MSCs can have both regenerative and anti-inflammatory paracrine effects by which white matter deficits can be restored. The exact working mechanism of MSCs in different pathologies, however, seems to be dependent on the underlying pathophysiology of the disease and the administration route.

Mesenchymal Stem Cells in Term Neonatal Brain Pathologies

Neonatal Hypoxic-Ischemia Encephalopathy

Aside from the abundance of studies showing the potency of MSC therapy in adult white matter disease, the evidence supporting MSC treatment in neonatal brain injury has grown steadily over the years. A vast amount of preclinical research has focused on a relatively prevalent form of neonatal brain injury, hypoxic-ischemic encephalopathy (HIE) in the term infant.

Hypoxic-ischemic encephalopathy can be the result of perinatal asphyxia, in which a birth-related event, such as shoulder dystocia or collapse of the umbilical cord, leads to inadequate cerebral blood flow and oxygenation (Douglas-Escobar and Weiss, 2015). The decreased cerebral perfusion sets in motion a temporal sequence of detrimental insults, eventually leading to neuronal cell death in the cerebral cortex or basal ganglia and thalami (Douglas-Escobar and Weiss, 2015). In addition to gray matter injury, the white matter is also affected in HIE (Silbereis et al., 2010). Previous work in a 9-day-old (postnatal day 9, P9; for human gestation equivalence, see **Figure 1**) mouse model of HIE performed at our center showed that MSC therapy improved functional outcome and reduced lesion size following HIE by stimulating endogenous repair of the brain. Intracranial mouse BM-MSC transplantation was shown to boost neurogenesis, OL formation and reduced white matter loss (van Velthoven et al., 2010a). Further reduction in myelin loss was achieved with a second intracranial dose of MSCs, but this second MSC dose did not further increase oligodendrogenesis (van Velthoven et al., 2010c). The effect of MSC therapy on white matter integrity in the mouse HIE model was further investigated using DTI, showing normalization of FA values in the cortex and corpus callosum in MSC-treated animals. These results were confirmed by restored histological MBP intensity and pattern in similar brain areas (van Velthoven et al., 2012b). Other research by van Velthoven et al. (2011) provided evidence underlining the adaptive potential of the MSCs' secretome, reporting multiple gene expression changes in growth factors and cytokines in MSCs, which are believed to be pivotal for cerebral cell survival, proliferation and differentiation, in response to the HIE milieu. Moreover, it was shown that MSCs are unlikely to integrate into the brain, as <1% of the cells could be detected 18 days after the last MSC administration (van Velthoven et al., 2011). In contrast to these findings, Park et al. (2013) reported that human AD-derived MSCs differentiated into MBP-expressing OLs following intracranial transplantation in their rat model of inflammatory HIE. In addition, the MSCs were shown to

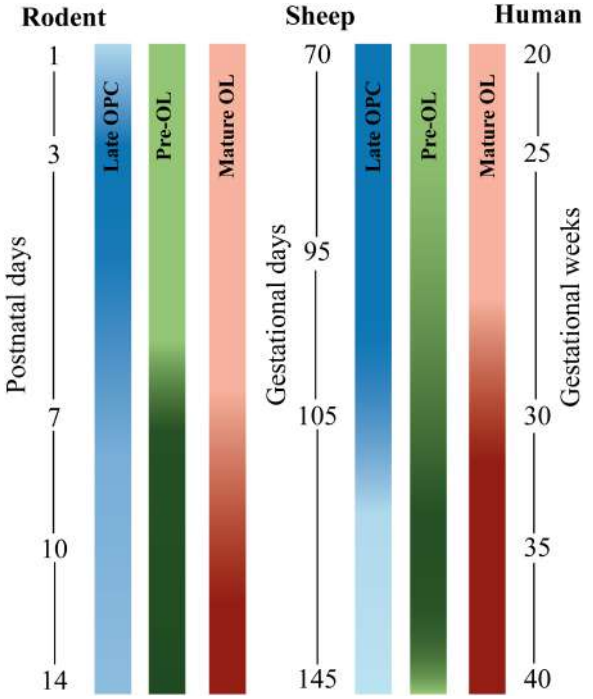


FIGURE 1 | Developmental timeline comparing oligodendrocyte (OL) stage-specific development in different species. Blue bars depict late OL precursor cells (OPC), green bars depict pre-myelinating precursors (pre-OLs), and red bars depict mature OLs. From left to right: rodent, sheep, and human. The postnatal window in rodent OL development between postnatal day (P1–P14) corresponds to the latter half of human gestation [data are based on (Craig et al., 2003) and (Salmaso et al., 2014)]. Fetal sheep OL development between 70 and 145 gestational days (GD) approximately corresponds with late second and third human trimester [data based on (Back et al., 2012)]. Human OL development is based on data from Back et al. (2001). The intensity of the bar indicates the peak of OL development. Note that OL development of human extreme preterms (24–28 weeks of gestation) roughly corresponds to rodent P2 to P5 and ovine 90–95 GD. Also note that sheep and human OL development are roughly comparable, whereas rodent OL development is slightly different regarding time-window of OL development and composition of OL subtypes per postnatal/gestational age (Craig et al., 2003).

aid endogenous preservation of myelin by producing trophic factors and decreasing pro-inflammatory cytokines (Park et al., 2013). It is, however, important to note the differences between these two preclinical HIE models. The rat model of Park et al. (2013) displayed severe cystic WMI while the mouse model of van Velthoven et al. (2010a) displayed moderate neuronal loss and more global myelin deficits. A different study using a near-term (P7) mouse model of HIE, displaying demyelination, neuronal and OL loss, alterations in OL development and axonal damage, showed a positive effect of intraventricular human amniotic fluid stem cells (AFSCs) administration (directly following hypoxia) with a marked reduction in MBP loss after treatment. AFSCs were shown to express an important MSC marker, CD73. However, the protective effect was only observed in AFSCs with a spindle-shaped cytoplasm, while AFSCs with a rod-shaped cytoplasm were not capable to prevent myelination deficits (Corcelli et al., 2018). In addition to improvement of functional outcome or lesion size, multiple studies reported a reduction in cerebral inflammation following MSC therapy, confirming the immunomodulatory properties of MSCs (van Velthoven et al., 2010a; Donega et al., 2014b; Gu et al., 2015, 2016; Ding et al., 2017). While most studies focus on short-term outcome of MSC therapy in HIE, our study using a 14 months post-HIE follow-up found long lasting improvements of functional outcome and myelination in intranasally mouse BM-MSC-treated mice compared to vehicle-treated littermates. Moreover, pathological analysis of multiple organs did not reveal an increase in neoplasia following MSC treatment after this long-term follow up, indicating that intranasal MSC treatment was safe (Donega et al., 2015).

Based on these promising results, more recent studies have focused on the efficacy of MSC therapy in combination with clinical hypothermia, the only recommended clinical intervention in HIE to date. Herz et al. (2018) hypothesized that hypothermia immediately after HIE induction (32°C during 4 h) and subsequent intranasal mouse BM-MSC treatment 3 days later would lead to augmented neuroprotection and improvement of neurological outcome in P9 mice. However, while both single therapies improved behavioral outcome and MBP protein levels, combination therapy abolished these protective effects. Additional *in vitro* and *in vivo* experiments revealed that hypothermia might alter the microenvironment in the brain, negatively impacting the potential of the MSC secretome (Herz et al., 2018). In contrast, another recent report showed positive effects of combination therapy in a rat model of HIE (Park et al., 2015; Ahn et al., 2018a). In their P7 rat model of HIE the combination of hypothermia, started 6 h after HIE induction (32°C during 24 h), plus intraventricular human umbilical cord blood-derived MSCs (UCB-MSCs; MSCs selected from the cord blood) at the time of hypothermia induction, improved functional recovery and attenuation of inflammation, measured by a reduction in optical density of the macrophage lineage marker ED-1 and cerebrospinal fluid (CSF) concentrations of pro-inflammatory cytokines, compared to either therapy alone (Park et al., 2015). Moreover, in a subsequent study these authors reported a broader therapeutic window of UCB-MSCs, as MSC treatment directly after a 48 h period of hypothermia (32°C, started 3 h after the HI insult) attenuated HIE associated brain injury compared to MSC treatment without prior hypothermia (Ahn et al., 2018a). It is, however, important to note that these studies exhibit large methodological differences in their HIE model, mode of administration and cell source. Most importantly, while Herz et al. (2018) treated animals 3 days after hypothermia, both other studies administer MSCs during or directly after cooling of the animals. Even though the additive effect of MSC therapy following therapeutic hypothermia in HIE is still up for discussion, multiple phase I/II clinical trials are currently underway or have recently been completed (NCT01962233, NCT02434965, NCT02881970, NCT02612155, and NCT03635450). A pioneer trial on human umbilical cord blood cell (UCBC; contains MSCs but also other cells from cord blood) treatment following therapeutic hypothermia in neonates with HIE showed that intravenous autologous UCBC treatment within 72 h postnatal is feasible and was not associated with any significant short-term adverse events in a small group of patients (Cotten et al., 2014). However, it is important to note that umbilical cord blood can contain a variety of cells, including MSCs (Pimentel-Coelho et al., 2010).

Perinatal Arterial Ischemic Stroke

Another important type of ischemic brain injury in term neonates is perinatal arterial ischemic stroke (PAIS), a cerebrovascular accident predominantly involving the middle cerebral artery (MCA), which is associated with serious morbidity in term neonates (Kirton and deVeber, 2009). Even though PAIS differs in pathology and symptoms from stroke in adults, white matter deficits are present in both conditions. While the body of evidence supporting MSC therapy in PAIS is less profound compared to its adult counterpart and compared to neonatal HIE, there are some studies providing evidence for the therapeutic potential of MSCs in PAIS. A recent review from our group summarized the findings of all available studies focusing on the potential of MSC therapy in *in vivo* experimental models of PAIS (Wagenaar et al., 2018). While the amount of evidence is limited, some studies that mimic PAIS by (transient) MCA occlusion do report attenuation of WM loss following intranasal rat BM-MSC therapy on either MRI-DTI parameters or by using histology (van Velthoven et al., 2013, 2017). A clinical trial studying the safety and feasibility of intranasal allogenic BM-MSC administration in PAIS patients will start at our center in the near future (PASSIoN; NCT03356821).

Mesenchymal Stem Cells in Preterm Brain Injury

The evidence provided supports MSCs in their ability to protect and regenerate the white matter after numerous types of injury in both the adult and neonatal brain. MSCs are shown to effectively stimulate OL survival, maturation and subsequent (re)myelination. Treatment options to combat preterm dWMI are currently lacking, as treatment possibilities such as hypothermia used in term HIE patients have shown to be ineffective or inapplicable in the preterm population (Deng, 2010). Nevertheless, preclinical studies investigating the potential of MSC treatment in specifically the diffuse form of preterm WMI

are still limited. However, stem cell therapy for other pathologies in the preterm brain, such as intraventricular hemorrhage (IVH) and cystic PVL, have received some attention over the years.

Intraventricular Hemorrhage

Apart from being susceptible to impaired WM maturation, preterm neonates are also prone to develop an IVH. Severe IVH (grade III/IV), in which the germinal matrix hemorrhage breaks through the ependymal lining into the ventricular system, followed by post-hemorrhagic ventricular dilatation (PHVD) or even secondary venous infarction, can result in serious neurological morbidity (Brouwer et al., 2012; Payne et al., 2013; Park et al., 2017). While the exact pathophysiology remains unclear, severe IVH is associated with damage to the (periventricular) white matter and cortical neuron dysfunction (Park et al., 2017). Pioneering studies of intraventricular human UCB-MSC therapy in a rat model of severe IVH revealed attenuation of the inflammatory response, reduction in apoptosis, restoration of corpus callosum thickness and improvement of myelination following MSC therapy (Ahn et al., 2013). Moreover, the incidence of PVHD, an important cause of (secondary) injury to the periventricular white matter, was significantly reduced after MSC treatment. A follow-up study on the optimal route of human UCB-MSC administration showed that both intracranial and intravenous administration were equally effective to reduce inflammation, reduce corpus callosum thinning and boost myelination following severe IVH (Ahn et al., 2015). The effectiveness of intravenous administration of human WJ-MSCs was confirmed by Mukai et al. (2017), who reported attenuation of hypomyelination and periventricular apoptosis following MSC therapy in a mouse model of severe IVH. Additional studies on the optimal timing of stem cell therapy showed a relatively limited window of treatment, as intraventricular human UCB-MSC therapy was shown solely to be effective when administered at 2 days after IVH compared to a 7 day interval (Park et al., 2016). The beneficial effect of UCB-MSCs in severe IVH was shown to be in part mediated by MSC-secreted brain-derived neurotrophic factor (BDNF) (Ahn et al., 2017). These promising results initiated the first clinical trial on MSC therapy in severe IVH in preterm infants, in which both a low and high dose of intraventricular UCB-MSCs were found to be safe and feasible (Ahn et al., 2018b). To evaluate the therapeutic potential a phase II trial is currently being executed (NCT02890953).

Cystic PVL

An early study of MSC therapy for preterm WMI by Chen et al. (2010) used a rat model of cystic PVL. In this model, bilateral injection of excitotoxic ibotenic acid (IBA) into the white matter of P5 rats leads to myelin loss, along with transient cyst formation, microglia activation, and cerebral palsy-like behavioral deficits (Chen et al., 2008). Animals were treated with neonatal rat BM-MSCs by unilateral intracerebral injection at 1 day post-PVL. Important to note with this study was that control PVL animals received an injection with cell-free MSC-CM. MSCs were shown to migrate to both lesioned hemispheres, increased endogenous glial cell proliferation and led to improved myelination and motor outcome compared to control PVL rats. Even though injection with MSCs was more effective than cell-free MSC-CM administration, conclusions on the possible (limited) effects of MSC-CM could not be made due to the lack of a suitable vehicle-treated control group (Chen et al., 2010). Similar results were obtained by Zhu et al. (2014) who induced PVL by ligation of the left common carotid artery, followed by 4 h of hypoxia (6% O_2) in P3 rats. Directly following PVL induction, rats received a daily intraperitoneal injection with human WJ-MSCs for 3 consecutive days. MSC treatment improved functional outcome in an open field test, reduced microglia and astrocyte activity and raised the amount of MBP-positive staining in the white matter. A more recent study induced PVL-like injury by an intraperitoneal lipopolysaccharide (LPS) injection (15 mg/kg) in P4 rats and demonstrated that intraperitoneal human WJ-MSC treatment significantly reduced pro-inflammatory cytokine expression in the brain and reversed the LPS-induced decrease in MBP-positive area (Morioka et al., 2017).

Diffuse WMI

While the studies on cystic PVL support the regenerative and anti-inflammatory capacities of MSCs, as discussed the most common form of preterm WMI is not focal necrosis but diffuse (non-cystic) WMI (Back, 2017). The effect of human WJ-MSC in diffuse WMI was studied by Mueller et al. (2017). In this study an intraperitoneal LPS injection (0.1 mg/kg) in P3 rat pups was followed by ligation of the left carotid artery combined with 40 min of hypoxia (8% O_2) the next day (P4). At P11 the animals received intracranial WJ-MSC treatment. WJ-MSC transplantation led to improvement in locomotor activity and less myelin loss and astrocyte activation. While these results are promising, intracranial MSC administration lacks clinical applicability. A recent study investigated intranasal delivery of human WJ-MSCs in a rat model of dWMI induced by intraperitoneal injection of LPS (0.1 mg/kg) at P2 and left carotid artery ligation and hypoxia at P3 (Oppliger et al., 2016). Neonatal rats treated intranasally with MSCs showed a reduction in myelination deficits and gliosis compared to vehicle-treated dWMI littermates. This model was associated with pre-OL depletion that could not be reversed by MSC therapy. Interestingly, the authors were able to identify two phenotypes of mature OLs. In vehicle-treated dWMI animals they found an increase in OLs with a MBP-positive and Ki67-negative perikaryon (mature non-proliferating OLs), with weak MBP-positive extensions. In MSC-treated dWMI animals, many MBP-positive and Ki67-negative OLs were also shown, but in contrast, these cells showed bright, thick and elaborate MBP-positive cell processes, indicating myelination. Based on these observations, the authors hypothesized that the local cerebral environment resulting from the insult could hinder newly generated OLs to fully regain their function and proceed with remyelination. In line with that hypothesis, human WJ-MSCs could beneficially change this negative cerebral environment by secreting immunomodulatory or trophic factors, leading to proper maturation of OLs and subsequent increase in myelin production.

In addition to rodent models of dWMI, effectiveness of MSC therapy has been explored in larger animal models. Multiple research groups have set up preterm sheep models to study dWMI. These models encompass *in utero* surgery between 95 and 102 days of gestation (for human gestation equivalence, see **Figure 1**) with either transient umbilical cord occlusion (Jellema et al., 2013; Li et al., 2016, 2018) or intra-uterine LPS infusion (Paton et al., 2018) leading to myelination deficits and pronounced OL cell death in the fetal sheep. A pioneer study by Jellema et al. (2013) showed reduction in OL loss, demyelination and microgliosis on histological and MRI-DTI outcome following intravenous human BM-MSC therapy in the fetal lamb *in utero*, 1 h after umbilical cord occlusion. Moreover, MSC administration was shown to affect the peripheral immune response by inducing T-cell tolerance. In a similar sheep model, Li et al. (2016) found an increase in OL numbers, myelin density and decrease of microglia activation and cell death when allogenic ovine UCBCs were administered intra-uterine intravenously at 12 h following transient umbilical cord occlusion to the fetal lamb. Important to note is that UCBCs include MSCs, but also contain lymphocytes, monocytes, and hematopoietic and endothelial stem cells. Interestingly, the therapeutic window proved to be limited. UCBC administration at 12 h after umbilical cord occlusion was effective, however, UCBC treatment at 5 days after the insult was no longer effective (Li et al., 2016). Follow-up studies in the preterm sheep model using intravenous administration of allogenic ovine UCB-MSCs to the fetal lamb *in utero* at 12 h after the insult showed similar results of reduced demyelination through modulation of peripheral and cerebral inflammatory processes (Li et al., 2018). An innovative study by Paton et al. (2018) modeled dWMI in preterm sheep by inducing *in utero* inflammation, a key hallmark of dWMI pathophysiology. Inflammation was induced by intravenous LPS (150 ng) infusion to the fetal sheep *in utero*, during three consecutive days at 95 days (65%) of gestation. 6 h following the final LPS dose, fetal sheep were treated with intravenous human UCBC therapy. UCBC treatment was shown to reduce cerebral gliosis, neutrophil recruitment to the brain and apoptosis, and to restore total and mature OL numbers in the preterm sheep. A recent follow-up study, adopting a similar experimental setup, compared the potential of intravenous human UCBC and human WJ-MSC treatment. WJ-MSCs were shown to be superior in dampening of the (neuro)inflammatory response, defined by lower IL-1β concentrations in the CSF and reduction of glial fibrillary acidic protein (GFAP) coverage in the white matter. However, only UCBCs were capable of reducing OL apoptosis, as measured by an decrease in active caspase-3 staining and a higher number of mature MBP-positive cells. In depth analyses showed a reduction of insulin-like growth factor-1 (IGF-1) expression in the white matter of MSC-treated animals compared to UCBC-treated animals. The authors suggest that enhanced downregulation of IGF-1, a vital growth factor in OL lineage survival and development, could in part be responsible for the absent neuroprotective properties of the WJ-MSCs compared to UCBCs (Paton et al., 2019). All in all this study shows that the working mechanism, the secretome assembly and the response to the local environment of different stem cells (or cellular compositions when using UCBCs) might vary extensively thereby affecting the potency of the different stem cell paradigms. Whether variable beneficial effects of WJ-MSCs versus UCBCs will be found in other experimental models of WMI remains to be studied. Importantly, this study illustrates that to gain optimal neuroprotective or neuroregenerative effects of stem cell therapy it will be crucial to target both neuroinflammation and OL differentiation.

STRATEGIES TO OPTIMIZE MSC THERAPY IN PRETERM WMI

The evidence supporting the efficacy and safety of MSC treatment in white matter pathologies is slowly mounting. However, these studies use a wide variety of methodological approaches, varying in important characteristics such as the MSC source, mode of administration and treatment timing. Moreover, efforts to optimize MSC efficacy through cell modification or preconditioning have been made over the years. These important different approaches and various optimization strategies will be discussed below.

The Source of MSCs

The most optimal source to harvest MSCs is still unclear. While the majority of the early studies use BM-MSCs, the use of MSCs from other sources, such as AD-MSCs and MSCs derived from cord blood or Wharton's jelly has increased in recent years. BM- and AD-MSCs can be obtained from animals or humans of any age, with cell harvest from adipose tissue being the least invasive. However, studies on the effectiveness of AD-MSCs seem inconclusive. Even though some studies report positive findings of (intravenous or intracranial) AD-MSC treatment on white matter regeneration in animal models of adult stroke and neonatal HIE (Gutierrez-Fernandez et al., 2013a; Park et al., 2013; Otero-Ortega et al., 2015), a very recent study by Sugiyama et al. (2018) has raised some concerns. These authors compared therapy with intravenous rat BM-MSCs versus rat AD-MSCs in a P7 rat model of HIE. Whereas apoptosis and microgliosis were both attenuated in animals treated with BM-MSCs, AD-MSC therapy was not associated with any neuroprotective effects. Importantly, AD-MSC therapy was related to a higher rate of pulmonary complications and mortality.

Although BM- and AD-MSCs can be collected during the whole lifespan, there is evidence linking advanced age to inferior therapeutic potential of the cells (Stolzing et al., 2008; Scruggs et al., 2013; Kalaszczynska and Ferdyn, 2015). Young, undamaged stem cells from the umbilical cord can be obtained without any invasive procedures, and are believed to have higher proliferative potential compared to BM- or AD-MSCs (Park et al., 2018). Even though autologous UCB- or WJ-MSC harvest and culture might not (always) be feasible due to limitations in time, logistics or lead to high variability due to differences in the patient's clinical condition (for example low pH following birth asphyxia), allogenic UC-MSC therapy is thought to be equally safe (El Omar et al., 2014) and could lead to an off-the-shelf cellular therapeutic

strategy with reduced variability in stem cells between patients. WJ-MSCs might be most suitable for allogenic treatment as they are thought to be least immunogenic (El Omar et al., 2014). Moreover, while isolation of MSCs from cord blood was shown to have a low yield of cells, Wharton's jelly is shown to produce consistent, high yields of MSCs (Zeddou et al., 2010). Recent studies have investigated potential differences in MSC potency as a result of developmental age and maternal conditions in stem cells harvested from the umbilical cord of preterm versus healthy term born neonates (Li et al., 2017; Oppliger et al., 2017). Oppliger et al. (2017) studied *in vitro* neural progenitor cells (NPC) differentiation toward the OL lineage following co-culture with WJ-MSCs, either from a preterm- or term neonatal donor. These authors show that both WJ-MSCs derived from preterm and term deliveries were able to stimulate differentiation of NPCs toward the glial lineage. However, the stem cells differed in their potential to produce mature OLs, as only WJ-MSCs from term deliveries increased the expression of MBP *in vitro*. WJ-MSCs from preterm deliveries did induce an increase of GalC, an immature OL marker, but did not result in maturation of OLs. In line with the study by Oppliger et al. (2017), a recent *in vivo* study by Li et al. (2017) found differences in mode of action between preterm and term UCB-MSCs. In their fetal sheep model both term- and preterm intravenous ovine UCB-MSC therapy reduced preterm WMI by reducing OL cell death, myelin loss and microgliosis. Interestingly, the secondary mechanisms underlying this neuroprotective effect seemed to differ between the cell types. Whereas both preterm and term UCB-MSCs attenuated neuroinflammation, preterm UCB-MSC treatment led to a decrease of TNF-α, while term UCB-MSC therapy caused an increase in anti-inflammatory IL-10. Moreover, term UCB-MSC treatment led to a reduction of oxidative stress, measured by fetal malondiadehyde (MDA) plasma levels, while preterm UCB-MSC treatment did not influence MDA levels. Thus, based on the non-invasive nature of cell harvest, high proliferative potential and apparent superior capacity to produce fully differentiated OLs, we suggest that term WJ-MSCs will perhaps be the stem cell of choice for the treatment of dWMI.

Route of MSC Administration

Besides questioning the optimal source of MSCs, the most efficient route of MSC administration is also still up for debate. Early studies focused mainly on local intracranial methods of stem cell delivery. Even though intracerebral administration ensures direct and targeted delivery and a minimum loss of stem cells, it is an invasive procedure. In order to avoid intracerebral injections, multiple studies looked at systemic MSC administration routes: either intravenous or intra-arterial applications. Despite being the more convenient and less invasive option, intravenous MSC administration can lead to entrapment of cells in other organs, such as the spleen, kidney, liver, or lungs, leading to a large reduction of cell numbers delivered to the brain (Danielyan et al., 2009). Although cell delivery to the brain is impaired, one could speculate that peripherally lost MSCs could still benefit the preterm patient with multi-organ dysfunction, by possibly dampening peripheral inflammation in the gut or lungs. Moreover, entrapment of MSCs in the spleen or liver has been reported to suppress T-cell activation and to contribute to the inactivation of destructive peripheral immune responses (Kurtz, 2008; Jellema et al., 2013). While intra-arterial MSC injection leads to a higher number of cells in the brain than intravenous application, it can lead to harmful microvascular occlusions (van Velthoven et al., 2010b; Park et al., 2018; Sarmah et al., 2018; Zhang et al., 2018). Interestingly, although this review provides a large body of evidence reporting a beneficial effect of intravenous MSC therapy, a recent meta-analysis including 64 studies regarding adult ischemic stroke found that the effect size and thus therapeutic potential of the invasive intracerebral route was superior compared to other routes of administration (Sarmah et al., 2018). A similar conclusion was drawn by Park et al. (2018), who argued in favor of local delivery of stem cells as delivery in the direct microenvironment of the lesion enhanced the paracrine potential of stem cells. In contrast, a study by Zhang et al. (2018) comparing intracerebral, intravenous and intra-arterial rat BM-MSC therapy in an adult rat model of ischemic stroke reported superior functional recovery, synaptogenesis, neurogenesis and axonal remodeling following intra-arterial MSC delivery compared to the other two administration methods. However, when considering the most optimal route of administration of stem cells for a specific neurological condition, it is vital to take both the pathophysiology of the injury and the clinical condition of the patients into account. In dWMI, OL development is disrupted throughout the developing brain. Even though predilection sites exist in the preterm brain due to spatial and temporal patterns in OL development, the injury, as the term suggests, is diffuse (van Tilborg et al., 2018). Therefore, local delivery of stem cells would be challenging in dWMI, as lesions are spread throughout the brain. Moreover, since extreme preterm infants admitted to the NICU suffer from multiple serious, often life-threatening morbidities, invasive intracranial procedures to deliver stem cells would not be preferable in an unstable patient. More recently, focus has shifted on intranasal MSC administration: a method of cell delivery that is non-invasive, direct, rapid, safe, and which evades loss of cells in the periphery (Danielyan et al., 2009). The possible migration routes following intranasal MSC administration cells were nicely illustrated by Danielyan et al. (2009). In short, stem cells are thought to pass the cribriform plate and migrate toward the lesion site through the olfactory bulb and brain parenchyma, CSF, trigeminal nerve and meningeal circulation. Experimental studies in models of SAH, MS, neonatal HIE and dWMI all show a beneficial outcome following intranasal MSC therapy, promoting endogenous repair of the brain (van Velthoven et al., 2010b; Donega et al., 2015; Oppliger et al., 2016; Nijboer et al., 2018). When comparing the effectiveness of the intranasal route to the intracerebral route of mouse BM-MSC delivery in a P9 mouse model of neonatal HIE, van Velthoven et al. (2012a) reported very similar functional recovery in mice with HIE-related injury. It is important to note that the treatment window in both intranasal as well as systemic administration of MSCs is most likely limited, due to loss of chemotactic signaling and recovery of BBB integrity, complicating MSC migration (Nessler et al., 2013; Donega et al.,

2014a). Apart from a limited time window for cell migration, the optimal window of MSC efficacy in dWMI is still up for debate. Preclinical studies in adult ischemic stroke, severe IVH and neonatal stroke demonstrated superior therapeutic efficacy in early (<48 h after injury induction) versus late (>7 days after injury induction) MSC treatment (Kim et al., 2012; Wang et al., 2014; Park et al., 2016). Interestingly, van Velthoven et al. (2010a) reported a treatment window of at least 10 days following injury induction in the P9 HIE mouse model. However, while early treatment could potentially give superior efficacy, pinpointing the exact timeframe in which injury develops and thereby determining the optimal treatment timing in the preterm infant is challenging. Namely, preterm infants encounter various potentially damaging insults consecutively, and multiple insults increase the risk of myelination failure (Rezaie and Dean, 2002; Zhao et al., 2013). Currently, dWMI diagnosis is based on MRI around term-equivalent age, when myelination is advancing (de Vries et al., 2013). In recent years, identification of biomarkers to predict neonatal brain injury has received increasing attention. In these studies multiple biomarkers in the blood, such as S100B, GFAP and metabolites as well as non-invasive monitoring such as EEG and NIRS have been shown to predict HIE, IVH, PHVD, and PVL (Douglas-Escobar and Weiss, 2012; Stewart et al., 2013; Jin et al., 2015; Lee, 2017). However, biomarkers for early identification of dWMI are still lacking. Future research is needed to identify the population of preterm infants that will develop dWMI, ensuring timely treatment to prevent myelination failure.

Optimizing the MSC Secretome

Other efforts in order to optimize MSC therapy are being made by targeting the paracrine potential of MSCs. Methods aiming to boost the MSC secretome can roughly be subdivided in two approaches, (1) preconditioning of MSCs, and (2) MSC modification. The first approach aims to optimize MSC paracrine functioning by subjecting the cells to an "adverse" event *in vitro*. These events are believed to prime the stem cells, making them more responsive and efficient upon arrival at the lesion site (Cunningham et al., 2018). Some of these preconditioning studies aim at enhancing the anti-inflammatory potential of MSCs by priming the cells with pro-inflammatory cytokines such as IFN-γ and IL-1. In a recent study by Redondo-Castro et al. (2017), a short (5 min) preconditioning period of human BM-MSCs with IL-1 (both α and β) enhanced the anti-inflammatory potential of the cells as a result of increased granulocyte colony stimulation factor (G-CSF) production, leading to a reduction of pro-inflammatory IL-6 and TNF-α production by cultured mouse microglia. In contrast, priming of human BM-MSCs with TNF-α or IFN-γ did not enhance the MSC potential (Redondo-Castro et al., 2017). In contrast, Morioka et al. (2017) did report a beneficial effect of IFN-γ pretreatment of human WJ-MSCs. In their P4 rat model of cystic PVL (please see above for description of the model), 4-day i.p. treatment with supernatant of WJ-MSCs pre-treated with IFN-γ did result in an significant increase in MBP-positive area in the brain, while treatment with medium of MSCs that were not preconditioned did not display this regenerative potential. Importantly, IFN-γ was absent in the preconditioned MSC medium, while anti-inflammatory and immunomodulatory factors, namely human tumor necrosis factor-stimulated gene-6 (TSG-6) and indoleamine 2,3-dioxygenase (IDO) were increased. Apart from preconditioning of MSCs using inflammatory stimuli, hypoxic preconditioning of MSCs has been proposed to boost the cell migration and survival capacity of MSCs. A study in a mouse model of adult stroke (MCAO) demonstrated a superior effect in migratory capacity of MSCs, as well as functional recovery of the animals following intranasal hypoxic-preconditioned (HP) rat BM-MSC treatment compared to treatment with MSCs cultured under normoxic conditions. Both cell types were equally effective in preventing apoptosis (Wei et al., 2013). Follow-up studies using ICH and neonatal stroke mouse models confirmed the enhanced potential of HP-BM-MSCs for neuronal regeneration and cell homing (Sun et al., 2015; Wei et al., 2015). Another interesting strategy is to precondition MSCs using ischemic brain extracts. Chen et al. (2002) exposed human MSCs (of unknown origin) to brain protein extracts of either stroke (MCAO) rats or control animals and demonstrated changes in the MSCs' secretome between the conditions. MSCs exposed to ischemic brain extracts showed increased secretion of trophic factors including BDNF, nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF). While this method is maybe not the most applicable option for the clinic, triggering the MSCs and thereby boosting their secretome prior to cell administration could possibly have added beneficial effects, though this needs to be examined in future preclinical studies. Other preconditioning methods, though studied less intensively in (neonatal) WMI, include serum- or medium-preconditioning or priming of MSCs with melatonin, respectively leading to increased MSC survival and functioning, and cell proliferation in the ischemic micro-environment following ischemic brain injury (Tang et al., 2014; Kim et al., 2016).

A different method to boost the MSC secretome is inducing overexpression of (trophic or immunomodulatory) factors by means of genetic engineering. The beneficial effect of modified MSCs has been studied in multiple brain pathologies. A number of preclinical adult MCAO studies found enhancement of functional recovery and reduction of infarct size following treatment with modified BM-MSCs, either overexpressing BDNF, glial cell line-derived neurotrophic factor (GDNF), hypoxia-inducible factor 1a (Hif-1a) or IL-10 compared to treatment with naïve BM-MSCs (Kurozumi et al., 2005; Lv et al., 2017; Nakajima et al., 2017). Other studies, more focused on white matter regeneration following modified MSC therapy, find a similar superior treatment efficacy of modified MSCs versus naïve MSCs. For instance, Liu et al. (2010) showed enhanced reduction of myelin loss, measured by luxol fast staining, in a rat model of adult hemorrhagic stroke, following intracranial treatment with human WJ-MSCs overexpressing HGF compared to animals receiving naïve MSCs. In a cuprizone mouse model of MS, intracerebrally administered IL-13-overexpressing mouse BM-MSCs were shown to superiorly attenuate microgliosis, OL apoptosis and demyelination when compared to naïve BM-MSCs

(Le Blon et al., 2016). Another study in the MS field, using an EAE mouse model reported improved functional recovery, greater reduction of pro-inflammatory cytokines in peripheral blood and enhanced reduction of cleaved caspase 3-positive (i.e., apoptotic) cells following intracerebral treatment with human ciliary neurotrophic factor (CNTF)-overexpressing MSCs versus naïve MSCs (origin unknown) (Lu et al., 2009). A study performed in our center in a mouse model of neonatal HIE found that intranasal treatment with mouse BDNF- or sonic hedgehog-overexpressing BM-MSCs led to additional reduction of MBP area loss when compared to naïve MSCs or vehicle-treated animals (van Velthoven et al., 2014). Although this strategy of genetic engineering seems very promising, it is associated with some safety concerns. Viral integration in the MSCs' genome might boost tumorigenicity. For that reason the use of adenoviruses to deliver the gene of interest into the MSCs could be preferable, as these viruses do not integrate into the hosts DNA (Schäfer et al., 2016; Park et al., 2018). Even though caution is advised, a clinical phase 1/2a trial for patients with adult stroke studying intracranial application of Notch-1-transfected human BM-MSCs reported no safety concerns, in addition to a favorable outcome at 12 months post-treatment (Steinberg et al., 2016).

Cell-Free Approaches: Stem-Cell Conditioned Medium and Extracellular Vesicles

Additional support for the vital role of the MSC's secretome comes from studies using either MSC-CM, or extracellular vesicles (EVs) released by MSCs in the treatment of brain injury. CM is defined as medium in which MSCs are cultured during variable lengths of time before collection and is thought to contain all elements of the MSC secretome, both paracrine secreted trophic and anti-inflammatory factors plus EVs (Cunningham et al., 2018). MSCs are believed to secrete multiple types of EVs, including exosomes and microvesicles, which arise from the endosomal compartment and from the plasma membrane, respectively. Both types of EVs contain a range of different cargos, such as mitochondria, messenger RNA (mRNA) and regulatory microRNA (miRNA), cytokines, and other proteins.

Conditioned Medium

Jadasz et al. (2013) compared the effect of unconditioned medium versus rat BM-MSC-CM (conditioned during 72 h) on the differentiation potential of primary cultured rat OPCs. Exposure to CM resulted in a boost of OPC maturation compared to unconditioned medium, measured by upregulation of myelin expression, increased MBP protein levels and immunopositive staining, and downregulation of important inhibitory signals. Similarly, when exposed to rat BM-MSC-CM conditioned during 72 h, rat NSCs differentiated toward the oligodendroglial lineage, shown by an increased MBP and CNPase gene expression compared to standard NSC medium, even when NSCs were challenged with growth factor withdrawal or were exposed to an astrogenic stimulus (Steffenhagen et al., 2012). A few *in vivo* studies also report a positive effect of CM therapy in experimental models of WMI. Bai et al. (2012) showed an increase in functional recovery and a reduction in demyelination, as measured by luxol fast blue staining, following intravenous human BM-MSC-CM treatment in a mouse model of autoimmune EAE. By studying the protein content of the CM, the authors discovered an important role of HGF in recovery of myelination. Exogenous intravenous HGF treatment resulted in recovery of myelination as well, while antibodies aimed against HGF or its receptor blocked the regenerative effects of both HGF and CM treatment. However, another study compared intracerebral rat BM-MSCs injections together with MSC-CM (conditioned during 24 h) injections into the lesion, and demonstrated a superior effect of live MSCs on the regeneration of the white matter in a rat model of cPVL when compared to CM only (Chen et al., 2010). The latter study indicates that continuous trophic factor or vesicle production by using actual MSCs, harboring a "regenerative niche" during several days, might be vital for optimal white matter regeneration.

Extracellular Vesicles

More recently, studies have focused on the use of EVs for white matter repair. A recent review by Cunningham et al. (2018) provides an excellent overview of the use of EVs in preclinical MCAO models, reporting positive effects of EVs on WM regeneration following adult stroke. In one of these studies, the authors reported white matter repair in adult rat subcortical stroke model as a result of a single intravenous administration of rat AD-MSC-EVs. The EV infusion led to an increase in expression of both CNPase and myelin oligodendrocyte glycoprotein (MOG), which are (early) mature OL markers, restored axonal myelination and improved mean axial diffusivity on DTI compared to vehicle-treated controls (Otero-Ortega et al., 2017). Moreover, another study using a mouse model of progressive MS (i.e., Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease) demonstrated that intravenously administered human AD-MSC-EVs were capable of improving functional outcome, attenuated neuroinflammation and boosted myelin expression in the mouse brain (Laso-Garcia et al., 2018). Similar findings have been reported in the field of preterm WMI. A pioneer study by Ophelders et al. (2016) using an ovine model of preterm WMI showed a reduction in seizure activity and partial protection against HI-induced myelination deficits following intravenous human BM-MSC-EVs therapy. However, EV treatment did not reduce OL apoptosis or cerebral inflammation. The authors suggest that while MSCs are able to "sense" the micro-environment and polarize toward an anti-inflammatory phenotype, EVs are static and therefore might lack immunomodulatory capabilities (Ophelders et al., 2016). A recent study by Drommelschmidt et al. (2017) did report reduction of gliosis following intraperitoneal human BM-MSC-EVs in an inflammatory model of preterm WMI (0.25 mg/kg LPS in P3 Wistar rats). Aside from reducing neuroinflammation, EV therapy reduced hypomyelination measured with MBP staining, and restored FA values up to SHAM control levels measured by DTI. Even though these studies all report (partial)

neuroprotective of regenerative effects of MSC-EV therapy, a direct comparison between MSC and MSC-EV therapy in dWMI has not been made. To the best of our current knowledge, only one study directly compared the efficacy of MSC-EV and MSC therapy directly in a mouse model of brain injury. Doeppner et al. (2015) reported comparable therapeutic effects of human BM-MSC treatment and human BM-MSC-EV in an adult MCAO mouse model. Both therapies potently promoted functional recovery and neurogenesis following stroke induction. It is, however, noteworthy that EV therapy failed to reduce cerebral immune cell infiltration whereas MSC therapy was capable of reducing leukocyte influx (Doeppner et al., 2015). Thus, both these authors and Ophelders et al. (2016) provide evidence for a more potent anti-inflammatory response of MSC therapy compared to EV treatment. The exosome content responsible for the observed regenerative effects is yet to be elucidated. An interesting *in vitro* study by Xiao et al. (2018) suggests an important role for a specific miRNA, miR-134.

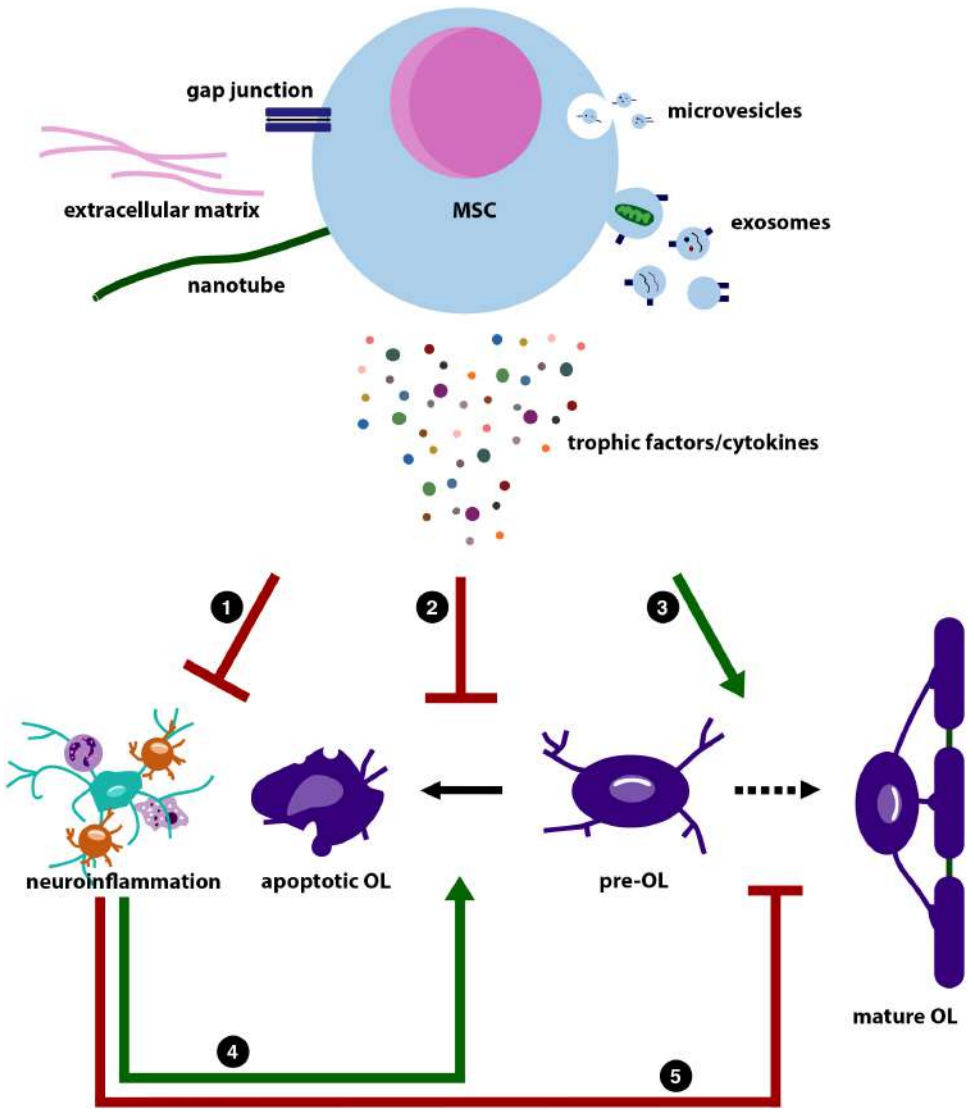


FIGURE 2 | Illustration summarizing the possible working mechanisms of mesenchymal stem cells (MSCs). This illustration is based on the literature discussed in this review. MSCs are believed to exert their regenerative potential through reciprocal transport via gap junctions (blue), production of extracellular matrix proteins (pink), such as laminin, and transport via nanotubes (green). In addition, MSCs are able to release microvesicles (top, small vesicles) and exosomes (bottom, larger vesicles). These vesicles contain a mix of miRNA, cytokines and trophic factors, and mitochondria. MSCs can also directly have paracrine effects on neighboring cells by release of trophic factors and cytokines. Direct cell contact and the MSCs' secretome can (1) dampen (red inhibitory arrow) the immune response in the periphery and central nervous system (CNS) (astrocyte in green; microglia in orange; neutrophil left top; and macrophage right bottom). The MSCs' secretome can also (2) directly inhibit apoptosis of pre-myelinating OL (pre-OL; irregular shaped purple cell), whereas it can also (3) directly stimulate (green stimulatory arrow) pre-OL differentiation toward myelinating mature OLs (middle and right purple cells). As neuroinflammation will (4) increase pre-OL apoptosis, and (5) inhibit pre-OL maturation [lower stimulatory arrow (green) and lower inhibitory arrow (red), respectively], MSCs can also indirectly reduce pre-OL apoptosis and stimulate pre-OL maturation by dampening neuroinflammation.

This miRNA was, among 8 other candidates, found in rat BM-MSC exosomes and has been shown to inhibit OL apoptosis in a primary rat OPC culture after oxygen and glucose deprivation, by targeting caspase 8 (Xiao et al., 2018).

In conclusion, CM or EVs seem potent alternatives to whole MSCs to restore myelination deficits in multiple animals models of brain injury. For an overview on the possible mechanisms of action of MSCs including cell-free approaches, see **Figure 2**. The use of these cell-free strategies to treat dWMI could prove to have superior clinical applicability compared to live cell administration, that theoretically could raise some safety concerns. However, solid future research comparing the efficacy of CM- or EV-based treatments to conventional MSC therapy in dWMI is needed.

CONCLUDING REMARKS

Currently, a large body of evidence supports a role for MSCs to protect and restore damage to the white matter of the brain. Studies in the field of adult stroke, MS and multiple neonatal brain pathologies underline the anti-inflammatory, immunomodulatory and trophic properties of MSCs, most likely mediated by the MSC secretome. However, several challenges have to be overcome when translating experimental data of MSC treatment to the preterm dWMI field, and eventually toward clinical application.

Even though evidence supporting the beneficial potential of MSCs in boosting (re)myelination following injury is mounting, the number of preclinical studies supporting the efficacy of MSC therapy in dWMI remains limited. This limited amount of evidence is important to consider, as the pathophysiology underlying preterm dWMI is substantially different from other neonatal or adult brain pathologies. In (neonatal) stroke and HIE, the loss of WM volume is, at least partially, the result of loss of white matter (most likely secondary to gray matter loss) with a pronounced role of OL cell death, while in MS immune system dysfunction leads to demyelination (Compston and Coles, 2002; Gutierrez-Fernandez et al., 2013b; Mifsud et al., 2014). Even in other preterm white matter pathologies, such as IVH and cystic PVL, WM loss is most likely more a result of OL apoptosis rather than impaired OL maturation as proposed in dWMI (Volpe et al., 2011; Buser et al., 2012). Thus, more research is needed on MSC therapy in clinically relevant models of specifically preterm dWMI, preferably in both rodents and larger species.

Another challenge for future clinical application is determining the best MSC treatment strategy. Due to methodological differences in experimental design, including MSC origin and mode of administration the optimal treatment protocol is currently unclear. Based on present literature, intranasally applied term WJ-MSCs might prove to be most optimal candidate, due to non-invasive cell harvest and a clinically applicable non-invasive administration route combined with excellent cell homing and paracrine properties of the cells (Li et al., 2017; Oppliger et al., 2017). However, to substantiate this statement, additional preclinical studies in models of dWMI, with a back-to-back comparison of the efficacy of multiple cell origins and routes of administration are needed.

In this review a number of strategies has been discussed to further optimize MSC therapy, all aimed to promote or adapt the anti-inflammatory and trophic factors within the MSC secretome. These options all seem promising, particularly hypoxic preconditioning and MSC genetic modification, but lack sufficient evidence in the dWMI field at present. More importantly, while CM and EV studies underline the vital role of paracrine signaling in MSC-mediated WMI recovery, the specific beneficial mediators of the MSC secretome remain unclear. Insight in the trophic and immunomodulatory factors, and other regulators (such as miRNA) in the MSC secretome underlying the boost of myelination of the preterm brain would not only provide a good basis for MSC optimization (i.e., overexpression studies) but also pave the way for potential cell-free treatment options, such as a cocktail of preferred beneficial growth factors. Cell-free strategies could be the more clinically desirable option, as these alternatives can be easily stored without any concerns on cell viability or safety. However, when taking into consideration the outcome of current CM and EV studies, it is still questionable whether these alternatives will truly replace the need of a whole cell-based therapy. Based on the evidence provided in this review, a regenerative niche harboring continuous (at least days-long) secretion of trophic factors, or possibly direct cell contact between MSCs and neural progenitors is more desirable than transient treatment with MSC derivatives. Therefore, additional research comparing the efficacy of cell-free (either EV, CM, or, growth factor cocktails) alternatives to whole-cell MSC therapy in dWMI models is urgently needed. Moreover in future, combination therapies of MSCs with other regenerative strategies, such as specific trophic factor supplementation, might prove to even further benefit the injured preterm brain.

Despite the fact that there are still quite some challenges to overcome before optimal clinical translation, this review shows that treatment with MSCs or its derivatives is a near-future favorable and promising novel regenerative treatment strategy to improve the prospects and quality of life for preterm infants suffering from dWMI.

AUTHOR CONTRIBUTIONS

JV and MV performed the literature search including the reading of selected literature. JV drafted the manuscript in collaboration with CN. CdT, FH, and MB revised the manuscript.

FUNDING

This work was supported by Brain Foundation Netherlands.

REFERENCES

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Ahn, J.-Y., and Park, W. S. (2017). Pivotal role of brain-derived neurotrophic factor secreted by mesenchymal stem cells in severe intraventricular hemorrhage in newborn rats. *Cell Transplant.* 26, 145–156. doi: 10.3727/096368916x692861

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., and Park, W. S. (2018a). Hypothermia broadens the therapeutic time window of mesenchymal stem cell transplantation for severe neonatal hypoxic ischemic encephalopathy. *Sci. Rep.* 8:7665. doi: 10.1038/s41598-018-25902-x

Ahn, S. Y., Chang, Y. S., Sung, S. I., and Park, W. S. (2018b). Mesenchymal stem cells for severe intraventricular hemorrhage in preterm infants: phase I dose-escalation clinical trial. *Stem Cells Transl. Med.* 7, 847–856. doi: 10.1002/sctm.17-0219

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Yoo, H. S., Im, G. H., et al. (2015). Optimal route for mesenchymal stem cells transplantation after severe intraventricular hemorrhage in newborn rats. *PLoS One* 10:e0132919. doi: 10.1371/journal.pone.0132919

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Yoo, H. S., Lee, J. H., et al. (2013). Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. *Stroke* 44, 497–504. doi: 10.1161/strokeaha.112.679092

Back, S. A. (2015). Brain injury in the preterm infant: new horizons for pathogenesis and prevention. *Pediatr. Neurol.* 53, 185–192. doi: 10.1016/j.pediatrneurol.2015.04.006

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathol.* 134, 331–349. doi: 10.1007/s00401-017-1718-6

Back, S. A., Luo, N. L., Borenstein, N. S., Levine, J. M., Volpe, J. J., Kinney, H. C., et al. (2001). Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J. Neurosci.* 21, 1302–1312.

Back, S. A., and Miller, S. P. (2014). Brain injury in premature neonates: a primary cerebral dysmaturation disorder? *Ann. Neurol.* 75, 469–486. doi: 10.1002/ana.24132

Back, S. A., Riddle, A., Dean, J., and Hohimer, A. R. (2012). The instrumented fetal sheep as a model of cerebral white matter injury in the premature infant. *Neurotherapeutics* 9, 359–370. doi: 10.1007/s13311-012-0108-y

Back, S. A., Riddle, A., and McClure, M. M. (2007). Maturation-dependent vulnerability of perinatal white matter in premature birth. *Stroke* 38(2 Suppl.), 724–730. doi: 10.1161/01.STR.0000254729.27386.05

Bai, L., Lennon, D. P., Caplan, A. I., DeChant, A., Hecker, J., Kranso, J., et al. (2012). Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. *Nat. Neurosci.* 15, 862–870. doi: 10.1038/nn.3109

Bai, L., Lennon, D. P., Eaton, V., Maier, K., Caplan, A. I., Miller, S. D., et al. (2009). Human bone marrow-derived mesenchymal stem cells induce Th2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis. *Glia* 57, 1192–1203. doi: 10.1002/glia.20841

Bedini, G., Bersano, A., Zanier, E. R., Pischiutta, F., and Parati, E. A. (2018). Mesenchymal stem cell therapy in intracerebral haemorrhagic stroke. *Curr. Med. Chem.* 25, 2176–2197. doi: 10.2174/0929867325666180111101410

Bennet, L., Dhillon, S., Lear, C. A., van den Heuij, L., King, V., Dean, J. M., et al. (2018). Chronic inflammation and impaired development of the preterm brain. *J. Reprod. Immunol.* 125, 45–55. doi: 10.1016/j.jri.2017.11.003

Billiards, S. S., Haynes, R. L., Folkerth, R. D., Borenstein, N. S., Trachtenberg, F. L., Rowitch, D. H., et al. (2008). Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia. *Brain Pathol.* 18, 153–163. doi: 10.1111/j.1750-3639.2007.00107.x

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A.-B., Narwal, R., et al. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 379, 2162–2172. doi: 10.1016/S0140-6736(12)60820-4

Bradl, M., and Lassmann, H. (2010). Oligodendrocytes: biology and pathology. *Acta Neuropathol.* 119, 37–53. doi: 10.1007/s00401-009-0601-5

Brouwer, A. J., van Stam, C., Uniken Venema, M., Koopman, C., Groenendaal, F., and de Vries, L. S. (2012). Cognitive and neurological outcome at the age of 5-8 years of preterm infants with post-hemorrhagic ventricular dilatation requiring neurosurgical intervention. *Neonatology* 101, 210–216. doi: 10.1159/000331797

Brown, M. K., and DiBlasi, R. M. (2011). Mechanical ventilation of the premature neonate. *Respiratory Care* 56, 1298–1313. doi: 10.4187/respcare.01429

Buser, J. R., Maire, J., Riddle, A., Gong, X., Nguyen, T., Nelson, K., et al. (2012). Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. *Ann. Neurol.* 71, 93–109. doi: 10.1002/ana.22627

Chen, A., Dimambro, N., and Clowry, G. J. (2008). A comparison of behavioural and histological outcomes of periventricular injection of ibotenic acid in neonatal rats at postnatal days 5 and 7. *Brain Res.* 1201, 187–195. doi: 10.1016/j.brainres.2008.01.066

Chen, A., Siow, B., Blamire, A. M., Lako, M., and Clowry, G. J. (2010). Transplantation of magnetically labeled mesenchymal stem cells in a model of perinatal brain injury. *Stem Cell Res.* 5, 255–266. doi: 10.1016/j.scr.2010.08.004

Chen, X., Li, Y., Wang, L., Katakowski, M., Zhang, L., Chen, J., et al. (2002). Ischemic rat brain extracts induce human marrow stromal cell growth factor production. *Neuropathology* 22, 275–279.

Ciccarelli, O., Barkhof, F., Bodini, B., De Stefano, N., Golay, X., Nicolay, K., et al. (2014). Pathogenesis of multiple sclerosis: insights from molecular and metabolic imaging. *Lancet Neurol.* 13, 807–822. doi: 10.1016/s1474-4422(14)70101-2

Cohen, J. A. (2013). Mesenchymal stem cell transplantation in multiple sclerosis. *J. Neurol. Sci.* 333, 43–49. doi: 10.1016/j.jns.2012.12.009

Compston, A., and Coles, A. (2002). Multiple sclerosis. *Lancet* 359, 1221–1231. doi: 10.1016/s0140-6736(02)08220-x

Corcelli, M., Hawkins, K., Vlahova, F., Hunjan, A., Dowding, K., De Coppi, P., et al. (2018). Neuroprotection of the hypoxic-ischemic mouse brain by human CD117(+)CD90(+)CD105(+) amniotic fluid stem cells. *Sci. Rep.* 8:2425. doi: 10.1038/s41598-018-20710-9

Correale, J., Gaitan, M. I., Ysrraelit, M. C., and Fiol, M. P. (2017). Progressive multiple sclerosis: from pathogenic mechanisms to treatment. *Brain* 140, 527–546. doi: 10.1093/brain/aww258

Cotten, C. M., Murtha, A. P., Goldberg, R. N., Grotegut, C. A., Smith, P. B., Goldstein, R. F., et al. (2014). Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *J. Pediatr.* 164:973–979.e971. doi: 10.1016/j.jpeds.2013.11.036

Craig, A., Ling Luo, N., Beardsley, D. J., Wingate-Pearse, N., Walker, D. W., Hohimer, A. R., et al. (2003). Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Exp. Neurol.* 181, 231–240.

Cristofanilli, M., Harris, V. K., Zigelbaum, A., Goossens, A. M., Lu, A., Rosenthal, H., et al. (2011). Mesenchymal stem cells enhance the engraftment and myelinating ability of allogeneic oligodendrocyte progenitors in dysmyelinated mice. *Stem Cells Dev.* 20, 2065–2076. doi: 10.1089/scd.2010.0547

Cruz-Martinez, P., Gonzalez-Granero, S., Molina-Navarro, M. M., Pacheco-Torres, J., Garcia-Verdugo, J. M., Geijo-Barrientos, E., et al. (2016). Intraventricular injections of mesenchymal stem cells activate endogenous functional remyelination in a chronic demyelinating murine model. *Cell Death Dis.* 7:e2223. doi: 10.1038/cddis.2016.130

Cunningham, C. J., Redondo-Castro, E., and Allan, S. M. (2018). The therapeutic potential of the mesenchymal stem cell secretome in ischaemic stroke. *J. Cereb. Blood Flow Metab.* 38, 1276–1292. doi: 10.1177/0271678x18776802

Danielyan, L., Schafer, R., von Ameln-Mayerhofer, A., Buadze, M., Geisler, J., Klopfer, T., et al. (2009). Intranasal delivery of cells to the brain. *Eur. J. Cell Biol.* 88, 315–324. doi: 10.1016/j.ejcb.2009.02.001

De Miguel, M. P., Fuentes-Julian, S., Blazquez-Martinez, A., Pascual, C. Y., Aller, M. A., Arias, J., et al. (2012). Immunosuppressive properties of mesenchymal stem cells: advances and applications. *Curr. Mol. Med.* 12, 574–591.

de Vries, L. S., Benders, M. J. N. L., and Groenendaal, F. (2013). Imaging the premature brain: ultrasound or MRI? *Neuroradiology* 55, 13–22. doi: 10.1007/s00234-013-1233-y

Deng, W. (2010). Neurobiology of injury to the developing brain. *Nat. Rev. Neurol.* 6, 328–336. doi: 10.1038/nrneurol.2010.53

Ding, H., Zhang, H., Ding, H., Li, D., Yi, X., Ma, X., et al. (2017). Transplantation of placenta-derived mesenchymal stem cells reduces hypoxic-ischemic brain

damage in rats by ameliorating the inflammatory response. *Cell Mol. Immunol.* 14, 693–701. doi: 10.1038/cmi.2015.99

Doeppner, T. R., Herz, J., Gorgens, A., Schlechter, J., Ludwig, A. K., Radtke, S., et al. (2015). Extracellular vesicles improve post-stroke neuroregeneration and prevent postischemic immunosuppression. *Stem Cells Transl. Med.* 4, 1131–1143. doi: 10.5966/sctm.2015-0078

Donega, V., Nijboer, C. H., Braccioli, L., Slaper-Cortenbach, I., Kavelaars, A., van Bel, F., et al. (2014a). Intranasal administration of human MSC for ischemic brain injury in the mouse: in vitro and in vivo neuroregenerative functions. *PLoS One* 9:e112339. doi: 10.1371/journal.pone.0112339

Donega, V., Nijboer, C. H., van Tilborg, G., Dijkhuizen, R. M., Kavelaars, A., and Heijnen, C. J. (2014b). Intranasally administered mesenchymal stem cells promote a regenerative niche for repair of neonatal ischemic brain injury. *Exp. Neurol.* 261, 53–64. doi: 10.1016/j.expneurol.2014.06.009

Donega, V., Nijboer, C. H., van Velthoven, C. T., Youssef, S. A., de Bruin, A., van Bel, F., et al. (2015). Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse. *Pediatr. Res.* 78, 520–526. doi: 10.1038/pr.2015.145

Douglas-Escobar, M., and Weiss, M. D. (2012). Biomarkers of brain injury in the premature infant. *Front. Neurol.* 3:185. doi: 10.3389/fneur.2012.00185

Douglas-Escobar, M., and Weiss, M. D. (2015). Hypoxic-ischemic encephalopathy: a review for the clinician. *JAMA Pediatrics* 169, 397–403. doi: 10.1001/jamapediatrics.2014.3269

Drommelschmidt, K., Serdar, M., Bendix, I., Herz, J., Bertling, F., Prager, S., et al. (2017). Mesenchymal stem cell-derived extracellular vesicles ameliorate inflammation-induced preterm brain injury. *Brain Behav. Immun.* 60, 220–232. doi: 10.1016/j.bbi.2016.11.011

Dulamea, A. O. (2015). The potential use of mesenchymal stem cells in stroke therapy–From bench to bedside. *J. Neurol. Sci.* 352, 1–11. doi: 10.1016/j.jns.2015.03.014

El Omar, R., Beroud, J., Stoltz, J. F., Menu, P., Velot, E., and Decot, V. (2014). Umbilical cord mesenchymal stem cells: the new gold standard for mesenchymal stem cell-based therapies? *Tissue Eng. Part B Rev.* 20, 523–544. doi: 10.1089/ten.TEB.2013.0664

Emery, B. (2010). Regulation of oligodendrocyte differentiation and myelination. *Science* 330, 779–782. doi: 10.1126/science.1190927

Favrais, G., van de Looij, Y., Fleiss, B., Ramanantsoa, N., Bonnin, P., Stoltenburg-Didinger, G., et al. (2011). Systemic inflammation disrupts the developmental program of white matter. *Ann. Neurol.* 70, 550–565. doi: 10.1002/ana.22489

Freeman, M. R., and Rowitch, D. H. (2013). Evolving concepts of gliogenesis: a look way back and ahead to the next 25 years. *Neuron* 80, 613–623. doi: 10.1016/j.neuron.2013.10.034

Fyfe, K. L., Yiallourou, S. R., Wong, F. Y., and Horne, R. S. (2014). The development of cardiovascular and cerebral vascular control in preterm infants. *Sleep Med. Rev.* 18, 299–310. doi: 10.1016/j.smrv.2013.06.002

Garg, N., and Smith, T. W. (2015). An update on immunopathogenesis, diagnosis, and treatment of multiple sclerosis. *Brain Behav.* 5:e00362. doi: 10.1002/brb3.362

Genc, B., Bozan, H. R., Genc, S., and Genc, K. (2018). Stem cell therapy for multiple sclerosis. *Adv. Exp. Med. Biol.* doi: 10.1007/5584_2018_247 [Epub ahead of print].

Gharibi, T., Ahmadi, M., Seyfizadeh, N., Jadidi-Niaragh, F., and Yousefi, M. (2015). Immunomodulatory characteristics of mesenchymal stem cells and their role in the treatment of multiple sclerosis. *Cell Immunol.* 293, 113–121. doi: 10.1016/j.cellimm.2015.01.002

Gluckman, P., Klempt, N., Guan, J., Mallard, C., Sirimanne, E., Dragunow, M., et al. (1992). A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem. Biophys. Res. Commun.* 182, 593–599.

Gu, Y., He, M., Zhou, X., Liu, J., Hou, N., Bin, T., et al. (2016). Endogenous IL-6 of mesenchymal stem cell improves behavioral outcome of hypoxic-ischemic brain damage neonatal rats by supressing apoptosis in astrocyte. *Sci. Rep.* 6:18587. doi: 10.1038/srep18587

Gu, Y., Zhang, Y., Bi, Y., Liu, J., Tan, B., Gong, M., et al. (2015). Mesenchymal stem cells suppress neuronal apoptosis and decrease IL-10 release via the TLR2/NFkappaB pathway in rats with hypoxic-ischemic brain damage. *Mol. Brain* 8:65. doi: 10.1186/s13041-015-0157-3

Gutierrez-Fernandez, M., Rodriguez-Frutos, B., Ramos-Cejudo, J., Teresa Vallejo-Cremades, M., Fuentes, B., Cerdan, S., et al. (2013a). Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke. *Stem Cell Res. Ther.* 4:11. doi: 10.1186/scrt159

Gutierrez-Fernandez, M., Rodriguez-Frutos, B., Ramos-Cejudo, J., Otero-Ortega, L., Fuentes, B., and Diez-Tejedor, E. (2013b). Stem cells for brain repair and recovery after stroke. *Expert. Opin. Biol. Ther.* 13, 1479–1483. doi: 10.1517/14712598.2013.824420

Hagberg, H., Gressens, P., and Mallard, C. (2012). Inflammation during fetal and neonatal life: implications for neurologic and neuropsychiatric disease in children and adults. *Ann. Neurol.* 71, 444–457. doi: 10.1002/ana.22620

Hayakawa, K., and Lo, E. H. (2016). Brain-peripheral cell crosstalk in white matter damage and repair. *Biochim. Biophys. Acta* 1862, 901–908. doi: 10.1016/j.bbadis.2015.08.006

Herz, J., Koster, C., Reinboth, B. S., Dzietko, M., Hansen, W., Sabir, H., et al. (2018). Interaction between hypothermia and delayed mesenchymal stem cell therapy in neonatal hypoxic-ischemic brain injury. *Brain Behav. Immun.* 70, 118–130. doi: 10.1016/j.bbi.2018.02.006

Jacobs, S. A., Roobrouck, V. D., Verfaillie, C. M., and Van Gool, S. W. (2013). Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. *Immunol. Cell Biol.* 91, 32–39. doi: 10.1038/icb.2012.64

Jadasz, J. J., Aigner, L., Rivera, F. J., and Kury, P. (2012). The remyelination Philosopher's Stone: stem and progenitor cell therapies for multiple sclerosis. *Cell Tissue Res.* 349, 331–347. doi: 10.1007/s00441-012-1331-x

Jadasz, J. J., Kremer, D., Gottle, P., Tzekova, N., Domke, J., Rivera, F. J., et al. (2013). Mesenchymal stem cell conditioning promotes rat oligodendroglial cell maturation. *PLoS One* 8:e71814. doi: 10.1371/journal.pone.0071814

Jakovcevski, I., Filipovic, R., Mo, Z., Rakic, S., and Zecevic, N. (2009). Oligodendrocyte development and the onset of myelination in the human fetal brain. *Front. Neuroanat.* 3:5. doi: 10.3389/neuro.05.005.2009

Jaramillo-Merchan, J., Jones, J., Ivorra, J. L., Pastor, D., Viso-Leon, M. C., Armengol, J. A., et al. (2013). Mesenchymal stromal-cell transplants induce oligodendrocyte progenitor migration and remyelination in a chronic demyelination model. *Cell Death Dis.* 4:e779. doi: 10.1038/cddis.2013.304

Jellema, R. K., Wolfs, T. G., Lima Passos, V., Zwanenburg, A., Ophelders, D. R., Kuypers, E., et al. (2013). Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia. *PLoS One* 8:e73031. doi: 10.1371/journal.pone.0073031

Jiang, Q., Zhang, Z. G., Ding, G. L., Silver, B., Zhang, L., Meng, H., et al. (2006). MRI detects white matter reorganization after neural progenitor cell treatment of stroke. *Neuroimage* 32, 1080–1089.

Jin, C., Londono, I., Mallard, C., and Lodygensky, G. A. (2015). New means to assess neonatal inflammatory brain injury. *J. Neuroinflamm.* 12:180. doi: 10.1186/s12974-015-0397-2

Johnson, S., Fawke, J., Hennessy, E., Rowell, V., Thomas, S., Wolke, D., et al. (2009). Neurodevelopmental disability through 11 years of age in children born before 26 weeks of gestation. *Pediatrics* 124, e249–e257.

Kalaszczynska, I., and Ferdyn, K. (2015). Wharton's jelly derived mesenchymal stem cells: future of regenerative medicine? Recent findings and clinical significance. *Biomed. Res. Int.* 2015:430847. doi: 10.1155/2015/430847

Kassis, I., Grigoriadis, N., Gowda-Kurkalli, B., Mizrachi-Kol, R., Ben-Hur, T., Slavin, S., et al. (2008). Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. *Arch. Neurol.* 65, 753–761. doi: 10.1001/archneur.65.6.753

Kassis, I., Vaknin-Dembinsky, A., and Karussis, D. (2011). Bone marrow mesenchymal stem cells: agents of immunomodulation and neuroprotection. *Curr. Stem Cell Res. Ther.* 6, 63–68.

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., and Richardson, W. D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat. Neurosci.* 9, 173–179. doi: 10.1038/nn1620

Khwaja, O., and Volpe, J. J. (2008). Pathogenesis of cerebral white matter injury of prematurity. *Arch. Dis. Child. Fetal Neonatal Ed.* 93, F153–F161. doi: 10.1136/adc.2006.108837

Kim, E. H., Kim, D. H., Kim, H. R., Kim, S. Y., Kim, H. H., and Bang, O. Y. (2016). Stroke serum priming modulates characteristics of mesenchymal stromal cells by controlling the expression miRNA-20a. *Cell Transplant.* 25, 1489–1499. doi: 10.3727/096368916x690430

Kim, E. S., Ahn, S. Y., Im, G. H., Sung, D. K., Park, Y. R., Choi, S. H., et al. (2012). Human umbilical cord blood–derived mesenchymal stem cell transplantation attenuates severe brain injury by permanent middle cerebral artery occlusion in newborn rats. *Pediatr. Res.* 72:277. doi: 10.1038/pr.2012.71

Kirton, A., and deVeber, G. (2009). Advances in perinatal ischemic stroke. *Pediatr. Neurol.* 40, 205–214. doi: 10.1016/j.pediatrneurol.2008.09.018

Knuesel, I., Chicha, L., Britschgi, M., Schobel, S. A., Bodmer, M., Hellings, J. A., et al. (2014). Maternal immune activation and abnormal brain development across CNS disorders. *Nat. Rev. Neurol.* 10, 643–660. doi: 10.1038/nrneurol.2014.187

Kobolak, J., Dinnyes, A., Memic, A., Khademhosseini, A., and Mobasheri, A. (2016). Mesenchymal stem cells: identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial micro-engineering of their niche. *Methods* 99, 62–68. doi: 10.1016/j.ymeth.2015.09.016

Kurozumi, K., Nakamura, K., Tamiya, T., Kawano, Y., Ishii, K., Kobune, M., et al. (2005). Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. *Mol. Ther.* 11, 96–104. doi: 10.1016/j.ymthe.2004.09.020

Kurtz, A. (2008). Mesenchymal stem cell delivery routes and fate. *Int. J. Stem Cells* 1, 1–7.

Lakshminrusimha, S., Manja, V., Mathew, B., and Suresh, G. K. (2014). Oxygen targeting in preterm infants: a physiological interpretation. *J. Perinatol.* 35:8.

Laroni, A., Novi, G., Kerlero de Rosbo, N., and Uccelli, A. (2013). Towards clinical application of mesenchymal stem cells for treatment of neurological diseases of the central nervous system. *J. Neuroimmune Pharmacol.* 8, 1062–1076. doi: 10.1007/s11481-013-9456-6

Larroque, B., Ancel, P. Y., Marret, S., Marchand, L., Andre, M., Arnaud, C., et al. (2008). Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study. *Lancet* 371, 813–820. doi: 10.1016/s0140-6736(08)60380-3

Laso-Garcia, F., Ramos-Cejudo, J., Carrillo-Salinas, F. J., Otero-Ortega, L., Feliu, A., Gomez-de Frutos, M., et al. (2018). Therapeutic potential of extracellular vesicles derived from human mesenchymal stem cells in a model of progressive multiple sclerosis. *PLoS One* 13:e0202590. doi: 10.1371/journal.pone.0202590

Le Blon, D., Guglielmetti, C., Hoornaert, C., Quarta, A., Daans, J., Dooley, D., et al. (2016). Intracerebral transplantation of interleukin 13-producing mesenchymal stem cells limits microgliosis, oligodendrocyte loss and demyelination in the cuprizone mouse model. *J. Neuroinflamm.* 13:288. doi: 10.1186/s12974-016-0756-7

Lee, Y. A. (2017). White matter injury of prematurity: its mechanisms and clinical features. *J. Pathol. Transl. Med.* 51, 449–455. doi: 10.4132/jptm.2017.07.25

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Allison, B. J., et al. (2017). Term vs. preterm cord blood cells for the prevention of preterm brain injury. *Pediatr. Res.* 82, 1030–1038. doi: 10.1038/pr.2017.170

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., et al. (2016). Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells. *Exp. Neurol.* 283(Pt A), 179–187. doi: 10.1016/j.expneurol.2016.06.017

Li, J., Yawno, T., Sutherland, A. E., Gurung, S., Paton, M., McDonald, C., et al. (2018). Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia. *Exp. Neurol.* 308, 120–131. doi: 10.1016/j.expneurol.2018.07.006

Liang, X., Ding, Y., Zhang, Y., Tse, H. F., and Lian, Q. (2014). Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant.* 23, 1045–1059. doi: 10.3727/096368913x667709

Lin, H. Y., Liou, C. W., Chen, S. D., Hsu, T. Y., Chuang, J. H., Wang, P. W., et al. (2015). Mitochondrial transfer from Wharton's jelly-derived mesenchymal stem cells to mitochondria-defective cells recaptures impaired mitochondrial function. *Mitochondrion* 22, 31–44. doi: 10.1016/j.mito.2015.02.006

Linsell, L., Johnson, S., Wolke, D., O'Reilly, H., Morris, J. K., Kurinczuk, J. J., et al. (2018). Cognitive trajectories from infancy to early adulthood following birth before 26 weeks of gestation: a prospective, population-based cohort study. *Arch. Dis. Child.* 103, 363–370. doi: 10.1136/archdischild-2017-313414

Liu, A. M., Lu, G., Tsang, K. S., Li, G., Wu, Y., Huang, Z. S., et al. (2010). Umbilical cord-derived mesenchymal stem cells with forced expression of hepatocyte growth factor enhance remyelination and functional recovery in a rat intracerebral hemorrhage model. *Neurosurgery* 67, 357–365; discussion 365–356. doi: 10.1227/01.neu.0000371983.06278.b3

Liu, R., Zhang, Z., Lu, Z., Borlongan, C., Pan, J., Chen, J., et al. (2013). Human umbilical cord stem cells ameliorate experimental autoimmune encephalomyelitis by regulating immunoinflammation and remyelination. *Stem Cells Dev.* 22, 1053–1062. doi: 10.1089/scd.2012.0463

Liu, X. J., Zhang, J. F., Sun, B., Peng, H. S., Kong, Q. F., Bai, S. S., et al. (2009). Reciprocal effect of mesenchymal stem cell on experimental autoimmune encephalomyelitis is mediated by transforming growth factor-beta and interleukin-6. *Clin. Exp. Immunol.* 158, 37–44. doi: 10.1111/j.1365-2249.2009.03995.x

Lu, Z., Hu, X., Zhu, C., Wang, D., Zheng, X., and Liu, Q. (2009). Overexpression of CNTF in mesenchymal stem cells reduces demyelination and induces clinical recovery in experimental autoimmune encephalomyelitis mice. *J. Neuroimmunol.* 206, 58–69. doi: 10.1016/j.jneuroim.2008.10.014

Lv, B., Li, F., Han, J., Fang, J., Xu, L., Sun, C., et al. (2017). Hif-1alpha overexpression improves transplanted bone mesenchymal stem cells survival in rat MCAO stroke model. *Front. Mol. Neurosci.* 10:80. doi: 10.3389/fnmol.2017.00080

MacKay, D. F., Smith, G. C., Dobbie, R., and Pell, J. P. (2010). Gestational age at delivery and special educational need: retrospective cohort study of 407,503 schoolchildren. *PLoS Med.* 7:e1000289. doi: 10.1371/journal.pmed.1000289

Mahrouf-Yorgov, M., Augeul, L., Da Silva, C. C., Jourdan, M., Rigolet, M., Manin, S., et al. (2017). Mesenchymal stem cells sense mitochondria released from damaged cells as danger signals to activate their rescue properties. *Cell Death Differ.* 24, 1224–1238. doi: 10.1038/cdd.2017.51

Mifsud, G., Zammit, C., Muscat, R., Di Giovanni, G., and Valentino, M. (2014). Oligodendrocyte pathophysiology and treatment strategies in cerebral ischemia. *CNS Neurosci. Ther.* 20, 603–612. doi: 10.1111/cns.12263

Mitew, S., Hay, C. M., Peckham, H., Xiao, J., Koenning, M., and Emery, B. (2014). Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. *Neuroscience* 276, 29–47. doi: 10.1016/j.neuroscience.2013.11.029

Morando, S., Vigo, T., Esposito, M., Casazza, S., Novi, G., Principato, M. C., et al. (2012). The therapeutic effect of mesenchymal stem cell transplantation in experimental autoimmune encephalomyelitis is mediated by peripheral and central mechanisms. *Stem Cell Res. Ther.* 3:3. doi: 10.1186/scrt94

Morioka, C., Komaki, M., Taki, A., Honda, I., Yokoyama, N., Iwasaki, K., et al. (2017). Neuroprotective effects of human umbilical cord-derived mesenchymal stem cells on periventricular leukomalacia-like brain injury in neonatal rats. *Inflamm. Regen.* 37:1. doi: 10.1186/s41232-016-0032-3

Moster, D., Lie, R. T., and Markestad, T. (2008). Long-term medical and social consequences of preterm birth. *N. Engl. J. Med.* 359, 262–273. doi: 10.1056/NEJMoa0706475

Mueller, M., Oppliger, B., Joerger-Messerli, M., Reinhart, U., Barnea, E., Paidas, M., et al. (2017). Wharton's jelly mesenchymal stem cells protect the immature brain in rats and modulate cell fate. *Stem Cells Dev.* 26, 239–248. doi: 10.1089/scd.2016.0108

Mukai, T., Mori, Y., Shimazu, T., Takahashi, A., Tsunoda, H., Yamaguchi, S., et al. (2017). Intravenous injection of umbilical cord-derived mesenchymal stromal cells attenuates reactive gliosis and hypomyelination in a neonatal intraventricular hemorrhage model. *Neuroscience* 355, 175–187. doi: 10.1016/j.neuroscience.2017.05.006

Nakajima, M., Nito, C., Sowa, K., Suda, S., Nishiyama, Y., Nakamura-Takahashi, A., et al. (2017). Mesenchymal stem cells overexpressing Interleukin-10 promote neuroprotection in experimental acute ischemic stroke. *Mol. Ther. Methods Clin. Dev.* 6, 102–111. doi: 10.1016/j.omtm.2017.06.005

Nessler, J., Benardais, K., Gudi, V., Hoffmann, A., Salinas Tejedor, L., Janssen, S., et al. (2013). Effects of murine and human bone marrow-derived mesenchymal stem cells on cuprizone induced demyelination. *PLoS One* 8:e69795. doi: 10.1371/journal.pone.0069795

Nijboer, C. H., Kooijman, E., van Velthoven, C. T., van Tilborg, E., Tiebosch, I. A., Eijkelkamp, N., et al. (2018). Intranasal stem cell treatment as a novel therapy

for subarachnoid hemorrhage. *Stem Cells Dev.* 27, 313–325. doi: 10.1089/scd.2017.0148

Ophelders, D. R., Wolfs, T. G., Jellema, R. K., Zwanenburg, A., Andriessen, P., Delhaas, T., et al. (2016). Mesenchymal stromal cell-derived extracellular vesicles protect the fetal brain after hypoxia-ischemia. *Stem Cells Transl. Med.* 5, 754–763. doi: 10.5966/sctm.2015-0197

Oppliger, B., Joerger-Messerli, M., Mueller, M., Reinhart, U., Schneider, P., Surbek, D. V., et al. (2016). Intranasal delivery of umbilical cord-derived mesenchymal stem cells preserves myelination in perinatal brain damage. *Stem Cells Dev.* 25, 1234–1242. doi: 10.1089/scd.2016.0027

Oppliger, B., Joerger-Messerli, M. S., Simillion, C., Mueller, M., Surbek, D. V., and Schoeberlein, A. (2017). Mesenchymal stromal cells from umbilical cord Wharton's jelly trigger oligodendroglial differentiation in neural progenitor cells through cell-to-cell contact. *Cytotherapy* 19, 829–838. doi: 10.1016/j.jcyt.2017.03.075

Otero-Ortega, L., Gutierrez-Fernandez, M., Ramos-Cejudo, J., Rodriguez-Frutos, B., Fuentes, B., Sobrino, T., et al. (2015). White matter injury restoration after stem cell administration in subcortical ischemic stroke. *Stem Cell Res. Ther.* 6:121. doi: 10.1186/s13287-015-0111-4

Otero-Ortega, L., Laso-Garcia, F., Gomez-de Frutos, M. D., Rodriguez-Frutos, B., Pascual-Guerra, J., Fuentes, B., et al. (2017). White matter repair after extracellular vesicles administration in an experimental animal model of subcortical stroke. *Sci. Rep.* 7:44433. doi: 10.1038/srep44433

Paliwal, S., Chaudhuri, R., Agrawal, A., and Mohanty, S. (2018). Regenerative abilities of mesenchymal stem cells through mitochondrial transfer. *J. Biomed. Sci.* 25:31. doi: 10.1186/s12929-018-0429-1

Panda, S., Dohare, P., Jain, S., Parikh, N., Singla, P., Mehdizadeh, R., et al. (2018). Estrogen treatment reverses prematurity-induced disruption in cortical interneuron population. *J. Neurosci.* 38, 7378–7391. doi: 10.1523/jneurosci.0478-18.2018

Park, D., Lee, S. H., Bae, D. K., Yang, Y. H., Yang, G., Kyung, J., et al. (2013). Transplantation of human adipose tissue-derived mesenchymal stem cells restores the neurobehavioral disorders of rats with neonatal hypoxic-ischemic encephalopathy. *Cell Med.* 5, 17–28. doi: 10.3727/215517913x658936

Park, W. S., Ahn, S. Y., Sung, S. I., Ahn, J. Y., and Chang, Y. S. (2017). Mesenchymal stem cells: the magic cure for intraventricular hemorrhage? *Cell Transplant* 26, 439–448. doi: 10.3727/096368916x694193

Park, W. S., Ahn, S. Y., Sung, S. I., Ahn, J. Y., and Chang, Y. S. (2018). Strategies to enhance paracrine potency of transplanted mesenchymal stem cells in intractable neonatal disorders. *Pediatr. Res.* 83, 214–222. doi: 10.1038/pr.2017.249

Park, W. S., Sung, S. I., Ahn, S. Y., Sung, D. K., Im, G. H., Yoo, H. S., et al. (2016). Optimal timing of mesenchymal stem cell therapy for neonatal intraventricular hemorrhage. *Cell Transplant.* 25, 1131–1144. doi: 10.3727/096368915x689640

Park, W. S., Sung, S. I., Ahn, S. Y., Yoo, H. S., Sung, D. K., Im, G. H., et al. (2015). Hypothermia augments neuroprotective activity of mesenchymal stem cells for neonatal hypoxic-ischemic encephalopathy. *PLoS One* 10:e0120893. doi: 10.1371/journal.pone.0120893

Paton, M. C. B., Allison, B. J., Fahey, M. C., Li, J., Sutherland, A. E., Pham, Y., et al. (2019). Umbilical cord blood versus mesenchymal stem cells for inflammation-induced preterm brain injury in fetal sheep. *Pediatr. Res.* doi: 10.1038/s41390-019-0366-z [Epub ahead of print].

Paton, M. C. B., Allison, B. J., Li, J., Fahey, M. C., Sutherland, A. E., Nitsos, I., et al. (2018). Human umbilical cord blood therapy protects cerebral white matter from systemic LPS exposure in preterm fetal sheep. *Dev. Neurosci.* 40, 258–270. doi: 10.1159/000490943

Paton, M. C. B., McDonald, C. A., Allison, B. J., Fahey, M. C., Jenkin, G., and Miller, S. L. (2017). Perinatal brain injury as a consequence of preterm birth and intrauterine inflammation: designing targeted stem cell therapies. *Front. Neurosci.* 11:200. doi: 10.3389/fnins.2017.00200

Payne, A. H., Hintz, S. R., Hibbs, A. M., Walsh, M. C., Vohr, B. R., Bann, C. M., et al. (2013). Neurodevelopmental outcomes of extremely low-gestational-age neonates with low-grade periventricular-intraventricular hemorrhage. *JAMA Pediatr.* 167, 451–459. doi: 10.1001/jamapediatrics.2013.866

Perrone, S., Tataranno, L. M., Stazzoni, G., Ramenghi, L., and Buonocore, G. (2015). Brain susceptibility to oxidative stress in the perinatal period. *J. Matern. Fetal Neonatal Med.* 28(Suppl. 1), 2291–2295. doi: 10.3109/14767058.2013.796170

Pimentel-Coelho, P. M., Magalhaes, E. S., Lopes, L. M., deAzevedo, L. C., Santiago, M. F., and Mendez-Otero, R. (2010). Human cord blood transplantation in a neonatal rat model of hypoxic-ischemic brain damage: functional outcome related to neuroprotection in the striatum. *Stem Cells Dev.* 19, 351–358. doi: 10.1089/scd.2009.0049

Redondo-Castro, E., Cunningham, C., Miller, J., Martuscelli, L., Aoulad-Ali, S., Rothwell, N. J., et al. (2017). Interleukin-1 primes human mesenchymal stem cells towards an anti-inflammatory and pro-trophic phenotype in vitro. *Stem Cell Res. Ther.* 8:79. doi: 10.1186/s13287-017-0531-4

Rezaie, P., and Dean, A. (2002). Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. *Neuropathology* 22, 106–132.

Rivera, F. J., and Aigner, L. (2012). Adult mesenchymal stem cell therapy for myelin repair in multiple sclerosis. *Biol. Res.* 45, 257–268. doi: 10.4067/s0716-97602012000300007

Rivera, F. J., Couillard-Despres, S., Pedre, X., Ploetz, S., Caioni, M., Lois, C., et al. (2006). Mesenchymal stem cells instruct oligodendrogenic fate decision on adult neural stem cells. *Stem Cells* 24, 2209–2219. doi: 10.1634/stemcells.2005-0614

Robinson, S., Li, Q., Dechant, A., and Cohen, M. L. (2006). Neonatal loss of gamma-aminobutyric acid pathway expression after human perinatal brain injury. *J. Neurosurg.* 104(Suppl.), 396–408. doi: 10.3171/ped.2006.104.6.396

Salmaso, N., Jablonska, B., Scafidi, J., Vaccarino, F. M., and Gallo, V. (2014). Neurobiology of premature brain injury. *Nat. Neurosci.* 17, 341–346. doi: 10.1038/nn.3604

Sarmah, D., Agrawal, V., Rane, P., Bhute, S., Watanabe, M., Kalia, K., et al. (2018). Mesenchymal stem cell therapy in ischemic stroke: a meta-analysis of preclinical studies. *Clin. Pharmacol. Ther.* 103, 990–998. doi: 10.1002/cpt.927

Schäfer, R., Spohn, G., and Baer, P. C. (2016). Mesenchymal stem/stromal cells in regenerative medicine: can preconditioning strategies improve therapeutic efficacy. *Transfus. Med. Hemother.* 43, 256–267. doi: 10.1159/000447458

Scolding, N. J., Pasquini, M., Reingold, S. C., and Cohen, J. A. (2017). Cell-based therapeutic strategies for multiple sclerosis. *Brain* 140, 2776–2796. doi: 10.1093/brain/awx154

Scruggs, B. A., Semon, J. A., Zhang, X., Zhang, S., Bowles, A. C., Pandey, A. C., et al. (2013). Age of the donor reduces the ability of human adipose-derived stem cells to alleviate symptoms in the experimental autoimmune encephalomyelitis mouse model. *Stem Cells Transl. Med.* 2, 797–807. doi: 10.5966/sctm.2013-0026

Segovia, K. N., McClure, M., Moravec, M., Luo, N. L., Wan, Y., Gong, X., et al. (2008). Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann. Neurol.* 63, 520–530. doi: 10.1002/ana.21359

Silbereis, J. C., Huang, E. J., Back, S. A., and Rowitch, D. H. (2010). Towards improved animal models of neonatal white matter injury associated with cerebral palsy. *Dis. Model Mech.* 3, 678–688. doi: 10.1242/dmm.002915

Singh, D. K., Ling, E. A., and Kaur, C. (2018). Hypoxia and myelination deficits in the developing brain. *Int. J. Dev. Neurosci.* 70, 3–11. doi: 10.1016/j.ijdevneu.2018.06.012

Steffenhagen, C., Dechant, F. X., Oberbauer, E., Furtner, T., Weidner, N., Kury, P., et al. (2012). Mesenchymal stem cells prime proliferating adult neural progenitors toward an oligodendrocyte fate. *Stem Cells Dev.* 21, 1838–1851. doi: 10.1089/scd.2011.0137

Steinberg, G. K., Kondziolka, D., Wechsler, L. R., Lunsford, L. D., Coburn, M. L., Billigen, J. B., et al. (2016). Clinical outcomes of transplanted modified bone marrow-derived mesenchymal stem cells in stroke: a phase 1/2a study. *Stroke* 47, 1817–1824. doi: 10.1161/strokeaha.116.012995

Stewart, A., Tekes, A., Huisman, T. A., Jennings, J. M., Allen, M. C., Northington, F. J., et al. (2013). Glial fibrillary acidic protein as a biomarker for periventricular white matter injury. *Am. J. Obstet. Gynecol.* 209:27.e21-e27. doi: 10.1016/j.ajog.2013.02.049

Stoll, B. J., Hansen, N. I., Bell, E. F., Walsh, M. C., Carlo, W. A., Shankaran, S., et al. (2015). Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993-2012. *JAMA* 314, 1039–1051. doi: 10.1001/jama.2015.10244

Stolzing, A., Jones, E., McGonagle, D., and Scutt, A. (2008). Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech. Ageing Dev.* 129, 163–173. doi: 10.1016/j.mad.2007.12.002

Sugiyama, Y., Sato, Y., Kitase, Y., Suzuki, T., Kondo, T., Mikrogeorgiou, A., et al. (2018). Intravenous administration of bone marrow-derived mesenchymal stem cell, but not adipose tissue-derived stem cell, ameliorated the neonatal

hypoxic-ischemic brain injury by changing cerebral inflammatory state in rat. *Front. Neurol.* 9:757. doi: 10.3389/fneur.2018.00757

Sun, J., Wei, Z. Z., Gu, X., Zhang, J. Y., Zhang, Y., Li, J., et al. (2015). Intranasal delivery of hypoxia-preconditioned bone marrow-derived mesenchymal stem cells enhanced regenerative effects after intracerebral hemorrhagic stroke in mice. *Exp. Neurol.* 272, 78–87. doi: 10.1016/j.expneurol.2015.03.011

Tang, Y., Cai, B., Yuan, F., He, X., Lin, X., Wang, J., et al. (2014). Melatonin pretreatment improves the survival and function of transplanted mesenchymal stem cells after focal cerebral ischemia. *Cell Transplant.* 23, 1279–1291. doi: 10.3727/096368913x667510

Thompson, A. J., Baranzini, S. E., Geurts, J., Hemmer, B., and Ciccarelli, O. (2018). Multiple sclerosis. *Lancet* 391, 1622–1636. doi: 10.1016/s0140-6736(18)30481-1

Tibrewal, M., Cheng, B., Dohare, P., Hu, F., Mehdizadeh, R., Wang, P., et al. (2018). Disruption of interneuron neurogenesis in premature newborns and reversal with estrogen treatment. *J. Neurosci.* 38, 1100–1113. doi: 10.1523/jneurosci.1875-17.2017

Toyoshima, A., Yasuhara, T., and Date, I. (2017). Mesenchymal stem cell therapy for ischemic stroke. *Acta Med. Okayama* 71, 263–268. doi: 10.18926/amo/55302

Uccelli, A., Frassoni, F., and Mancardi, G. (2007). Stem cells for multiple sclerosis: promises and reality. *Regen. Med.* 2, 7–9. doi: 10.2217/17460751.2.1.7

van Tilborg, E., de Theije, C. G. M., van Hal, M., Wagenaar, N., de Vries, L. S., Benders, M. J., et al. (2018). Origin and dynamics of oligodendrocytes in the developing brain: implications for perinatal white matter injury. *Glia* 66, 221–238. doi: 10.1002/glia.23256

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., et al. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Prog. Neurobiol.* 136, 28–49. doi: 10.1016/j.pneurobio.2015.11.002

van Velthoven, C. T., Braccioli, L., Willemen, H. L., Kavelaars, A., and Heijnen, C. J. (2014). Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage. *Mol. Ther.* 22, 645–654. doi: 10.1038/mt.2013.260

van Velthoven, C. T., Dzietko, M., Wendland, M. F., Derugin, N., Faustino, J., Heijnen, C. J., et al. (2017). Mesenchymal stem cells attenuate MRI-identifiable injury, protect white matter, and improve long-term functional outcomes after neonatal focal stroke in rats. *J. Neurosci. Res.* 95, 1225–1236. doi: 10.1002/jnr.23954

van Velthoven, C. T., Kavelaars, A., and Heijnen, C. J. (2012a). Mesenchymal stem cells as a treatment for neonatal ischemic brain damage. *Pediatr. Res.* 71(4 Pt 2), 474–481. doi: 10.1038/pr.2011.64

van Velthoven, C. T., van de Looij, Y., Kavelaars, A., Zijlstra, J., van Bel, F., Huppi, P. S., et al. (2012b). Mesenchymal stem cells restore cortical rewiring after neonatal ischemia in mice. *Ann. Neurol.* 71, 785–796. doi: 10.1002/ana.23543

van Velthoven, C. T., Kavelaars, A., van Bel, F., and Heijnen, C. J. (2010a). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain Behav. Immun.* 24, 387–393. doi: 10.1016/j.bbi.2009.10.017

van Velthoven, C. T., Kavelaars, A., van Bel, F., and Heijnen, C. J. (2010b). Nasal administration of stem cells: a promising novel route to treat neonatal ischemic brain damage. *Pediatr. Res.* 68, 419–422. doi: 10.1203/PDR.0b013e3181f1c289

van Velthoven, C. T., Kavelaars, A., van Bel, F., and Heijnen, C. J. (2010c). Repeated mesenchymal stem cell treatment after neonatal hypoxia-ischemia has distinct effects on formation and maturation of new neurons and oligodendrocytes leading to restoration of damage, corticospinal motor tract activity, and sensorimotor function. *J. Neurosci.* 30, 9603–9611. doi: 10.1523/jneurosci.1835-10.2010

van Velthoven, C. T., Kavelaars, A., van Bel, F., and Heijnen, C. J. (2011). Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. *Brain Behav. Immun.* 25, 1342–1348. doi: 10.1016/j.bbi.2011.03.021

van Velthoven, C. T., Sheldon, R. A., Kavelaars, A., Derugin, N., Vexler, Z. S., Willemen, H. L., et al. (2013). Mesenchymal stem cell transplantation attenuates brain injury after neonatal stroke. *Stroke* 44, 1426–1432. doi: 10.1161/strokeaha.111.000326

Verney, C., Pogledic, I., Biran, V., Adle-Biassette, H., Fallet-Bianco, C., and Gressens, P. (2012). Microglial reaction in axonal crossroads is a hallmark of noncystic periventricular white matter injury in very preterm infants. *J. Neuropathol. Exp. Neurol.* 71, 251–264. doi: 10.1097/NEN.0b013e3182496429

Viscardi, R. M., Muhumuza, C. K., Rodriguez, A., Fairchild, K. D., Sun, C. C., Gross, G. W., et al. (2004). Inflammatory markers in intrauterine and fetal blood and cerebrospinal fluid compartments are associated with adverse pulmonary and neurologic outcomes in preterm infants. *Pediatr. Res.* 55, 1009–1017. doi: 10.1203/01.pdr.0000127015.60185.8a

Volkman, R., and Offen, D. (2017). Concise review: mesenchymal stem cells in neurodegenerative diseases. *Stem Cells* 35, 1867–1880. doi: 10.1002/stem.2651

Volpe, J. J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 8, 110–124. doi: 10.1016/s1474-4422(08)70294-1

Volpe, J. J. (2017). Confusions in nomenclature: "periventricular leukomalacia" and "white matter injury"-identical, distinct, or overlapping? *Pediatr. Neurol.* 73, 3–6. doi: 10.1016/j.pediatrneurol.2017.05.013

Volpe, J. J., Kinney, H. C., Jensen, F. E., and Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *Int. J. Dev. Neurosci.* 29, 423–440. doi: 10.1016/j.ijdevneu.2011.02.012

von Bahr, L., Batsis, I., Moll, G., Hagg, M., Szakos, A., Sundberg, B., et al. (2012). Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. *Stem Cells* 30, 1575–1578. doi: 10.1002/stem.1118

Wagenaar, N., de Theije, C. G. M., de Vries, L. S., Groenendaal, F., Benders, M., and Nijboer, C. H. A. (2018). Promoting neuroregeneration after perinatal arterial ischemic stroke: neurotrophic factors and mesenchymal stem cells. *Pediatr. Res.* 83, 372–384. doi: 10.1038/pr.2017.243

Wang, L. Q., Lin, Z. Z., Zhang, H. X., Shao, B., Xiao, L., Jiang, H. G., et al. (2014). Timing and dose regimens of marrow mesenchymal stem cell transplantation affect the outcomes and neuroinflammatory response after ischemic stroke. *CNS Neurosci. Ther.* 20, 317–326. doi: 10.1111/cns.12216

Wei, N., Yu, S. P., Gu, X., Taylor, T. M., Song, D., Liu, X.-F., et al. (2013). Delayed intranasal delivery of hypoxic-preconditioned bone marrow mesenchymal stem cells enhanced cell homing and therapeutic benefits after ischemic stroke in mice. *Cell Transplant.* 22, 977–991. doi: 10.3727/096368912X657251

Wei, Z. Z., Gu, X., Ferdinand, A., Lee, J. H., Ji, X., Ji, X. M., et al. (2015). Intranasal delivery of bone marrow mesenchymal stem cells improved neurovascular regeneration and rescued neuropsychiatric deficits after neonatal stroke in rats. *Cell Transplant.* 24, 391–402. doi: 10.3727/096368915x686887

Xiao, J., Yang, R., Biswas, S., Qin, X., Zhang, M., and Deng, W. (2015). Mesenchymal stem cells and induced pluripotent stem cells as therapies for multiple sclerosis. *Int. J. Mol. Sci.* 16, 9283–9302. doi: 10.3390/ijms16059283

Xiao, Y., Geng, F., Wang, G., Li, X., Zhu, J., and Zhu, W. (2018). Bone marrow-derived mesenchymal stem cells-derived exosomes prevent oligodendrocyte apoptosis through exosomal miR-134 by targeting caspase-8. *J. Cell Biochem.* doi: 10.1002/jcb.27519 [Epub ahead of print].

Xue, P., Wang, M., and Yan, G. (2018). Mesenchymal stem cell transplantation as an effective treatment strategy for ischemic stroke in Asia: a meta-analysis of controlled trials. *Ther. Clin. Risk Manag.* 14, 909–928. doi: 10.2147/tcrm.s161326

Yu, X., Wu, H., Zhao, Y., Guo, Y., Chen, Y., Dong, P., et al. (2018). Bone marrow mesenchymal stromal cells alleviate brain white matter injury via the enhanced proliferation of oligodendrocyte progenitor cells in focal cerebral ischemic rats. *Brain Res.* 1680, 127–136. doi: 10.1016/j.brainres.2017.12.019

Zaghloul, N., and Ahmed, M. (2017). Pathophysiology of periventricular leukomalacia: what we learned from animal models. *Neural Regen. Res.* 12, 1795–1796. doi: 10.4103/1673-5374.219034

Zappia, E., Casazza, S., Pedemonte, E., Benvenuto, F., Bonanni, I., Gerdoni, E., et al. (2005). Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106, 1755–1761. doi: 10.1182/blood-2005-04-1496

Zeddou, M., Briquet, A., Relic, B., Josse, C., Malaise, M. G., Gothot, A., et al. (2010). The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. *Cell Biol. Int.* 34, 693–701. doi: 10.1042/cbi20090414

Zhang, H. L., Xie, X. F., Xiong, Y. Q., Liu, S. M., Hu, G. Z., Cao, W. F., et al. (2018). Comparisons of the therapeutic effects of three different routes of bone

marrow mesenchymal stem cell transplantation in cerebral ischemic rats. *Brain Res.* 1680, 143–154. doi: 10.1016/j.brainres.2017.12.017

Zhang, J., Li, Y., Chen, J., Cui, Y., Lu, M., Elias, S. B., et al. (2005). Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. *Exp. Neurol.* 195, 16–26.

Zhang, Z., Li, Z., Deng, W., He, Q., Wang, Q., Shi, W., et al. (2016). Ectoderm mesenchymal stem cells promote differentiation and maturation of oligodendrocyte precursor cells. *Biochem. Biophys. Res. Commun.* 480, 727–733. doi: 10.1016/j.bbrc.2016.10.115

Zhao, J., Chen, Y., Xu, Y., and Pi, G. (2013). Effect of intrauterine infection on brain development and injury. *Int. J. Dev. Neurosci.* 31, 543–549. doi: 10.1016/j.ijdevneu.2013.06.008

Zhu, L. H., Bai, X., Zhang, N., Wang, S. Y., Li, W., and Jiang, L. (2014). Improvement of human umbilical cord mesenchymal stem cell transplantation on glial cell and behavioral function in a neonatal model of periventricular white matter damage. *Brain Res.* 1563, 13–21. doi: 10.1016/j.brainres.2014.03.030

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Vaes, Vink, de Theije, Hoebeek, Benders and Nijboer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 10 May 2019
doi: 10.3389/fphys.2019.00541

Ibuprofen Treatment Reduces the Neuroinflammatory Response and Associated Neuronal and White Matter Impairment in the Growth Restricted Newborn

Julie A. Wixey[1], Kishen R. Sukumar[1], Rinaldi Pretorius[1], Kah Meng Lee[2], Paul B. Colditz[1,3], S. Tracey Bjorkman[1] and Kirat K. Chand[1]*

[1] UQ Centre for Clinical Research, Faculty of Medicine, The University of Queensland, Brisbane, QLD, Australia, [2] Institute of Health Biomedical Innovation, Queensland University of Technology, Brisbane, QLD, Australia, [3] Perinatal Research Centre, Royal Brisbane and Women's Hospital, Brisbane, QLD, Australia

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Courtney Anne McDonald,
Hudson Institute of Medical Research,
Australia
Flora Wong,
Monash University, Australia
Jonathan James Hirst,
University of Newcastle, Australia

***Correspondence:**
Julie A. Wixey
j.wixey@uq.edu.au

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *24 January 2019*
Accepted: *17 April 2019*
Published: *10 May 2019*

Citation:
Wixey JA, Sukumar KR, Pretorius R, Lee KM, Colditz PB, Bjorkman ST and Chand KK (2019) Ibuprofen Treatment Reduces the Neuroinflammatory Response and Associated Neuronal and White Matter Impairment in the Growth Restricted Newborn. Front. Physiol. 10:541. doi: 10.3389/fphys.2019.00541

Intrauterine growth restriction (IUGR) is a condition where the fetus does not achieve optimal growth, commonly caused by placental insufficiency. The chronic decrease in blood flow restricts oxygen and nutrient supply to the fetus, which can damage numerous organ systems, with the fetal brain being particularly vulnerable. Although white matter and neuronal injury are evident in IUGR infants, the specific mechanisms underlying these changes are poorly understood. Inflammation is considered to be a main driver in exacerbating brain injury. Using a spontaneous piglet model of IUGR, we aim to determine whether administration of the anti-inflammatory drug ibuprofen will decrease inflammation at postnatal day 4 (P4). The treatment group received ibuprofen (20 mg/kg/day on day 1 and 10 mg/kg/day on days 2 and 3) in piglet formula during the morning feed each day and brains examined on P4. Markers of inflammation, apoptosis, cell proliferation, neuronal injury, and white matter injury were examined. Ibuprofen treatment ameliorated the increase in numbers of microglia and astrocytes in the parietal cortex and white matter tracts of the IUGR piglet brain on P4 as well as decreasing proinflammatory cytokines. Ibuprofen treatment prevented the reduction in apoptosis, neuronal cell counts, and myelin index in the IUGR piglets. Our findings demonstrate ibuprofen reduces the inflammatory response in the IUGR neonatal brain and concurrently reduces neuronal and white matter impairment.

Keywords: placental insufficiency, growth retardation, fetal growth restriction, astrocytes, microglia, ibuprofen, newborn brain injury

INTRODUCTION

Intrauterine growth restriction (IUGR) is a major pediatric concern associated with increased perinatal mortality and long-term morbidity. The fetal brain is particularly vulnerable to prolonged IUGR conditions with neuronal and white matter disturbances observed in clinical imaging studies (Tolsa et al., 2004; Esteban et al., 2010; Padilla et al., 2015). Large follow up studies on IUGR

infants have shown IUGR is associated with neurodevelopmental disabilities including lower academic performance, short-term memory deficits, and attention deficit disorders (Geva et al., 2006; Heinonen et al., 2010). With term born IUGR newborns having a 4- to 7-fold increase of developing cerebral palsy (Jarvis et al., 2003; Jacobsson et al., 2008). Currently there is no treatment available to minimize long-term adverse neurological outcomes in IUGR newborns. It is important to target key mechanisms of brain injury in the IUGR newborn to reduce or prevent long-term neurological dysfunction in these infants.

There is growing evidence of the critical role neuroinflammation plays in IUGR brain injury (Wixey et al., 2017, 2018). Recent studies in animal models of IUGR report an inflammatory response in the IUGR brain (Olivier et al., 2005, 2007; Tolcos et al., 2011; Campbell et al., 2012; Black et al., 2015; Castillo-Melendez et al., 2015; Pham et al., 2015; Rideau Batista Novais et al., 2016; Wixey et al., 2019). Neuroinflammation envelops a set of processes which include increased number of activated microglia, astrogliosis, increased production of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and decreased production of anti-inflammatory cytokines (Cai et al., 2006; Kremlev et al., 2007; Carty et al., 2008; Leonardo et al., 2008; Huang et al., 2009; Wixey et al., 2009, 2011a). In the newborn IUGR piglet a robust increase in inflammatory mediators such as activated microglia, astrocytes, and proinflammatory cytokines are associated with neuronal and white matter impairment (Wixey et al., 2019). Worsening brain injury is associated with increases in proinflammatory cytokines in a fetal IUGR guinea pig model (Guo et al., 2010). Furthermore, an increase in proinflammatory cytokines have been reported in the blood of IUGR infants (Mcelrath et al., 2013) and systemic inflammation in the small for gestational age infant is associated with adverse neurodevelopment at 2 years (Leviton et al., 2013). The opportunity to target inflammation to potentially reduce this brain impairment is appealing. As IUGR is detected around birth in majority of cases (Sovio et al., 2015), postnatal neuroprotective therapies may be invaluable. With inflammation present in the IUGR neonatal brain, an anti-inflammatory intervention may reduce adverse effects in the IUGR newborn.

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) with anti-inflammatory, anti-pyretic and analgesic properties. It is currently safely used in the preterm neonate to treat patent ductus arteriosus. Ibuprofen acts to inhibit cyclooxygenase 1 and 2 (COX-1 and -2) activity, that have various critical functions through production of prostaglandins, in regulating blood flow as well as inflammatory pathways. Ibuprofen has been shown to inhibit neuroinflammation and have neuroprotective effects in neonatal animal models of acute hypoxia-ischemia (HI) (Carty et al., 2011; Wixey et al., 2012). One week treatment of ibuprofen attenuates HI-induced increases COX-2 levels, proinflammatory cytokine levels and microglial activation in the neonatal HI rat brain (Wixey et al., 2012) whilst concurrently protecting the white matter and neurons from injury (Carty et al., 2011; Wixey et al., 2012). However, the potential neuroprotective effects of ibuprofen treatment for the IUGR newborn have not been examined in associated animal models.

In the present study we hypothesized that ibuprofen treatment can reduce inflammation, as well as neuronal and white matter impairment in the IUGR newborn. Using the preclinical piglet model of IUGR we assessed whether 3 days of oral ibuprofen treatment to the newborn IUGR piglet could not only reduce the neuroinflammatory response, but reduce cell death and neuronal and white matter impairment.

MATERIALS AND METHODS

Approval for this study was granted by The University of Queensland Animal Ethics Committee (MED/UQCCR/132/16/RBWH) and was carried out with respect to the National Health and Medical Research Council guidelines (Australia) and ARRIVE guidelines.

Newborn large white piglets (n = 24; <18 h) were collected from the UQ Gatton Piggery monitored and cared for at the Herston Medical Research Centre (HMRC) until day of euthanasia on postnatal day 4 (P4). Litter matched pairs were obtained from multiple sows (n = 10). Piglets were divided into 4 groups: normally grown (NG) (n = 6), IUGR (n = 6), NG + ibuprofen (n = 6) and IUGR + ibuprofen (n = 6); with equal males and females in each group. IUGR piglets were defined by birth bodyweight (<10th percentile on the day of birth) and confirmed by brain to liver weight ratio (B:L) $\geq$ 1 at postmortem (Bauer et al., 1998; Cox and Marton, 2009; Kalanjati et al., 2017). B:L is used to define asymmetric growth restriction in the IUGR newborn. The IUGR piglet mimics many human outcomes associated with IUGR including asymmetrical growth restriction with brain sparing (Bauer et al., 2003). Inadequate fetal growth in pigs is caused by placental insufficiency (Bauer et al., 2003) which is the most common cause of IUGR in the human population. Therefore, data obtained from the piglet model translates well to the human IUGR. Ibuprofen treatment groups received 20 mg/kg/day on day 1 and 10 mg/kg/day on days 2 and 3. This dosage is routinely used in the human preterm newborn to treat patent ductus arteriosis (Ohlsson et al., 2015). Ibuprofen was mixed with pig milk formula and delivered via an oro-gastric tube at 9 am each morning. On P4, piglets were euthanized via an intracardiac injection of sodium phenobarbital (650 mg/kg; Lethabarb, Virbac, Australia). Brain tissue was collected, weighed, hemisected and coronally sliced. The right hemisphere sections were immersion fixed in 4% paraformaldehyde as previously described (Kalanjati et al., 2011). The parietal cortex from the left hemisphere was snap frozen in liquid nitrogen and stored at −80°C for mRNA analysis.

Quantitative Polymerase Chain Reaction (qPCR)

RNA was isolated and purified using an RNeasy Tissue Mini Kit (Qiagen) from 30 mg parietal cortex. RNA yield and quality was determined using a NanoDrop spectrophotometer (ND-1000 system). A reverse transcription kit (RT^2 First Strand Kit; Qiagen) was used for cDNA synthesis. Synthesized cDNA was pooled for each group giving equal concentrations from each animal in the pooled sample. The pooled synthesized cDNA was combined

with RT^2 SYBR Green qPCR Mastermix (Qiagen) and loaded into the Pig Inflammatory Cytokines & Receptors RT^2 Profiler^{TM} PCR Array (Qiagen, Hilden, Germany). The qPCR reactions were performed using a Qiagen Rotor-Gene Q real-time cycler [10 min at 95°C, 40 cycles (15 s at 95°C; 1 min at 60°C)]. The amplified transcripts were quantified with the comparative CT method using actin, gamma 1 (ACTG1) mRNA expression levels for normalization. The same CT threshold value was used across all arrays to allow comparison between runs.

Immunohistochemistry

Brain sections from the parietal cortex of the right hemisphere (Pig stereotaxic map, A 5.5 mm; Felix et al., 1999) were embedded in paraffin and coronally sectioned 6 μm apart. Sections were affixed to Menzel Superfrost Plus adhesive slides and air-dried overnight at 37°C. All sections were dewaxed and rehydrated using standard protocols followed by heat induced epitope retrieval using 10 mM citrate buffer of pH 6 at 80°C for 10 min before cooling to room temperature (RT). A hydrophobic barrier was drawn around the tissue followed by non-specific blocking with 5% donkey serum in PBS with 0.5% Triton-X 100 for 1 h at RT. Cellular markers examined using immunohistochemistry were astrocytes [glial fibrillary acidic protein (GFAP); 1:1000, Z0334, Dako], microglia (ionized calcium binding adaptor molecule-1; Iba-1; 1:1000, ab5076; Wako Chemicals), neurons (NeuN; 1:1000; ab177487; Abcam Cambridge), proliferating cells (Ki67; 1:200, ab15580; Abcam) and apoptotic cells (cleaved Caspase-3; 1:500, #9661; Cell Signaling). Primary antibodies were incubated at 4°C for 20 h. Slides were washed in Tris-Buffered Saline followed by incubation with species-specific secondary fluorophores at RT for 1 h (Alexafluor 488, Alexafluor 568; 1:1000, Molecular Probes, Invitrogen Australia, Mount Waverley, VIC, Australia). Tissue was then washed, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and mounted with Prolong Gold antifade (Molecular Probes, Invitrogen Australia, Mount Waverley, VIC, Australia). Negative control sections without primary antibodies were processed in parallel. Staining was conducted in triplicates for all animals.

Luxol Fast Blue Staining

General myelination status of IUGR brains was assessed using Luxol Fast Blue (LFB) staining as previously described (Wixey et al., 2019). Tissue sections underwent standard dewaxing and rehydration followed by overnight immersion in LFB solution at 57°C. Sections were immersed in 95% ethanol and differentiated in 0.05% lithium carbonate followed by 70% ethanol until gray and white matter could be distinguished and nuclei decolorized. Tissue was processed and stained simultaneously to minimize variability of LFB staining.

Image Acquisition and Analysis

Analysis of immunolabeled sections was performed using Olympus BX41 light microscope with a DP70 camera. Pictomicrographs (881.2 μm × 663.5 μm) of gray matter (parietal cortex) and white matter [intragyral white matter (IGWM); subcortical white matter (SCWM); periventricular white matter (PVWM)] were captured for analysis. Four pictomicrographs were captured in each respective area for each animal in triplicate. All tissue was imaged and analyzed under blind conditions by KKC, JAW, KS and RP, and manual counts for NeuN, Caspase-3, Ki67, and Iba-1 were performed.

Microglia were manually counted with respect to morphology in cortical gray matter, IGWM, SCWM, and PVWM of the parietal cortex. Astrocytes in the WM were quantified using densitometry by thresholding the intensity of GFAP labeling using ImageJ (Image Processing and Analysis in Java; National Institutes of Health, Bethesda, MD, United States). Areal density was expressed as percentage of the whole WM for each region covered as previously described (Wixey et al., 2019). For LFB staining, slides were scanned using a Pannoramic SCAN II digital slide scanner (3D HISTECH, Ltd.) with a 20x Plan-apochromat objective and analyzed as previously reported (Wixey et al., 2019). In short, images were converted to high resolution 8-bit grayscale images and thresholded to determine staining intensity from 0 to 127 (0, white; 255, black). This range was divided into quartiles and the percent area stained (% area) for each was calculated. The median gray level of each quartile (14.5, 46.0, 78.5, and 111.0) was then multiplied by % area/100 in each quartile, to give the total myelin index.

Statistical Analysis

Two-way ANOVA with the *post hoc* Sidak analysis was used to determine differences between NG and IUGR animals under non-treated and ibuprofen-treated conditions (GraphPad Prism 7.0 software, San Diego, CA, United States). Results were expressed as mean ± SEM with statistical significance accepted at $p < 0.05$.

RESULTS

Mean body weight was significantly lower in both IUGR untreated piglets ($p < 0.0001$) and IUGR ibuprofen treated piglets ($p < 0.0001$) compared to untreated NG piglets (**Table 1**). There was no significant difference in body weight between the treated and untreated NG piglets ($p = 0.246$). Brain weight was significantly reduced only in the IUGR untreated group compared to the NG group ($p = 0.018$). The mean brain to liver weight ratio was significantly higher in both treated ($p < 0.0001$) and untreated IUGR piglets ($p < 0.0001$) compared to NG piglets indicating asymmetric growth restriction in the IUGR piglets. A small number of the NG ibuprofen treated piglets may have been slightly growth restricted due to the significant difference in liver weight in comparison to NG piglets ($p = 0.003$), however the mean brain to liver weight ratio was <1 for the NG ibuprofen treated group and not significantly different to NG piglets ($p = 0.269$; **Table 1**).

Inflammatory Response Following Ibuprofen Treatment in the IUGR Piglet Brain

We observed altered expression of both pro- and anti-inflammatory cytokines in the IUGR parietal cortex compared

TABLE 1 | Piglet bodyweight, brain weight, and liver weight.

	NG (n = 6)	IUGR (n = 6)	IUGR+ibuprofen (n = 6)	NG+ibuprofen (n = 6)
Bodyweight in grams (mean ± SEM)	1973 ± 157.4	†††866.7 ± 77.23****	†††1000 ± 39.67****	1643 ± 125.6
Brain weight in grams (mean ± SEM)	32.19 ± 1.10	†28.66 ± 0.63*	30.18 ± 0.49	32.29 ± 0.59
Liver weight in grams (mean ± SEM)	64.41 ± 4.89	†††19.1 ± 1.26****	††22.69 ± 2.09****	43.44 ± 4.76**
Brain:liver ratio (mean ± SEM)	0.51 ± 0.03	†††1.537 ± 0.11****	††1.383 ± 0.12****	0.797 ± 0.10

*Mean body weight was significantly lower in both IUGR untreated and ibuprofen treated piglets compared to untreated NG piglets. Brain weight was significantly reduced only in the IUGR untreated group compared to the NG group. The mean brain to liver weight ratio was significantly higher in both treated and untreated IUGR piglets compared to NG piglets indicating asymmetric growth restriction in the IUGR piglets. Values are the mean ± SEM. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ versus NG. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$ versus NG+ibuprofen.*

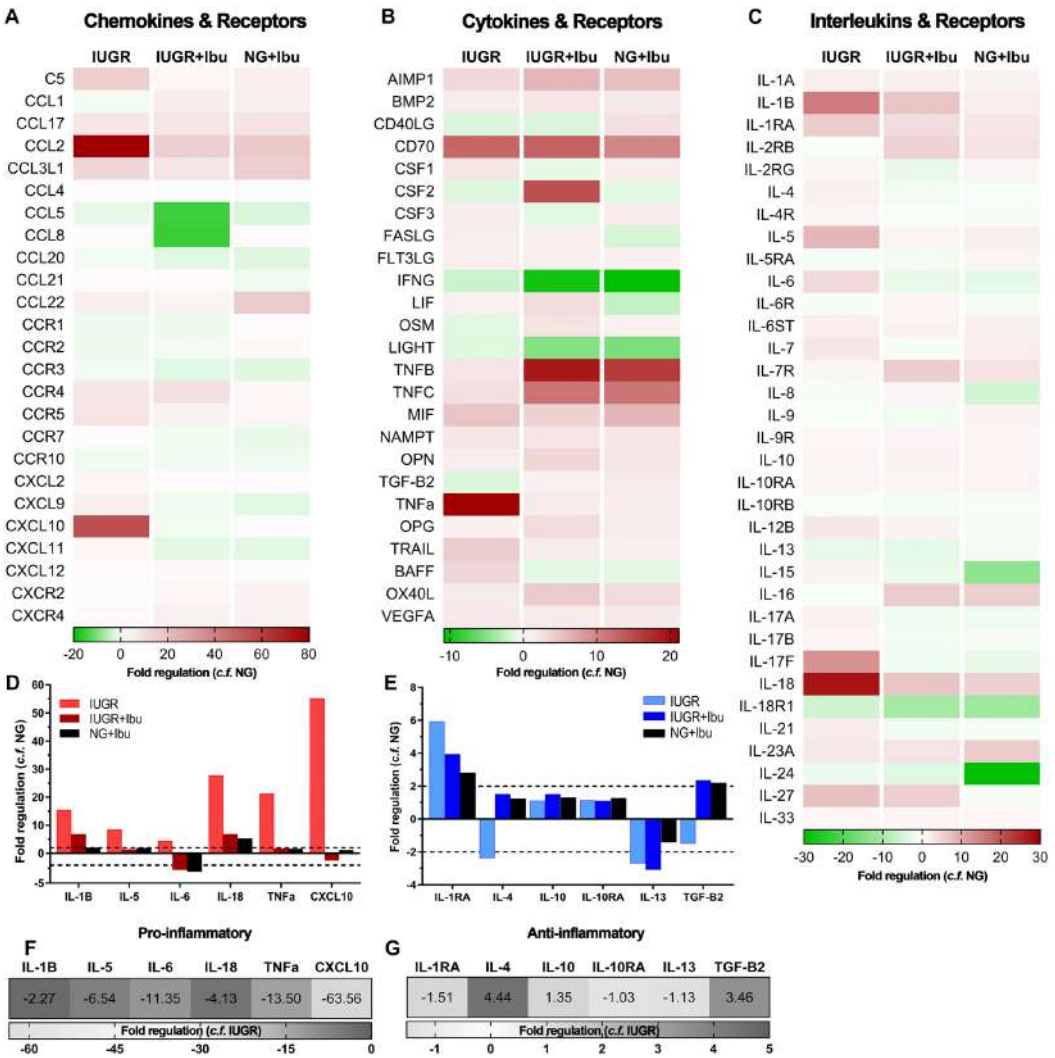


FIGURE 1 | Ibuprofen treatment attenuates expression of inflammatory mediators in IUGR piglet brains. Heat maps of inflammatory profiler arrays separated into chemokines and receptors **(A)**, cytokines and receptors **(B)**, and interleukins and receptors **(C)**. Heat maps demonstrate altered expression of pro- and anti-inflammatory mediators in the cortex of IUGR relative to untreated NG. Treatment with ibuprofen largely down-regulated inflammatory genes relative to untreated NG. Expression of well-characterized pro-inflammatory **(D)** and anti-inflammatory **(E)** genes relative to NG. Ibuprofen treatment in IUGR resulted in down-regulation of pro-inflammatory **(F)** and up-regulation of key anti-inflammatory genes **(G)** when compared with untreated IUGR.

with NG piglets using a PCR array panel of 84 inflammatory genes (**Figure 1**). Heat maps of fold regulation expression levels of chemokines, cytokines and interleukins compared with NG piglets are represented in **Figures 1A–C**. The proinflammatory mediators IL-1β, IL-5, IL-6, IL-18, TNFα, and C-X-C motif chemokine (CXCL10) showed high upregulation of expression

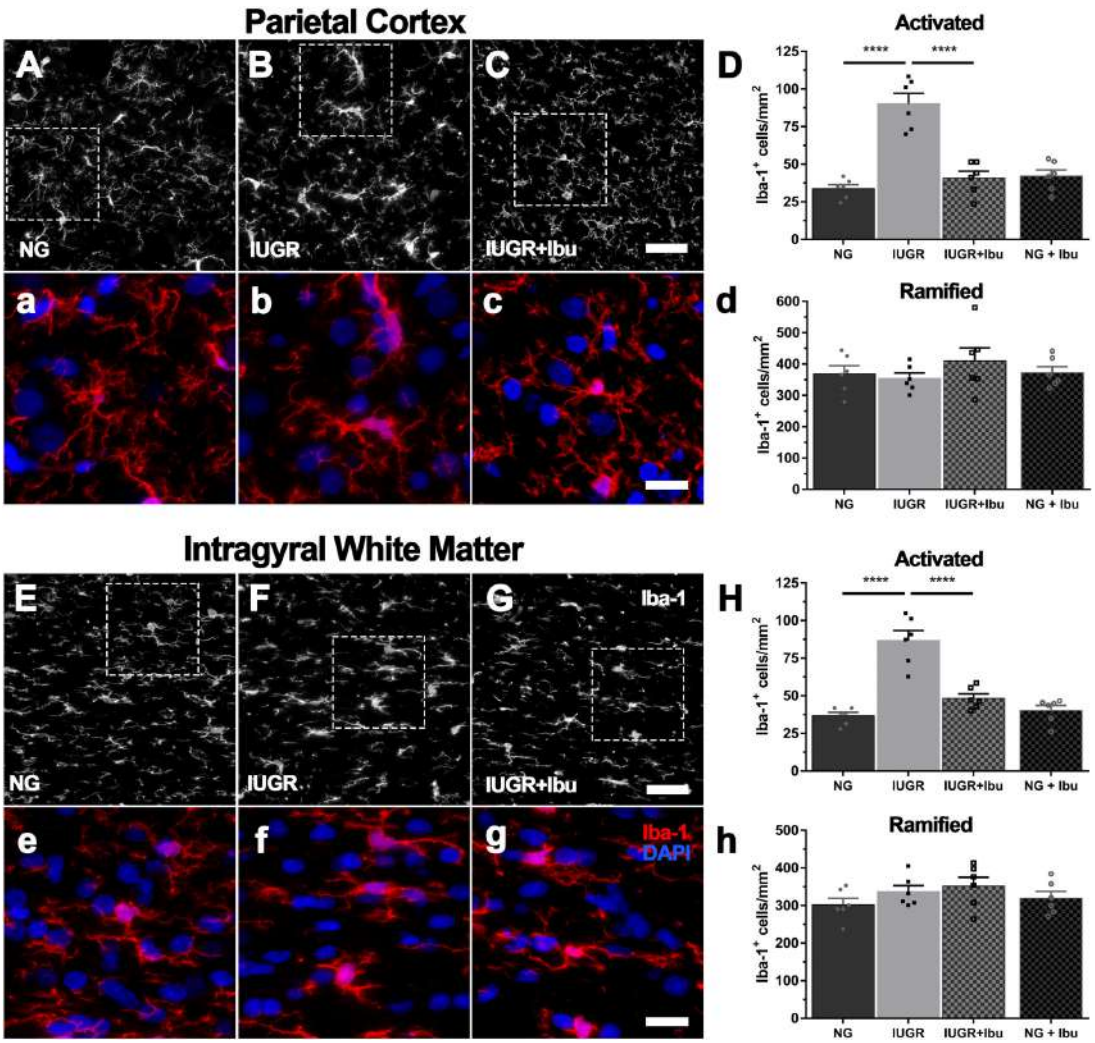


FIGURE 2 | Increased microglial activation in cortex and WM of IUGR. Representative immunofluorescent images of microglial immunolabeling in the PC **(A–C)** and IGWM **(E–G)** of untreated NG, IUGR, and treated IUGR brains. Microglia in untreated IUGR brains displayed dense cell bodies with truncated and thickened processes in both the PC **(B)** and IGWM **(F)**, indicative of microglial activation. NG brains presented microglia with predominantly ramified morphology with long thin extended processes (NG; **A,E**). Quantification based on morphology found significantly higher activated microglia in IUGR compared with NG in both the PC and IGWM (**D,H**; respectively) and no difference in the number of ramified microglia **(d,h)**. Ibuprofen treated IUGR **(C,G)** animals displayed morphology similar to that observed in untreated NG. IUGR animals had significantly lower activated microglial counts when compared with untreated IUGR in both the PC and IGWM **(D,H)**. Scale bars for representative images are 50 μm **(A–C,E–G)** and 20 μm **(a–c,e–g)**. For **(D,H)** n = 6 per group. Values are presented as mean ± SEM. Analysis was performed using Two-way ANOVA with Sidak *post hoc* test (****$p < 0.0001$).

in the IUGR brain when compared with NG (**Figure 1D**). Anti-inflammatory markers IL-4 and TGF-β displayed decreased expression in the IUGR brain compared to NG (**Figure 1E**). Following ibuprofen treatment a reduction in the fold expression of proinflammatory mediators IL-1β (−2.27), IL-5 (−6.54), IL-6 (−11.35), IL-18 (−4.13), TNFα (−13.50), and CXCL10 (−63.56) were evident in IUGR treated piglets compared to IUGR untreated piglets (**Figure 1F**). Ibuprofen treatment also ameliorated the reductions in the anti-inflammatory cytokines IL-4 (4.44) and TGF-β (3.46) (**Figure 1G**).

Iba-1-positive microglia in NG brains displayed light cell bodies with fine extended processes indicative of a resting state (**Figure 2a**). In comparison, many of the Iba-1-positive microglia in IUGR brains resembled the morphology of activated microglia with darker cell bodies and thickened retracted processes as previously described (Wixey et al., 2019). When the Iba-1-positive cells were counted based on morphology, a significant increase in activated microglia was evident in the parietal cortex (62.6%; $p < 0.0001$), IGWM (57.7%; $p < 0.0001$), SCWM (40.0%; NG: 44.85 ± 4.64; IUGR: 75.14 ± 2.96; $p = 0.0006$) and PVWM (49.7%; NG: 48.88 ± 4.23; IUGR: 97.27 ± 2.46; $p < 0.0001$) of IUGR piglets compared to NGs (**Figure 2**). No significant difference in numbers of Iba-1-positive ramified (resting) microglia were observed in any of the regions examined

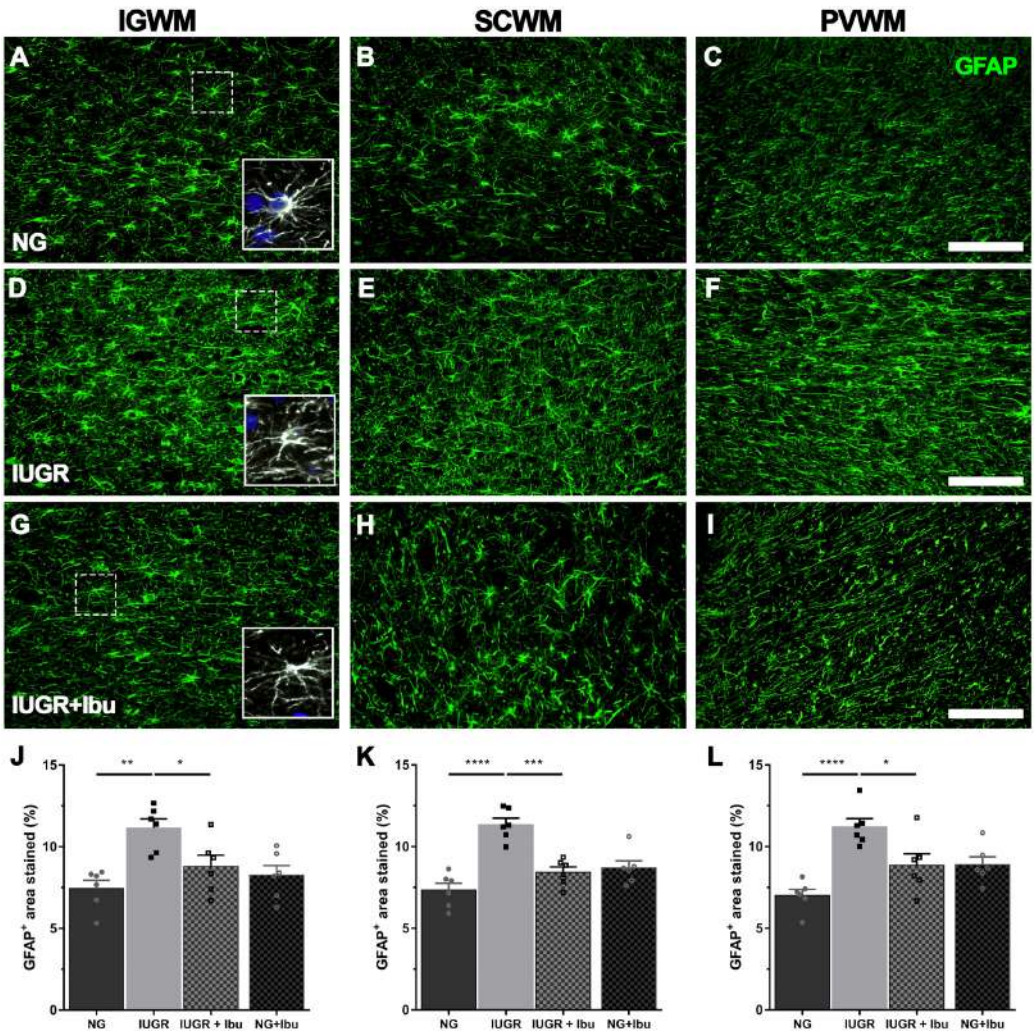


FIGURE 3 | Ibuprofen treatment prevents astrogliosis in white matter regions of IUGR brain. **(A)** Representative images of astrocytes labeled with GFAP in the intragyral (IGWM; first column), subcortical (SCWM; second column) and periventricular (PVWM; third column) white matter regions in P4 NG **(A–C)**, IUGR **(D–F)**, and Ibuprofen treated IUGR (IUGR+Ibu; **G–I**) brains. Astrocytes in NG displayed long extended processes and small cell bodies, typical of ramified astrocytes (see inserts; **A–C**). GFAP staining in IUGR white matter more abundant and showed morphology with enlarged cell bodies and thickened processes (see inserts **D–F**). Ibuprofen treated IUGR displayed ramified morphology (see inserts; **G–I**). Scale bar is 100 μm. Quantification of GFAP expression using densitometry showed an increase in GFAP expression in all white matter regions investigated IUGR when compared with NG brains (**J**, IGWM; **K**, SCWM; **L**, PVWM). Ibuprofen treated IUGR showed significantly lower GFAP density in comparison to untreated IUGR in all regions. No significant alterations in GFAP expression were observed in NG treated animals. For **(J–L)** $n = 6$ for all groups. Values are presented as mean ± SEM. Two-way ANOVA with Sidak *post hoc* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

in IUGR piglets compared to NGs (**Figure 2**). **Figure 2** graphs demonstrating parietal cortex and IGWM only as SCWM and PVWM results are similar. Data reported above.

Ibuprofen treatment significantly alleviated the increase in Iba-1-positive activated microglia in the parietal cortex (54.7%; $p < 0.0001$), IGWM (44.2%; $p < 0.0001$), SCWM (39.5%; IUGR 75.14 ± 2.96; IUGR+Ibu: 45.68 ± 4.87; $p = 0.0008$), and PVWM (52.4%; IUGR: 97.27 ± 2.46; IUGR+Ibu: 46.26 ± 4.02; $p < 0.0001$) in treated IUGR piglets compared to untreated IUGR piglets (**Figure 2**). Furthermore, the Iba-1-positive microglial morphology in the ibuprofen treated IUGR piglets resembled that of healthy microglia in NG piglets with the processes appearing finer and less dense (**Figure 2h**). Ibuprofen treatment did not affect the numbers of Iba-1-positive ramified microglia through all regions examined.

Glial fibrillary acidic protein-positive astrocytes were observed through the WM tracts of the parietal cortex. In the NG piglet brain GFAP-positive cells demonstrated multiple long branching processes from the cell body typical of normal astrocyte morphology. In the IUGR piglet brains many of the GFAP-positive astrocytes displayed morphology of a more reactive state with retracted processes and large cell bodies (**Figure 3**). GFAP-positive astrocyte density was significantly increased in the IUGR IGWM (33.2%; $p = 0.0013$), SCWM

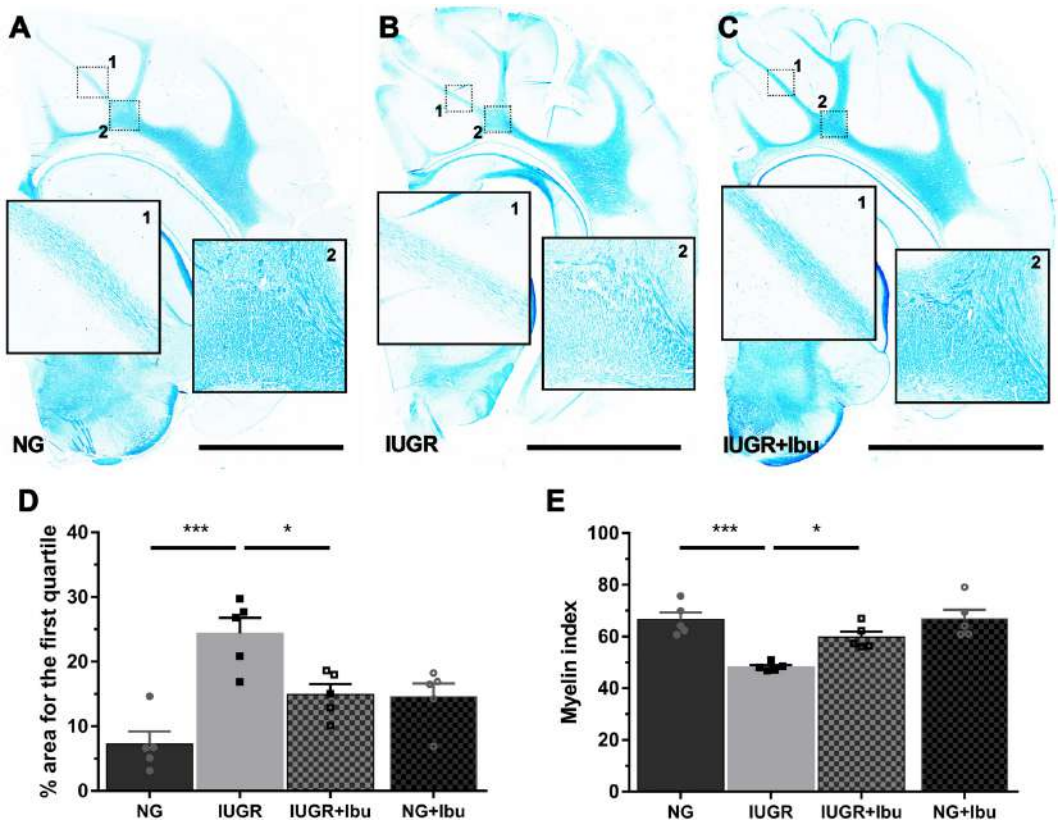


FIGURE 4 | Ibuprofen treatment improves myelination status in white matter of IUGR piglets. Representative images demonstrating high expression of myelin stained with Luxol fast blue (LFB) in untreated NG **(A)** compared with IUGR **(B)** brains (scale bar = 10 mm). The degree of LFB staining was consistent across all white matter (WM) regions in NG brains, while IUGR displayed overall lower levels with sparse staining evident across WM regions (compare B1&2 with A1&2). **(C)** Ibuprofen treated IUGR showed staining consistent with that observed in NG. **(D)** IUGR brains presented greater areas with significantly lower LFB staining, as indicated by percentage area for the first quartile. This was attenuated in IUGR treated with Ibuprofen. **(E)** IUGR displayed a decreased myelin status as shown by the lower myelin index in IUGR compared with NG. Ibuprofen treated animals maintained myelin status consistent with untreated NG. For **(D,E)** $n = 5$ for each group. Values are presented as mean ± SEM. Two-way ANOVA with Sidak *post hoc* test (*$p < 0.05$; ***$p < 0.001$).

(35.3%; $p < 0.0001$) and PVWM (37.5%; $p < 0.0001$) compared to NGs (**Figures 3J–L**). Ibuprofen treatment alleviated the GFAP-positive astrocyte increase in the IGWM (21.2%; $p = 0.0460$), SCWM (25.7%; $p = 0.0002$), and PVWM (21.0%; $p = 0.0229$) in IUGR treated piglets compared to untreated IUGR piglets (**Figure 3**). There was no significant difference between treated IUGR and NG piglets in any of the regions examined.

Myelination Status in White Matter in IUGR Piglet Brain Following Ibuprofen Treatment

Using Luxol Fast Blue (LFB) to observe myelination we observed organized white matter fiber tracts through the IGWM, SCWM, and PVWM in the NG piglet brains. In the IUGR piglet brain these tracts show lower staining intensity and regions with sparse staining (**Figure 4**). IUGR brains demonstrate significantly higher areas of WM in the first quartile (low intensity LFB staining), as indicated by the % area for the first quartile, when compared with NG (**Figure 4D**; $p = 0.001$). Furthermore, there was a significant reduction in myelin index in the IUGR piglet brain compared to NG (**Figure 4E**; $p = 0.0005$). Ibuprofen treatment ameliorated all of these disruptions in the IUGR piglet brain (**Figures 4D,E**), with white matter tracts displaying dense LFB staining similar to the myelin tracts in the NG piglet brain.

Neuronal Integrity in the IUGR Piglet Brain Following Ibuprofen Treatment

Using immunohistochemistry, we demonstrated a 30.2% reduction in NeuN-positive cells in the IUGR parietal cortex compared to NGs (**Figure 5D**; $p = 0.0011$). Qualitatively we observed a similar morphology to our recent observations of NeuN-positive cells in the IUGR brain with a smaller size and less defined neuronal labeling pattern (**Figure 5B**) (Wixey et al., 2019) relative to dense, clear healthy NGs (**Figure 5A**). Three days of ibuprofen treatment significantly ameliorated the decrease in NeuN-positive cell counts in the parietal cortex of IUGR treated piglets compared to untreated IUGR piglets (**Figure 5D**) with NeuN-positive counts 23.4% higher in ibuprofen treated IUGR piglets than untreated IUGR piglets (**Figure 5D**; $p = 0.0213$). There was no significant difference

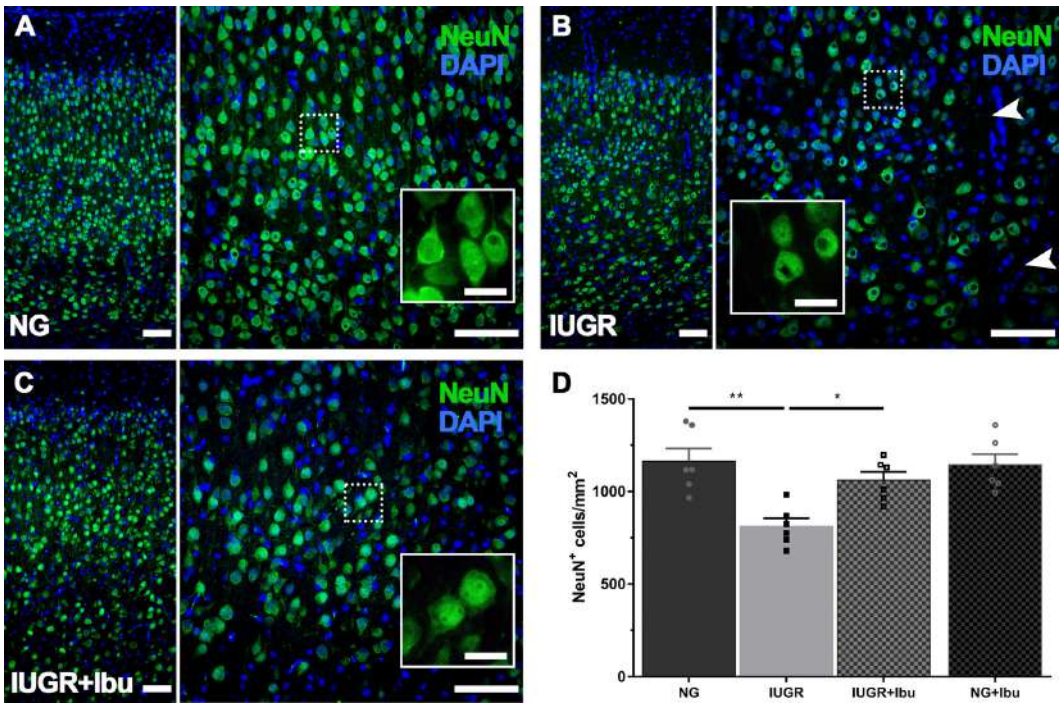


FIGURE 5 | Recovered expression of mature neurons following Ibuprofen treatment in IUGR brains. **(A)** Representative immunofluorescent staining of mature neurons using the neuron-specific nuclear marker NeuN (green) in the parietal cortex. NG brains displayed robust NeuN expression throughout the parietal cortex with large plump neurons (see insert). In comparison IUGR brains **(B)** showed regions void of neurons (see arrowheads), and altered NeuN immunolabeling (insert). Ibuprofen treated IUGR show similar staining patterns to untreated NG **(C)**. Scale bars = 100 μm for lower magnification images and 50 μm for high magnification inserts. **(D)** Quantification of cell counts found a decreased number of NeuN-positive labeled cells in IUGR compared with NG brains. IUGR+Ibu animals had higher NeuN-positive cells compared with untreated IUGR. For **(D)** $n = 6$ for each group. Values are presented as mean ± SEM. Two-way ANOVA with Sidak *post hoc* test (*$p < 0.05$; **$p < 0.005$).

in the number of NeuN-positive neurons between NGs and treated IUGR piglets. Furthermore the NeuN-positive neuronal morphology appeared more full bodied in the ibuprofen treated IUGR piglet brain, resembling the morphology of NeuN-positive neurons in the NG brain (**Figure 5C**).

Apoptosis and Cellular Proliferation in the IUGR Piglet Brain Following Ibuprofen Treatment

We previously demonstrated an increase in cellular apoptosis and reduction and cellular proliferation (Wixey et al., 2019) at P4 in the IUGR piglet parietal cortex and therefore proceeded to determine whether ibuprofen treatment could ameliorate these changes. In the current study we confirm the increase in caspase-3-positive cell counts in the IUGR piglet parietal cortex compared to NG ($p < 0.0001$; **Figures 6A,B**). Furthermore, ibuprofen treatment significantly prevented the increase in caspase-3-positive cell counts in the parietal cortex in the IUGR treated animals in comparison to untreated IUGR piglets ($p < 0.0001$; **Figures 6A,B**). No significant differences in caspase-3-positive cell counts were observed between NG and IUGR treated ($p = 0.3808$) and NG treated ($p = 0.8811$) piglets.

We observed a significant decrease in Ki67-positive cells in the IUGR brains in comparison to NG piglets ($p = 0.0021$; **Figures 6C,D**), in agreement with our previous findings (Wixey et al., 2019). However, ibuprofen treatment did not ameliorate this reduction in the IUGR piglet. Furthermore, we demonstrate a significant reduction in Ki67-positive cells in both the IUGR ibuprofen treated ($p = 0.0002$) and NG ibuprofen treated ($p = 0.0012$) piglet brains compared to untreated NG piglets (**Figures 6C,D**).

DISCUSSION

We present novel evidence that postnatal oral ibuprofen treatment reduces neuroinflammation and neuronal and white matter impairment in the newborn IUGR piglet. A repeated daily dose of ibuprofen for 3 days ameliorated reductions in myelin index, neuronal cell counts, and increases in caspase-3-positive cells at P4. The reductions in numbers of activated microglia, astrogliosis, and expression of a number of proinflammatory cytokines suggest that ibuprofen may reduce neuronal and white matter impairment by hindering the actions of neuroinflammatory mediators during the first few days following birth. These findings reveal a potential new application for ibuprofen to treat brain impairment associated with IUGR in the newborn. As ibuprofen is currently administered to the newborn for other purposes, these findings are encouraging, as

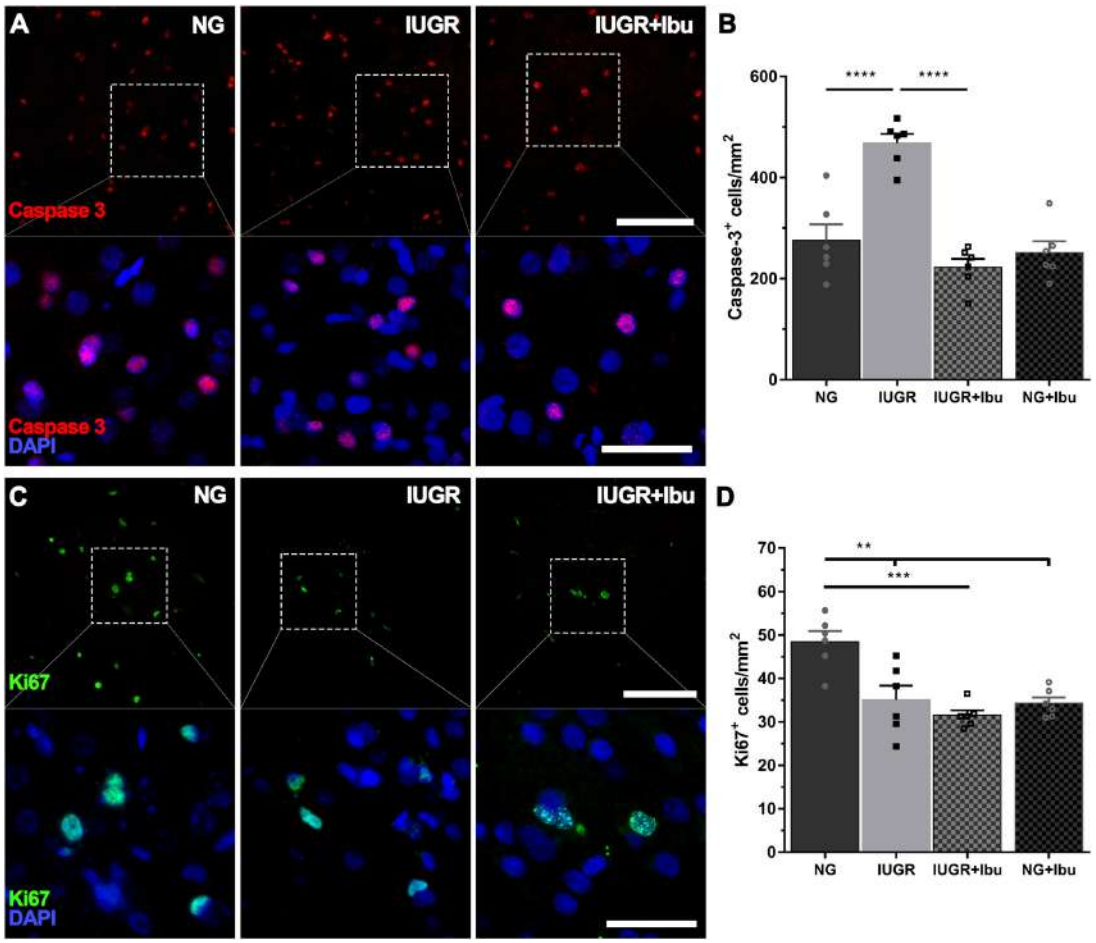


FIGURE 6 | Ibuprofen reduces apoptosis but has does not ameliorate cellular proliferation in IUGR. **(A)** Representative images of apoptotic cells (caspase 3-postive, red) in the parietal cortex of both untreated NG, IUGR, and IUGR+Ibu. **(B)** IUGR displayed higher apoptotic cell counts compared with NG. Ibuprofen significantly reduced the number of Caspase-3 cells in IUGR brains to levels comparable to NG. **(C)** Cellular proliferation was observed using Ki67. **(D)** Ki67 expression was significantly higher in untreated NG compared with all other groups. For **(A,C)** scale bar = 100 μm for low magnification and 50 μm for high magnification. For all data $n = 6$ for each group. Values are presented as mean ± SEM. Two-way ANOVA with Sidak *post hoc* test (**$p < 0.005$; ***$p < 0.001$; ****$p < 0.0001$).

there is currently no effective therapeutic intervention available to reduce neurological disorders in the IUGR newborn.

Brain Impairment in IUGR Newborn Piglets

Clinical MRI studies show reduced gray matter and white volume and structure in human IUGR newborns and in early infancy (Tolsa et al., 2004; Esteban et al., 2010; Padilla et al., 2014), with these disturbances being associated with adverse neurological outcomes in the IUGR infant (Batalle et al., 2012, 2013; Eixarch et al., 2016). At a microscopic level, IUGR animal studies also demonstrate these neuronal and white matter disturbances (Mallard et al., 2000; Guo et al., 2010; Mazur et al., 2010; Alves De Alencar Rocha et al., 2017; Kalanjati et al., 2017; Ruff et al., 2017; Wixey et al., 2019). The current study corroborates these findings demonstrating a reduction in neuronal cell counts and myelination in the newborn IUGR piglet brain. We further demonstrate reduced cellular proliferation and increased cell death in the IUGR piglet brain as previously reported in piglet and rat IUGR animal models (Pham et al., 2015; Wixey et al., 2019) suggesting brains of IUGR newborns are not just in a state of delayed neuronal maturation, but in a state of ongoing cellular injury following birth. Therefore there is a real potential to therapeutically treat, and in doing so, reduce this ongoing injury.

Dose of Ibuprofen

The dosage and treatment regime used in the current study was adopted from the human clinical situation where ibuprofen is administered to treat patent ductus arteriosus in the preterm neonate (Ohlsson et al., 2015). This dosage is much lower than other neonatal animal studies where high dosages of ibuprofen have been administered daily (50–100 mg/kg/day), that even though are neuroprotective (Carty et al., 2011; Wixey et al., 2012), may be toxic to other organs such as the renal and gastrointestinal tract (De Martino et al., 2017). Ibuprofen is a lipophilic compound that crosses the blood–brain barrier

(BBB) (Parepally et al., 2006; Kokki et al., 2007). Oral ibuprofen administration has an excellent absorption rate in the preterm newborn (Barzilay et al., 2012). A pharmacokinetic study of a single dose of oral ibuprofen (10 mg/kg) in 13 preterm infants showed all infants had detectable ibuprofen levels 1 h after administration, with ibuprofen levels peaking around 8 h and remaining plateau until 24 h (Barzilay et al., 2012). Thus, the daily dosage in the current study (20 mg/kg day 1 and 10 mg/kg days 2 and 3) would be sufficient to maintain pharmaceutical levels of ibuprofen throughout the 3 days.

Oral Ibuprofen Administration Improves IUGR Brain Outcomes

Inflammatory Markers

Previous studies have demonstrated ibuprofen's ability to target neuroinflammation by reducing numbers of activated microglia and levels of proinflammatory cytokines in the P3 HI rat brain (Carty et al., 2011; Wixey et al., 2012). Although these studies used much higher dosages of ibuprofen (50–100/kg/day) for a longer period of time (7 days) than used in the current study, similar responses were observed in the current study with reductions in numbers of activated microglia and proinflammatory cytokines following just 3 days of ibuprofen administration. Ibuprofen treatment has also been shown to have an effect on astrogliosis in an animal model of dementia (Sekiyama et al., 2012). In the current study we demonstrate not only a recovery of the density of astrocytes, but the morphology of the astrocytes in the IUGR treated brain were similar to NGs demonstrating ibuprofen's effect on the morphology of the astrocytes. Whether this is a direct or indirect effect on these glial cells requires further investigation.

Activated microglia are hallmark features of neuroinflammatory processes that occur after hypoxic events. Increased numbers of activated microglia are associated with brain injury in the HI neonatal rat model (Wixey et al., 2009, 2011b), and increased microglial numbers have been demonstrated in IUGR animal models (Olivier et al., 2007; Black et al., 2015; Pham et al., 2015; Wixey et al., 2019). Selectively blocking microglia (using the tetracycline drug minocycline) decreases not only the inflammatory response but also reduces both neuronal and white matter injury in the neonatal HI rat brain (Carty et al., 2008; Wixey et al., 2011a,b). In the current study we demonstrate a similar response with ibuprofen's ability to ameliorate the increase in numbers of activated microglia in the IUGR brain. Though activated microglia, broadly speaking, can exist in different forms: M1-like (pro-inflammatory) and M2-like (anti-inflammatory). We can assume the IUGR brain is in a proinflammatory state from the PCR inflammatory panel results and therefore ibuprofen is blocking the proinflammatory microglia rather than the anti-inflammatory microglia, however this remains to be fully elucidated. It is also important to note that complete blockade of microglial activity can exacerbate brain damage as seen in adult and neonatal HI injury models (Lalancette-Hebert et al., 2007; Faustino et al., 2011). Therapeutic interventions specifically blocking M1-like microglia and favoring M2-like microglia could be beneficial to protect the injured brain. Minocycline selectively blocks M1-like microglia and does not affect M2-like microglia expression (Kobayashi et al., 2013). However, minocycline is not the drug of choice to reduce brain injury in the newborn as it can have adverse effects when administered to neonates. Whether ibuprofen has the potential to selectively block M1-like activated microglia requires further investigation.

When microglia become activated they can release large amounts of proinflammatory cytokines which can be toxic to neurons and white matter in the newborn brain. Overexpression of IL-1β is associated with white matter damage in neonatal human brain; with specific co-localization to microglia and astrocytes (Girard et al., 2010). Variability in cytokine response was apparent in the IUGR brain as well as in response to ibuprofen treatment. A greater proportion of proinflammatory cytokines were increased in the IUGR brain with little changes to the anti-inflammatory cytokine expression; demonstrating the IUGR brain may mainly be in a proinflammatory state. This is further evident from our recent study demonstrating expression of IL-1β and TNF-α in neuronal and glial cells in P4 IUGR piglet brains (Wixey et al., 2019). The significant increases we observed in TNF-α, CXCL10, and IL-1β expression in the IUGR brain were alleviated following ibuprofen treatment. TNF-α, CXCL10, and IL-1β are common cytokines involved in the inflammatory response in the neonate. High levels of TNF-α, IL-1β, and CXCL10 are present in amniotic fluid from pregnancies complicated by infection compared to uninfected controls (Scott et al., 2012). An increase in multiple cytokines including TNF-α, IL-1β, CXCL10 are observed in a guinea pig model of IUGR with these increases relating to increased apoptosis and neuronal loss (Guo et al., 2010). A rat neonatal HI study demonstrated similar results to the current study with increases in CXCL10, TNF-α, and IL-1β (Jaworska et al., 2017). The levels of increase were similar with modest increases in TNF-α and IL-1β, and highly significant increases in CXCL10 as we observed. Furthermore, a neuroprotective strategy, sodium butyrate, suppressed the upregulation of CXCL10 at 48 h post-insult. However only at 6 days post-insult did the reduction from treatment occur for IL-1β expression, with no significant effect on TNF-α, although there was a tendency toward a decrease in expression (Jaworska et al., 2017).

Increased levels of TNF-α and IL-1β have been demonstrated in the blood of IUGR infants on day 14 following birth, which are not evident at birth (Mcelrath et al., 2013). IUGR infants tend to have low circulating levels of inflammatory proteins in their blood during the first four postnatal days (Hecht et al., 2011). Furthermore, increases in inflammatory cytokines collected during the first two postnatal weeks are correlated with adverse neurobehavioral outcomes in small for gestational age infants at 2 years (Leviton et al., 2013). Determining whether these increases in inflammatory cytokines are evident in the IUGR piglet blood would be advantageous to determine the potential for a correlation between inflammatory markers in blood and brain. If proven, we could detect and determine the extent of brain injury and thus response to treatment. Furthermore, it is yet to be determined whether the inflammation is originating from the blood or brain. As increases in inflammatory markers

are only evident in the IUGR human blood 2 weeks after birth (Mcelrath et al., 2013) it is plausible inflammation is originating from the brain and these inflammatory markers may be released into the blood due to BBB breakdown; as BBB disruption occurs in the IUGR brain (Castillo-Melendez et al., 2015). Future studies should include a focus on protein levels of cytokines in both the blood and cerebral spinal fluid to assess both the brain environment and other factors that may be contributing to this inflammatory state in the IUGR newborn.

Neuronal and White Matter Impairment

We have previously shown sustained neuronal and white matter impairment in the IUGR brain up to postnatal day 7 (Kalanjati et al., 2017; Wixey et al., 2019). In the current study we found that 3 days of ibuprofen treatment from day of birth was sufficient to alleviate the decrease in neuronal cell counts and myelination disruption in the IUGR piglet brain, therefore ceasing this injurious pattern. Whether 3 days of treatment is enough to evoke long lasting neuroprotection remains to be determined. Yet the rapid neuronal and white matter recovery observed in the current study suggests administering ibuprofen on day of birth may afford not only neuroprotection, but other mechanisms are at play. A body of evidence states alterations in myelination in the IUGR brain arise due to stalling of oligodendroglial cell maturation (Tolcos et al., 2011). This block in maturation occurs at a premyelinating stage of oligodendrocyte development, resulting in reduced density of mature myelinating oligodendrocytes. Whether ibuprofen treatment can 'unblock' the oligodendroglial cell maturation arrest in the IUGR brain is unknown. However a study in the neonatal HI rat model shows 1 week of ibuprofen treatment alleviated the loss of O4-positive premyelinating oligodendrocytes and O1-positive immature oligodendrocytes progenitor cells (Carty et al., 2011). Therefore the possibility of a similar response in the IUGR brain may be likely. Furthermore, whether a similar phenomenon is apparent for the neuronal population, i.e., a developmental stall in the immature neurons may occur in the IUGR brain and ibuprofen releases this effect, may also be plausible. Further immunohistochemistry studies on oligodendrocyte lineage markers, immature neuronal markers and mechanistic studies would be useful to decipher the role of ibuprofen in neuronal and white matter recovery.

Exploring ibuprofen's effects on Ki67-cellular proliferation would also be useful to determine whether this significant reduction following treatment in both the IUGR and NG piglets may have a detrimental impact in the long term. No other adverse outcomes following treatment were observed in these piglets therefore this decrease may not exert a physiological impact. It would be beneficial to determine the cell type of these proliferative cells as we have previously shown very rare colocalization of Ki67-positive cells with NeuN-positive cells (Wixey et al., 2019). We have unpublished evidence that Ki67 co-localizes with Iba-1-positive microglial cells in the parietal cortex. The decrease in Ki67-positive cells we observed in the current study may be due to a decrease in the activated microglia in these treated animals. However, whether this cell type is the population decreasing in the current study requires in depth exploration with double labeling of multiple cellular phenotypes. This will determine whether this decrease in Ki67-cellular proliferation is or is not a concerning aspect to treatment. Examining other regions of the brain rich in proliferative cells (such as the subventricular zone) and later time points may explain our current findings. Furthermore, follow up studies examining another common marker for proliferating cells (BrdU – which detects cells during DNA replication) may answer these questions. Yet, just 3 days of ibuprofen treatment was enough to cease the cycle of cellular injury. The highly significant increases in caspase-3-positive cell counts were completely ameliorated in the IUGR brain following ibuprofen treatment. Interestingly, there is emerging evidence that ibuprofen directly (independent of COX actions) inhibits caspases and cell death (Aranda et al., 2017; Smith et al., 2017). Determining which caspase-3-positive cell types were affected in the IUGR brain following treatment would be advantageous to determine in more detail, ibuprofen's response in the brain. These cells may be a combination of microglia, astrocytes and neurons, however further detailed investigations are warranted.

Putative Mechanisms of Ibuprofen Protection of IUGR Brain Impairment

While the role of ibuprofen as a COX inhibitor is well-established, other targets may contribute to its neuroprotective effects. Recent reports have demonstrated ibuprofen's additional targets such as caspases, Rho activity, and peroxisome proliferator-activated receptor-γ (PPAR-γ) (Heneka et al., 1999; Landreth and Heneka, 2001; Asanuma and Miyazaki, 2006; Rocha et al., 2015; Smith et al., 2017).

Ibuprofen is a potent agonist of the PPAR-γ. PPAR-γ plays a role in the modulation of immune responses through suppression of proinflammatory gene expression (Villapol, 2018). Ibuprofen has been shown to exert neuroprotective effects by inhibiting gene expression of inflammatory mediators (microglia, TNF-α, IL-6, and iNOS) through the activation of PPAR-γ (Heneka et al., 1999; Landreth and Heneka, 2001; Asanuma and Miyazaki, 2006). Ibuprofen has also been found to be an astroglial inhibitor, whereby decreasing GFAP and iNOS expression (Lim et al., 2000; Heneka et al., 2005). Ibuprofen administration diminishes reactive astrogliosis in dementia animal models (Lim et al., 2000; Heneka et al., 2005; Sekiyama et al., 2012). Its action on astrocytes is seemingly dependent on RhoA activity (Rocha et al., 2015). RhoA is known to play a role in astrocyte reactivity, and *in vitro* inhibition of RhoA with ibuprofen treatment diminishes astrogliosis (Rocha et al., 2015). Rho activation also plays a critical role in limiting axonal regeneration following CNS injuries and therapeutically inhibiting Rho via ibuprofen treatment is an important target for axonal repair in injured CNS neurons (Madura et al., 2011). A recent finding demonstrates physiologically relevant concentrations of ibuprofen impedes caspase catalysis, reducing inflammation and cell death both *in vitro* and *in vivo* (Smith et al., 2017). The study showed that caspase inhibition is COX independent and represents a new anti-inflammatory target as caspases play a pivotal role in inflammation and

cell death (Smith et al., 2017). It is likely that both COX and caspase pathways are simultaneously modulated following ibuprofen treatment, each contributing to the anti-inflammatory mechanism. As we demonstrated a significant decrease in the number of casapase-3 cells in the IUGR brain, this could be due to a direct effect of ibuprofen on apoptosis and inflammation.

Limitations

Although the results from the current study are promising, there are a few limitations to this study. The piglet brains were examined immediately after treatment ceased and therefore we were unable to adequately assess potential long-term adverse effects or long-term benefits of the 3 days of ibuprofen treatment. Surviving the piglets for a longer time period after treatment has ceased would be beneficial to explore this avenue of research. Determining the type of proliferating and apoptotic cells would also prove beneficial, especially in regard to the treatment response of the proliferating cells. There are multiple cellular phenotypes in the brain, however further examination of oligodendrocyte lineage markers and immature neuronal markers would assist with understanding the major neurodevelopmental networks of the IUGR newborn brain and their response to treatment.

CONCLUSION

Currently, there are limited treatments to prevent neurological impairment in the IUGR infant. Understanding the impact of inflammation in the IUGR brain is critical for the development of therapies to improving neurodevelopmental outcomes in IUGR newborns. As evidenced in this study, ibuprofen may be effective at protecting the IUGR newborn brain and be a promising therapeutic option. Long-term studies testing the efficacy of early ibuprofen treatment would assist in determining the potential clinical application of ibuprofen as an intervention for protecting the newborn brain. It is notable that in an adult rodent model, 6 days of ibuprofen treatment protects neurons from ischemic-induced injury for up to 4 weeks post-insult (Park et al., 2005). Exploring interventions that specifically target inflammatory processes could not only reduce white matter and neuronal injury in the brain but may provide neuroprotection through effects on central and systemic inflammation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ARRIVE guidelines. The protocol was approved by The University of Queensland Animal Ethics Committee (MED/UQCCR/132/16/RBWH).

AUTHOR CONTRIBUTIONS

JW was involved in attaining funding, experimental designs, conducting animal experiments, critical revision, and drafting of the manuscript. KS and RP conducted the animal experiments, laboratory aspects, and collated and analyzed the data. KL undertook Luxol Fast Blue staining, analysis, and interpretation. PC was involved in attaining funding, critical revision, and editing the manuscript. SB was involved in attaining funding, critical revision, and editing the manuscript. KC was involved in attaining funding and responsible for all laboratory aspects of the project, data analysis, interpretation, and editing the manuscript. All authors read and approved the final manuscript.

FUNDING

A Royal Brisbane and Women's Hospital Research Grant, Children's Hospital Foundation Innovation (Grant No. 50217) and National Health and Medical Research Council Project (Grant No. APP1147545) supported this work. SB was supported by a Lion's Medical Research Fellowship.

ACKNOWLEDGMENTS

The authors acknowledge the Research Histology Facility from IHBI (QUT). The authors would also like to thank Dr. Stephanie Miller and Kate Goasdoue for assistance with animal experimentation.

REFERENCES

Alves De Alencar Rocha, A. K., Allison, B. J., Yawno, T., Polglase, G. R., Sutherland, A. E., Malhotra, A., et al. (2017). Early- versus late-onset fetal growth restriction differentially affects the development of the fetal sheep brain. *Dev. Neurosci.* 39, 141–155. doi: 10.1159/000456542

Aranda, J. V., Salomone, F., Valencia, G. B., and Beharry, K. D. (2017). Non-steroidal anti-inflammatory drugs in newborns and infants. *Pediatr. Clin. North Am.* 64, 1327–1340. doi: 10.1016/j.pcl.2017.08.009

Asanuma, M., and Miyazaki, I. (2006). Nonsteroidal anti-inflammatory drugs in Parkinson's disease: possible involvement of quinone formation. *Expert Rev. Neurother.* 6, 1313–1325. doi: 10.1586/14737175.6.9.1313

Barzilay, B., Youngster, I., Batash, D., Keidar, R., Baram, S., Goldman, M., et al. (2012). Pharmacokinetics of oral ibuprofen for patent ductus arteriosus closure in preterm infants. *Arch. Dis. Child Fetal. Neonatal. Ed.* 97, F116–F119. doi: 10.1136/adc.2011.215160

Batalle, D., Eixarch, E., Figueras, F., Munoz-Moreno, E., Bargallo, N., Illa, M., et al. (2012). Altered small-world topology of structural brain networks in infants with intrauterine growth restriction and its association with later neurodevelopmental outcome. *Neuroimage* 60, 1352–1366. doi: 10.1016/j.neuroimage.2012.01.059

Batalle, D., Munoz-Moreno, E., Figueras, F., Bargallo, N., Eixarch, E., and Gratacos, E. (2013). Normalization of similarity-based individual brain networks from gray matter MRI and its association with neurodevelopment in infants with intrauterine growth restriction. *Neuroimage* 83, 901–911. doi: 10.1016/j.neuroimage.2013.07.045

Bauer, R., Walter, B., Brust, P., Fuchtner, F., and Zwiener, U. (2003). Impact of asymmetric intrauterine growth restriction on organ function in newborn piglets. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 110(Suppl. 1), S40–S49.

Bauer, R., Walter, B., Hoppe, A., Gaser, E., Lampe, V., Kauf, E., et al. (1998). Body weight distribution and organ size in newborn swine (*sus scrofa* domestica) – a study describing an animal model for asymmetrical intrauterine growth

retardation. *Exp. Toxicol. Pathol.* 50, 59–65. doi: 10.1016/s0940-2993(98)80071-7

Black, A. M., Armstrong, E. A., Scott, O., Juurlink, B. J., and Yager, J. Y. (2015). Broccoli sprout supplementation during pregnancy prevents brain injury in the newborn rat following placental insufficiency. *Behav. Brain Res.* 291, 289–298. doi: 10.1016/j.bbr.2015.05.033

Cai, Z., Lin, S., Fan, L.-W., Pang, Y., and Rhodes, P. G. (2006). Minocycline alleviates hypoxic-ischemic injury to developing oligodendrocytes in the neonatal rat brain. *Neuroscience* 137, 425–435. doi: 10.1016/j.neuroscience.2005.09.023

Campbell, L. R., Pang, Y., Ojeda, N. B., Zheng, B., Rhodes, P. G., and Alexander, B. T. (2012). Intracerebral lipopolysaccharide induces neuroinflammatory change and augmented brain injury in growth-restricted neonatal rats. *Pediatr. Res.* 71, 645–652. doi: 10.1038/pr.2012.26

Carty, M. L., Wixey, J. A., Colditz, P. B., and Buller, K. M. (2008). Post-hypoxia-ischemia minocycline treatment attenuates neuroinflammation and white matter injury in the neonatal rat; a comparison of two different dose regimens. *Int. J. Dev. Neurosci.* 26, 477–485. doi: 10.1016/j.ijdevneu.2008.02.005

Carty, M. L., Wixey, J. A., Reinebrant, H. E., Gobe, G., Colditz, P. B., and Buller, K. M. (2011). Ibuprofen inhibits neuroinflammation and attenuates white matter damage following hypoxia–ischemia in the immature rodent brain. *Brain Res.* 1402, 9–19. doi: 10.1016/j.brainres.2011.06.001

Castillo-Melendez, M., Yawno, T., Allison, B. J., Jenkin, G., Wallace, E. M., and Miller, S. L. (2015). Cerebrovascular adaptations to chronic hypoxia in the growth restricted lamb. *Int. J. Dev. Neurosci.* 45, 55–65. doi: 10.1016/j.ijdevneu.2015.01.004

Cox, P., and Marton, T. (2009). Pathological assessment of intrauterine growth restriction. *Best Pract. Res. Clin. Obstet. Gynaecol.* 23, 751–764. doi: 10.1016/j.bpobgyn.2009.06.006

De Martino, M., Chiarugi, A., Boner, A., Montini, G., and De' Angelis, G. L. (2017). Working towards an appropriate use of ibuprofen in children: an evidence-based appraisal. *Drugs* 77, 1295–1311. doi: 10.1007/s40265-017-0751-z

Eixarch, E., Munoz-Moreno, E., Bargallo, N., Batalle, D., and Gratacos, E. (2016). Motor and cortico-striatal-thalamic connectivity alterations in intrauterine growth restriction. *Am. J. Obstet. Gynecol.* 214, e721–e729. doi: 10.1016/j.ajog.2015.12.028

Esteban, F. J., Padilla, N., Sanz-Cortes, M., De Miras, J. R., Bargallo, N., Villoslada, P., et al. (2010). Fractal-dimension analysis detects cerebral changes in preterm infants with and without intrauterine growth restriction. *Neuroimage* 53, 1225–1232. doi: 10.1016/j.neuroimage.2010.07.019

Faustino, J. V., Wang, X., Johnson, C. E., Klibanov, A., Derugin, N., Wendland, M. F., et al. (2011). Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. *J. Neurosci.* 31, 12992–13001. doi: 10.1523/JNEUROSCI.2102-11.2011

Felix, B., Leger, M. E., Albe-Fessard, D., Marcilloux, J. C., Rampin, O., and Laplace, J. P. (1999). Stereotaxic atlas of the pig brain. *Brain Res. Bull.* 49, 1–137.

Geva, R., Eshel, R., Leitner, Y., Valevski, A. F., and Harel, S. (2006). Neuropsychological outcome of children with intrauterine growth restriction: a 9-year prospective study. *Pediatrics* 118, 91–100. doi: 10.1542/peds.2005-2343

Girard, S., Sebire, G., and Kadhim, H. (2010). Proinflammatory orientation of the interleukin 1 system and downstream induction of matrix metalloproteinase 9 in the pathophysiology of human perinatal white matter damage. *J. Neuropathol. Exp. Neurol.* 69, 1116–1129. doi: 10.1097/NEN.0b013e3181f971e4

Guo, R., Hou, W., Dong, Y., Yu, Z., Stites, J., and Weiner, C. P. (2010). Brain injury caused by chronic fetal hypoxemia is mediated by inflammatory cascade activation. *Reprod. Sci.* 17, 540–548. doi: 10.1177/1933719110364061

Hecht, J. L., Fichorova, R. N., Tang, V. F., Allred, E. N., Mcelrath, T. F., Leviton, A., et al. (2011). Relationship between neonatal blood protein concentrations and placenta histologic characteristics in extremely low ga newborns. *Pediatr. Res.* 69, 68–73. doi: 10.1203/PDR.0b013e3181fed334

Heinonen, K., Raikkonen, K., Pesonen, A. K., Andersson, S., Kajantie, E., Eriksson, J. G., et al. (2010). Behavioural symptoms of attention deficit/hyperactivity disorder in preterm and term children born small and appropriate for gestational age: a longitudinal study. *BMC Pediatr.* 10:91. doi: 10.1186/1471-2431-10-91

Heneka, M. T., Feinstein, D. L., Galea, E., Gleichmann, M., Wullner, U., and Klockgether, T. (1999). Peroxisome proliferator-activated receptor gamma agonists protect cerebellar granule cells from cytokine-induced apoptotic cell death by inhibition of inducible nitric oxide synthase. *J. Neuroimmunol.* 100, 156–168. doi: 10.1016/s0165-5728(99)00192-7

Heneka, M. T., Sastre, M., Dumitrescu-Ozimek, L., Hanke, A., Dewachter, I., Kuiperi, C., et al. (2005). Acute treatment with the PPARgamma agonist pioglitazone and ibuprofen reduces glial inflammation and Abeta1-42 levels in APPV717I transgenic mice. *Brain* 128, 1442–1453. doi: 10.1093/brain/awh452

Huang, Z., Liu, J., Cheung, P. Y., and Chen, C. (2009). Long-term cognitive impairment and myelination deficiency in a rat model of perinatal hypoxic-ischemic brain injury. *Brain Res.* 1301, 100–109. doi: 10.1016/j.brainres.2009.09.006

Jacobsson, B., Ahlin, K., Francis, A., Hagberg, G., Hagberg, H., and Gardosi, J. (2008). Cerebral palsy and restricted growth status at birth: population-based case-control study. *BJOG* 115, 1250–1255. doi: 10.1111/j.1471-0528.2008.01827.x

Jarvis, S., Glinianaia, S. V., Torrioli, M. G., Platt, M. J., Miceli, M., Jouk, P. S., et al. (2003). Cerebral palsy and intrauterine growth in single births: European collaborative study. *Lancet* 362, 1106–1111. doi: 10.1016/s0140-6736(03)14466-2

Jaworska, J., Ziemka-Nalecz, M., Sypecka, J., and Zalewska, T. (2017). The potential neuroprotective role of a histone deacetylase inhibitor, sodium butyrate, after neonatal hypoxia-ischemia. *J. Neuroinflamm.* 14:34. doi: 10.1186/s12974-017-0807-8

Kalanjati, V. P., Miller, S. M., Ireland, Z., Colditz, P. B., and Bjorkman, S. T. (2011). Developmental expression and distribution of GABA(A) receptor alpha1-, alpha3- and beta2-subunits in pig brain. *Dev. Neurosci.* 33, 99–109. doi: 10.1159/000326630

Kalanjati, V. P., Wixey, J. A., Miller, S. M., Colditz, P. B., and Bjorkman, S. T. (2017). GABAA receptor expression and white matter disruption in intrauterine growth restricted piglets. *Int. J. Dev. Neurosci.* 59, 1–9. doi: 10.1016/j.ijdevneu.2017.02.004

Kobayashi, K., Imagama, S., Ohgomori, T., Hirano, K., Uchimura, K., Sakamoto, K., et al. (2013). Minocycline selectively inhibits M1 polarization of microglia. *Cell Death Dis.* 4:e525. doi: 10.1038/cddis.2013.54

Kokki, H., Kumpulainen, E., Lehtonen, M., Laisalmi, M., Heikkinen, M., Savolainen, J., et al. (2007). Cerebrospinal fluid distribution of ibuprofen after intravenous administration in children. *Pediatrics* 120, e1002–e1008. doi: 10.1542/peds.2007-0064

Kremlev, S. G., Roberts, R. L., and Palmer, C. (2007). Minocycline modulates chemokine receptors but not interleukin-10 mRNA expression in hypoxic-ischemic neonatal rat brain. *J. Neurosci. Res.* 85, 2450–2459. doi: 10.1002/jnr.21380

Lalancette-Hebert, M., Gowing, G., Simard, A., Weng, Y. C., and Kriz, J. (2007). Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. *J. Neurosci.* 27, 2596–2605. doi: 10.1523/jneurosci.5360-06.2007

Landreth, G. E., and Heneka, M. T. (2001). Anti-inflammatory actions of peroxisome proliferator-activated receptor gamma agonists in Alzheimer's disease. *Neurobiol. Aging* 22, 937–944. doi: 10.1016/s0197-4580(01)00296-2

Leonardo, C. C., Eakin, A. K., Ajmo, J. M., Collier, L. A., Pennypacker, K. R., Strongin, A. Y., et al. (2008). Delayed administration of a matrix metalloproteinase inhibitor limits progressive brain injury after hypoxia-ischemia in the neonatal rat. *J. Neuroinflamm.* 5:34. doi: 10.1186/1742-2094-5-34

Leviton, A., Fichorova, R. N., O'shea, T. M., Kuban, K., Paneth, N., Dammann, O., et al. (2013). Two-hit model of brain damage in the very preterm newborn: small for gestational age and postnatal systemic inflammation. *Pediatr. Res.* 73, 362–370. doi: 10.1038/pr.2012.188

Lim, G. P., Yang, F., Chu, T., Chen, P., Beech, W., Teter, B., et al. (2000). Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J. Neurosci.* 20, 5709–5714. doi: 10.1523/jneurosci.20-15-05709.2000

Madura, T., Tomita, K., and Terenghi, G. (2011). Ibuprofen improves functional outcome after axotomy and immediate repair in the peripheral nervous system. *J. Plast Reconstr. Aesthet. Surg.* 64, 1641–1646. doi: 10.1016/j.bjps.2011.07.014

Mallard, C., Loeliger, M., Copolov, D., and Rees, S. (2000). Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig

following intrauterine growth-restriction. *Neuroscience* 100, 327–333. doi: 10.1016/s0306-4522(00)00271-2

Mazur, M., Miller, R. H., and Robinson, S. (2010). Postnatal erythropoietin treatment mitigates neural cell loss after systemic prenatal hypoxic-ischemic injury. *J. Neurosurg. Pediatr.* 6, 206–221. doi: 10.3171/2010.5.PEDS1032

Mcelrath, T. F., Allred, E. N., Van Marter, L., Fichorova, R. N., Leviton, A., and Investigators, E. S. (2013). Perinatal systemic inflammatory responses of growth-restricted preterm newborns. *Acta Paediatr.* 102, e439–e442. doi: 10.1111/apa.12339

Ohlsson, A., Walia, R., and Shah, S. S. (2015). Ibuprofen for the treatment of patent ductus arteriosus in preterm or low birth weight (or both) infants. *Coch. Database Syst. Rev.* 18:CD003481.

Olivier, P., Baud, O., Bouslama, M., Evrard, P., Gressens, P., and Verney, C. (2007). Moderate growth restriction: deleterious and protective effects on white matter damage. *Neurobiol. Dis.* 26, 253–263. doi: 10.1016/j.nbd.2007.01.001

Olivier, P., Baud, O., Evrard, P., Gressens, P., and Verney, C. (2005). Prenatal ischemia and white matter damage in rats. *J. Neuropathol. Exp. Neurol.* 64, 998–1006. doi: 10.1097/01.jnen.0000187052.81889.57

Padilla, N., Alexandrou, G., Blennow, M., Lagercrantz, H., and Aden, U. (2015). Brain growth gains and losses in extremely preterm infants at term. *Cereb. Cortex* 25, 1897–1905. doi: 10.1093/cercor/bht431

Padilla, N., Junque, C., Figueras, F., Sanz-Cortes, M., Bargallo, N., Arranz, A., et al. (2014). Differential vulnerability of gray matter and white matter to intrauterine growth restriction in preterm infants at 12 months corrected age. *Brain Res.* 1545, 1–11. doi: 10.1016/j.brainres.2013.12.007

Parepally, J. M., Mandula, H., and Smith, Q. R. (2006). Brain uptake of nonsteroidal anti-inflammatory drugs: ibuprofen, flurbiprofen, and indomethacin. *Pharm. Res.* 23, 873–881. doi: 10.1007/s11095-006-9905-5

Park, E. M., Cho, B. P., Volpe, B. T., Cruz, M. O., Joh, T. H., and Cho, S. (2005). Ibuprofen protects ischemia-induced neuronal injury via up-regulating interleukin-1 receptor antagonist expression. *Neuroscience* 132, 625–631. doi: 10.1016/j.neuroscience.2005.01.021

Pham, H., Duy, A. P., Pansiot, J., Bollen, B., Gallego, J., Charriaut-Marlangue, C., et al. (2015). Impact of inhaled nitric oxide on white matter damage in growth-restricted neonatal rats. *Pediatr. Res.* 77, 563–569. doi: 10.1038/pr.2015.4

Rideau Batista Novais, A., Pham, H., Van De Looij, Y., Bernal, M., Mairesse, J., Zana-Taieb, E., et al. (2016). Transcriptomic regulations in oligodendroglial and microglial cells related to brain damage following fetal growth restriction. *Glia* 64, 2306–2320. doi: 10.1002/glia.23079

Rocha, D. N., Ferraz-Nogueira, J. P., Barrias, C. C., Relvas, J. B., and Pego, A. P. (2015). Extracellular environment contribution to astrogliosis-lessons learned from a tissue engineered 3D model of the glial scar. *Front. Cell Neurosci.* 9:377. doi: 10.3389/fncel.2015.00377

Ruff, C. A., Faulkner, S. D., Rumajogee, P., Beldick, S., Foltz, W., Corrigan, J., et al. (2017). The extent of intrauterine growth restriction determines the severity of cerebral injury and neurobehavioural deficits in rodents. *PLoS One* 12:e0184653. doi: 10.1371/journal.pone.0184653

Scott, G. M., Chow, S. S., Craig, M. E., Pang, C. N., Hall, B., Wilkins, M. R., et al. (2012). Cytomegalovirus infection during pregnancy with maternofetal transmission induces a proinflammatory cytokine bias in placenta and amniotic fluid. *J. Infect. Dis.* 205, 1305–1310. doi: 10.1093/infdis/jis186

Sekiyama, K., Fujita, M., Sekigawa, A., Takamatsu, Y., Waragai, M., Takenouchi, T., et al. (2012). Ibuprofen ameliorates protein aggregation and astrocytic gliosis, but not cognitive dysfunction, in a transgenic mouse expressing dementia with Lewy bodies-linked P123H beta-synuclein. *Neurosci. Lett.* 515, 97–101. doi: 10.1016/j.neulet.2012.03.037

Smith, C. E., Soti, S., Jones, T. A., Nakagawa, A., Xue, D., and Yin, H. (2017). Non-steroidal anti-inflammatory drugs are caspase inhibitors. *Cell Chem. Biol.* 24, 281–292. doi: 10.1016/j.chembiol.2017.02.003

Sovio, U., White, I. R., Dacey, A., Pasupathy, D., and Smith, G. C. S. (2015). Screening for fetal growth restriction with universal third trimester ultrasonography in nulliparous women in the pregnancy outcome prediction (POP) study: a prospective cohort study. *Lancet* 386, 2089–2097. doi: 10.1016/S0140-6736(15)00131-2

Tolcos, M., Bateman, E., O'dowd, R., Markwick, R., Vrijsen, K., Rehn, A., et al. (2011). Intrauterine growth restriction affects the maturation of myelin. *Exp. Neurol.* 232, 53–65. doi: 10.1016/j.expneurol.2011.08.002

Tolsa, C. B., Zimine, S., Warfield, S. K., Freschi, M., Sancho Rossignol, A., Lazeyras, F., et al. (2004). Early alteration of structural and functional brain development in premature infants born with intrauterine growth restriction. *Pediatr. Res.* 56, 132–138. doi: 10.1203/01.pdr.0000128983.54614.7e

Villapol, S. (2018). Roles of peroxisome proliferator-activated receptor gamma on brain and peripheral inflammation. *Cell Mol. Neurobiol.* 38, 121–132. doi: 10.1007/s10571-017-0554-5

Wixey, J. A., Chand, K. K., Colditz, P. B., and Bjorkman, S. T. (2017). Review: neuroinflammation in intrauterine growth restriction. *Placenta* 54, 117–124. doi: 10.1016/j.placenta.2016.11.012

Wixey, J. A., Chand, K. K., Pham, L., Colditz, P. B., and Bjorkman, S. T. (2018). Therapeutic potential to reduce brain injury in growth restricted newborns. *J. Physiol.* 596, 5675–5686. doi: 10.1113/JP275428

Wixey, J. A., Lee, K. M., Miller, S. M., Goasdoue, K., Colditz, P. B., Bjorkman, S. T., et al. (2019). Neuropathology in intrauterine growth restricted newborn piglets is associated with glial activation and proinflammatory status in the brain. *J. Neuroinflamm.* 16:5. doi: 10.1186/s12974-018-1392-1

Wixey, J. A., Reinebrant, H. E., and Buller, K. M. (2011a). Inhibition of neuroinflammation prevents injury to the serotonergic network after hypoxia-ischemia in the immature rat brain. *J. Neuropathol. Exp. Neurol.* 70, 23–35. doi: 10.1097/NEN.0b013e3182020b7b

Wixey, J. A., Reinebrant, H. E., Spencer, S. J., and Buller, K. M. (2011b). Efficacy of post-insult minocycline administration to alter long-term hypoxia-ischemia-induced damage to the serotonergic system in the immature rat brain. *Neuroscience* 182, 184–192. doi: 10.1016/j.neuroscience.2011.03.033

Wixey, J. A., Reinebrant, H. E., and Buller, K. M. (2012). Post-insult ibuprofen treatment attenuates damage to the serotonergic system after hypoxia-ischemia in the immature rat brain. *J. Neuropathol. Exp. Neurol.* 71, 1137–1148. doi: 10.1097/NEN.0b013e318277d4c7

Wixey, J. A., Reinebrant, H. E., Carty, M. L., and Buller, K. M. (2009). Delayed P2X4R expression after hypoxia-ischemia is associated with microglia in the immature rat brain. *J. Neuroimmunol.* 212, 35–43. doi: 10.1016/j.jneuroim.2009.04.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Wixey, Sukumar, Pretorius, Lee, Colditz, Bjorkman and Chand. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

REVIEW
published: 15 May 2019
doi: 10.3389/fphys.2019.00599

Reduced Neurosteroid Exposure Following Preterm Birth and Its' Contribution to Neurological Impairment: A Novel Avenue for Preventative Therapies

Julia C. Shaw[1,2], Mary J. Berry[3,4], Rebecca M. Dyson[3,4], Gabrielle K. Crombie[1,2], Jonathan J. Hirst[1,2†] and Hannah K. Palliser[1,2†]*

[1] School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle, NSW, Australia, [2] Mothers and Babies Research Centre, Hunter Medical Research Institute, University of Newcastle, Newcastle, NSW, Australia, [3] Department of Paediatrics and Child Health, University of Otago, Wellington, Wellington, New Zealand, [4] Centre for Translational Physiology, University of Otago, Wellington, Wellington, New Zealand

OPEN ACCESS

Edited by:
Carina Mallard, University of Gothenburg, Sweden

Reviewed by:
Eridan Rocha Ferreira, University of Gothenburg, Sweden
Robert Galinsky, Hudson Institute of Medical Research, Australia

***Correspondence:**
Julia C. Shaw
Julia.c.shaw@newcastle.edu.au

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *13 November 2018*
Accepted: *26 April 2019*
Published: *15 May 2019*

Citation:
Shaw JC, Berry MJ, Dyson RM, Crombie GK, Hirst JJ and Palliser HK (2019) Reduced Neurosteroid Exposure Following Preterm Birth and Its' Contribution to Neurological Impairment: A Novel Avenue for Preventative Therapies. Front. Physiol. 10:599. doi: 10.3389/fphys.2019.00599

Children born preterm are at an increased risk of developing cognitive problems and neuro-behavioral disorders such as attention deficit hyperactivity disorder (ADHD) and anxiety. Whilst neonates born at all gestational ages, even at term, can experience poor cognitive outcomes due to birth-complications such as birth asphyxia, it is becoming widely known that children born preterm in particular are at significant risk for learning difficulties with an increased utilization of special education resources, when compared to their healthy term-born peers. Additionally, those born preterm have evidence of altered cerebral myelination with reductions in white matter volumes of the frontal cortex, hippocampus and cerebellum evident on magnetic resonance imaging (MRI). This disruption to myelination may underlie some of the pathophysiology of preterm-associated brain injury. Compared to a fetus of the same post-conceptional age, the preterm newborn loses access to *in utero* factors that support and promote healthy brain development. Furthermore, the preterm *ex utero* environment is hostile to the developing brain with a myriad of environmental, biochemical and excitotoxic stressors. Allopregnanolone is a key neuroprotective fetal neurosteroid which has promyelinating effects in the developing brain. Preterm birth leads to an abrupt loss of the protective effects of allopregnanolone, with a dramatic drop in allopregnanolone concentrations in the preterm neonatal brain compared to the fetal brain. This occurs in conjunction with reduced myelination of the hippocampus, subcortical white matter and cerebellum; thus, damage to neurons, astrocytes and especially oligodendrocytes of the developing nervous system can occur in the vulnerable developmental window prior to term as a consequence reduced allopregnanolone. In an effort to prevent preterm-associated brain injury a number of therapies have been considered, but to date, other than antenatal magnesium sulfate and corticosteroid therapy, none have become part of standard clinical care for vulnerable infants. Therefore, there remains an urgent need for improved therapeutic options to prevent brain injury in preterm neonates. The

actions of the placentally derived neurosteroid allopregnanolone on $GABA_A$ receptor signaling has a major role in late gestation neurodevelopment. The early loss of this intrauterine neurotrophic support following preterm birth may be pivotal to development of neurodevelopmental morbidity. Thus, restoring the *in utero* neurosteroid environment for preterm neonates may represent a new and clinically feasible treatment option for promoting better trajectories of myelination and brain development, and therefore reducing neurodevelopmental disorders in children born preterm.

Keywords: neurosteroid, preterm birth, GABAA receptor (GABAAR), myelin, ganaxolone

INTRODUCTION

Preterm birth is the leading cause of death and neurodevelopmental related disability in early life (Goldenberg et al., 2008). In resource rich nations such as Australia, the incidence of moderate-late preterm birth specifically now accounts for ∼80% of all preterm births (Cheong and Doyle, 2012; Frey and Klebanoff, 2016). These neonates have a high survival rate and a low incidence of gross neuroanatomical damage on routine clinical imaging; however, there is increasing evidence of microcystic white matter injury when assessed using MRI. Even amongst those infants who appear well at the time of hospital discharge, and are free of gross neuroanatomical lesions, there remains a high burden of later cognitive difficulties and neurodevelopmental disorders such as anxiety and attention deficit hyperactivity disorder (ADHD) (Ananth and Vintzileos, 2006; Chyi et al., 2008; Moster et al., 2008; Petrini et al., 2009; Loe et al., 2011; Baron et al., 2012; Cheong and Doyle, 2012; Potijk et al., 2012). The long-term individual, familial and socio-economic burden of these issues remains profound; with rates of preterm birth at around 10%, and with increasing numbers of children surviving, there is an urgent need to develop novel therapeutic options to mitigate, or prevent, the ongoing neurological burden of preterm birth.

Myelination of white matter tracts continues throughout late gestation and following birth in areas such as the hippocampus and cerebellum: reduction in brain volumes and functionality of these regions are evident in children that were born preterm (Rivkin, 1997; Rees and Inder, 2005; Rees et al., 2008; Volpe, 2008). In particular myelination by mature oligodendrocytes is ongoing throughout this late gestation stage and is vulnerable to insults and excitotoxic damage associated with early exposure to the *ex utero* environment (Arnold and Trojanowski, 1996; Back et al., 2002; Matsusue et al., 2014). *In utero*, the fetal neurosteroid allopregnanolone is responsible for protection from neurological insults, modulating fetal behavior leading to the onset of a 'sleep-like state,' and promoting myelination through its action on the inhibitory $GABA_A$ receptors of the central nervous system (CNS) (Nicol et al., 1998; Nguyen et al., 2003; Herd et al., 2007). Importantly, recent studies suggest that behavioral and cognitive outcomes are tightly linked with gestational age (Berry et al., 2018). Any decrement in gestation, even across 'early term' (37/38 weeks' gestation) is associated with, on a population basis, impaired cognitive and developmental outcomes compared to outcomes found in children born at full term (39–40 weeks gestational age) (Berry et al., 2018).

Birth is necessarily associated with the loss of the fetus from the placenta-maternal unit, and therefore separation from any trophic factors derived from either mother or placenta. Preterm birth results in the premature loss of placentally supplied allopregnanolone during a period when it is critical for optimal neurodevelopment (Kelleher et al., 2013). Whilst neurosteroid therapy has been evaluated for the treatment of traumatic brain injury (TBI) and epilepsy (Nohria and Giller, 2007; Wright et al., 2007; Xiao et al., 2008; Reddy and Rogawski, 2012), therapeutic use of neurosteroids following preterm birth requires further evaluation.

NEUROLOGICAL OUTCOMES OF PRETERM BIRTH

Despite only comprising 10% of births, preterm birth is the leading cause of death and neurodevelopmental related disability in neonates, accounting for up to 50% of neonatal deaths (Simmons et al., 2010). Furthermore, the ongoing morbidity risks of preterm birth remain unacceptably high with up to 50% of survivors developing some form of long-term neurodevelopmental disability (Mathews et al., 2002; Ananth and Vintzileos, 2006). Cerebral white matter injury in the preterm infant varies based on gestational age at the time of birth. Historically, injury following early preterm birth was characterized by intraventricular hemorrhage and, or, periventricular leukomalacia (PVL) (Volpe, 2001, 2009). In survivors of early preterm birth weighing <1,500 g approximately 10% develop cerebral palsy as a result of these gross insults and necrosis (Volpe, 2003). However, with improvements in perinatal care these gross structural lesions are now far less common, whereas diffuse white matter injury (DWMI) demonstrable on MRI, but not routine screening cranial ultrasound, is increasingly recognized as the key contributor to the pathophysiology of preterm-associated brain injury. It is now established that impaired cognition, sensory and psychological functioning in children born preterm is associated with DWMI (van Tilborg et al., 2016). Furthermore, DWMI is a recognized risk factor for the development of neurobehavioral disorders such as autism-spectrum disorders and ADHD (van Tilborg et al., 2016). The underlying pathophysiological mechanisms of DWMI are poorly understood but are suggested

to be due to immature oligodendrocyte arrest resulting in impaired myelination.

In infants that were born <32 weeks' gestation, it has recently been shown that reductions in white matter volume in areas such as the fornix and the cingulum observed by MRI at the time of birth remained present until 19 years of age and were associated with impairments in memory functions (Caldinelli et al., 2017). The Stockholm Neonatal Project has also recently published the results of a longitudinal trial following infants born <36 weeks' gestation up until 18 years of age when they undertook psychological assessment including general intelligence and executive functioning measures. Significantly poorer outcomes were observed for preterm children in areas such as IQ, attention, working memory, and cognitive flexibility (Vollmer et al., 2017). Most importantly, however, is that the executive functioning deficits did not correlate with reductions in white matter or gray matter volumes evident by MRI following birth, but the microstructure of white matter tracts was altered at adolescence. Thus, this study found that following preterm birth, and in the absence of obvious perinatal brain injury, the alterations observed in white matter microstructure during adolescence correlate with executive function and general cognitive abilities. Furthermore, it suggested that disruption to neural pathways, as opposed to reductions in brain volume, is involved in the impairment of neurodevelopment following preterm birth. In addition to established preterm birth related disorders, such as cerebral palsy, there is now a growing body of evidence suggesting that preterm infants from moderate-late preterm pregnancies are much more likely to develop neurodevelopmental morbidities and learning disorders that become apparent at school age, with anxiety and ADHD being the most commonly diagnosed (Linnet et al., 2006; Chyi et al., 2008; Moster et al., 2008; Petrini et al., 2009; Lindstrom et al., 2011; Loe et al., 2011; Baron et al., 2012; Cheong and Doyle, 2012; Potijk et al., 2012; Berry et al., 2018).

Attention deficit hyperactivity disorder is characterized by a deficit in behavioral inhibition, inattention, impulsivity and social difficulties, and in a Norwegian cohort of preterm/low birth weight children at 5 years old was more commonly diagnosed in males (Elgen et al., 2014). In the same cohort, the females were more likely to be diagnosed with anxiety (Elgen et al., 2014) highlighting that the behavioral outcomes of preterm birth occur in a sex-dependent manner. In a large Danish cohort children born at 34–36 weeks' gestation (moderate-late preterm range) had an 80% increased risk of being diagnosed with ADHD compared to children born after 37 weeks' gestation, a larger percentage of these were also male (Linnet et al., 2006). Furthermore, in a Swedish cohort, the amount of ADHD medication purchased for ex-premature school-aged males was more than three times as much than for females, and the amount purchased increased by degree of immaturity at birth (Lindstrom et al., 2011). In addition to anxiety and ADHD, incidences of autism and depression are also increased following preterm birth. Children in the United States that were born moderate-late preterm have been reported to have twice the incidence of autism at 10 years of age (Schendel and Bhasin, 2008). Parent-reported mental health rates in the United States are also higher for ex-preterm children than the general population for children and adolescents, with a prevalence of 22.9% compared to 15.5% in the general pediatric population (Singh et al., 2013). This study also revealed that ex-preterm children have 61% higher risk of having serious emotional/behavioral problems; specifically, a 33% higher chance of developing depression and a 58% higher chance of developing anxiety in childhood and adolescence (Singh et al., 2013).

School-related problems also arise in children following preterm birth, with those born preterm needing more special educational support, having an increased risk of repeating a grade and lower overall reading and mathematics scores compared to children born at full term (Chyi et al., 2008). These findings appear to be consistent internationally with numerous cohort studies observing that moderate-late ex-premature children have a 1.3- to 2.8-fold increased risk for requiring special education, and a 1.3- to 2.2-fold increased risk of repeating grades at ages 5–10 (Huddy et al., 2001; Morse et al., 2009; van Baar et al., 2009; Gurka et al., 2010). Furthermore, another study identified reading, writing, and spelling difficulties in 9- to 11-year-old ex-premature children compared to those born full term (Kirkegaard et al., 2006). Even at just 3–4 years of age impairments to visuospatial processing, spatial working memory, and sustained attention have been documented following preterm birth where major neurological deficits were not present at the time of birth (Vicari et al., 2004).

The direct impact of preterm birth on cognitive function is hard to quantify as it is confounded by many of the complex socio-economic, environmental and other factors that precipitated preterm birth in the first place. Additionally, given the plasticity of the developing brain, the timing of cognitive assessment needs to recognize the prognostic limitations of early assessment, especially for those born at extremes of gestational age. Studies comparing cognitive delays in toddlers at 2 years of age do not find any significant difference between preterm and term when corrected for prematurity (Cheatham et al., 2006; Darlow et al., 2009; Romeo et al., 2010; Woythaler et al., 2011). Alternatively, studies based in Swedish, American, and French cohorts found that 5- to 10-year old ex-premature children are twice as likely to score <85 on an IQ (intelligence quotient) test than term born children and that this is correlated with the gestational age at birth, with those being born more preterm at the highest risk of severe cognitive impairments (Schermann and Sedin, 2004; Marret et al., 2007; Talge et al., 2010). However, a much larger and comprehensive longitudinal American study in late-preterm ex-preterm 4- to 15-year olds found no significant differences in IQ based on 11 different cognitive tests at every age group (Gurka et al., 2010). These results suggest that intellectual disability may not be as prevalent following late-preterm birth as other negative outcomes such as behavioral disorders and poor school performance, suggesting that poor school performance may reflect behavioral disruptions that impact ability to pay attention and learn during class, rather than a result of reduced cognitive capacity.

EXPOSURE TO THE *ex utero* ENVIRONMENT AND ASSOCIATED DAMAGE

Preterm birth abruptly removes the newborn from the supportive *in utero* environment experienced by a fetus of the same post-conceptional age. Organ maturation and function throughout the body changes tempo dramatically in response to this premature separation from the maternoplacental unit. Brain development requires neurotrophic and gliotrophic support during this time and so is vulnerable following preterm birth as it loses placental steroid support, the supply of precursors for fetal neurosteroid production, and other placentally supplied nutrients. In addition, premature loss of these steroids exposes the developing brain to increased stimulation and excitotoxic damage. Damage to oligodendrocytes of the developing nervous system can occur during this vulnerable developmental window prior to term gestation.

The oligodendrocyte development lineage is sensitive to premature exposure to the external environment, leading to injury by chemical and mechanical damage. This involves increased levels of reactive oxygen species following the rise in excitation after preterm birth and early exposure to the *ex utero* environment (Antony et al., 2004; Blasko et al., 2009). Demyelinated regions in relapsing remitting multiple sclerosis undergo remyelination but residual impaired motor coordination may remain (Dutta et al., 2011). Similarly, after preterm birth myelination continues and animal studies have shown less marked deficits at the equivalent of childhood, compared to the reduced myelination seen at term equivalent age. Despite this 'catch up' *ex utero* myelination, these children experience impaired learning ability and motor coordination, suggesting a similar causal pathway (Rees and Inder, 2005). Furthermore, reductions in myelination are apparent in a rat model of ADHD (Lindahl et al., 2008), and decreases in the white matter volumes of vulnerable regions such as the hippocampus and cerebellum are evident on magnetic resonance imaging (MRI) comparing term and preterm neonates (Counsell et al., 2003).

TREATMENT OPTIONS FOR PREVENTING POOR NEUROLOGICAL OUTCOMES

In an effort to ameliorate or prevent preterm-associated brain damage a number of therapies have been adopted. However, despite the increasing body of evidence highlighting the increased neurodevelopmental vulnerability at all gestational ages below full term (39–40 weeks' gestation) no targeted therapies are available in the perinatal period to those infants born late preterm (34–36 weeks). For the less mature infants, maternal magnesium sulfate has been shown to reduce cerebral palsy in extreme preterm neonates, but the number needed to treat remains high, highlighting the need for other therapeutic approaches. A Cochrane systematic review of five large trials comprising 6,145 babies found that the incidence of cerebral palsy in preterm neonates dropped from 5 to 3.4% following antenatal magnesium sulfate therapy (Doyle et al., 2009). A recent study where magnesium sulfate was given between 6 days and 12 h before unilateral hypoxic ischemia in neonatal rats identified that maximal neuronal protection was achieved by treatment only 24 h before the insult (Koning et al., 2017), which may be sufficient in some instances of preterm birth. Although promising, a limitation of this therapy is the need for antenatal rather than postnatal treatment, especially considering that more than 50% of preterm births are spontaneous and thus antenatal therapy cannot be initiated with appropriate timing. Human and animal studies have demonstrated a lack of neurological improvement following postnatal magnesium sulfate therapy in the context of chorioamnionitis induced preterm birth and asphyxia associated with preterm labor (Kamyar et al., 2016; Galinsky et al., 2017), thus although magnesium sulfate offers some therapeutic benefit, it is not, in itself sufficient to reduce preterm-related morbidity and rather may be suitable as an adjunct therapy.

The antioxidant melatonin has also been investigated for its neuroprotective benefits in animal models due to its roles in modulating neuroinflammation and reducing reactive oxygen species (Colella et al., 2016). In neonatal stroke and hemorrhage rat models pre-treatment with melatonin reduced the neuroinflammation and damage associated with stroke, whilst post-treatment reduced the amount of tissue death and improved cognitive and sensorimotor outcomes (Lekic et al., 2011; Villapol et al., 2011). However, despite entering clinical trials there are few conclusive results available, with a Cochrane systematic review finding no randomized trials published as yet (Wilkinson et al., 2016). Therefore, the long-term benefit of this treatment for neurobehavioral outcomes awaits the result of further randomized control trials.

Controlled therapeutic hypothermia in late preterm and term infants with hypoxic ischaemic encephalopathy has demonstrated well-established benefits, such as reduced mortality and decreased long-term neurodevelopmental disability, if implemented within 6 h of the insult occurring (Jacobs et al., 2013; Laptook, 2017). The physiological instability and vulnerability of the preterm infant means that therapeutic hypothermia is unlikely to be an appropriate intervention in this cohort. Even a small decrement in gestational age (to 34–35 week GA infants with HIE) at initiation of hypothermia was associated with an increase in over-cooling (Laptook, 2017) and other hypothermia-associated complications in 90% of the preterm group versus 81.3% in the term cohort (Rao et al., 2017). In this small retrospective cohort study, 66.7% of the preterm neonates that received hypothermia therapy had evidence of white matter injury, whilst just 25% of the term neonates with HIE showed signs of white matter injury following an asphyxial insult managed with therapeutic hypothermia. These results are difficult to interpret, however, due to the lack of a normothermic preterm-control group. Importantly, all deaths following the hypothermia therapy were in the preterm group, highlighting their increased vulnerability compared to the term neonates. Similarly, a small retrospective cohort analysis between 2007 and 2015 of preterm infants 33–35 weeks' gestation who

received whole body hypothermia revealed that 50% experienced mortality or moderate to severe neurodevelopmental impairment as a result of the therapy (Herrera et al., 2018). Currently there is an ongoing clinical trial implementing whole-body cooling in American preterm neonates born at 33–35 GA with moderate to severe neonatal encephalopathy, but as it is still in the recruiting stage results are not yet available (*ClinicalTrials.gov Identifier: NCT01793129*). The American Academy of Pediatrics committee advises that hypothermia should not be undertaken on preterm neonates due to the associated risks, unless it is performed in a research setting (Committee et al., 2014). These findings suggest that hypothermia may limit key developmental processes in the immediate postnatal period and this may limit its use in all but late preterm neonates, pending the outcome of the current clinical trial. Thus, the development of further adjunct therapy seems essential to improving neurodevelopmental outcome in the preterm infant.

PLACENTAL CONTRIBUTION TO *in utero* BRAIN DEVELOPMENT

The placenta plays an essential role in ensuring fetal neurodevelopment occurs correctly by secreting growth regulating factors including neurosteroid hormones throughout pregnancy (**Figure 1**). Neurosteroids are endogenous steroids that rapidly alter neuronal excitability through interaction with ligand-gated ion channels and other cell surface receptors. In late gestation, the fetus is maintained in a 'sleep-like' state, characterized by low levels of arousal-like activity. This ensures that excitation of the brain is limited, engendering a level of protection from excessive excitation and ultimately allowing sufficient energy for demanding developmental processes such as myelination to occur (Nicol et al., 1998; Nguyen et al., 2003). This fetal 'sleep' state is maintained by an elevated level of the neurosteroid allopregnanolone, and decreasing the synthesis of this neurosteroid has been shown to increase the excitation of the brain, potentially disrupting or delaying brain developmental processes (Yawno et al., 2007; Kelleher et al., 2011b). A reduction in the normal fetal neurosteroid environment is thus associated with adverse outcomes, such as the occurrence of potentially damaging seizures which can lead to destructive and permanent alterations in neurodevelopment (Yawno et al., 2011). Following preterm birth there is a premature reduction in the supply of neurosteroids, including progesterone and its neuroactive metabolite allopregnanolone, resulting in an already vulnerable premature neonate being exposed to the *ex utero* environment without neuroprotection.

Importance of the Fetal Neurosteroid Allopregnanolone for Brain Development

Reductions in white matter is suggested to be a key component in the development of neurobehavioral disorders in children that are born preterm (Rees and Inder, 2005) and may stem from the birth-associated loss of allopregnanolone, as the pro-myelinating effects of this neurosteroid are evident *in vitro* on rat cerebellar slice cultures (Ghoumari et al., 2003). Allopregnanolone induced protection against cell death has been demonstrated in an *in vivo* mouse model of neurodegeneration (Liao et al., 2009) and in a sheep model of acute fetal hypoxia which is also important in maintaining levels of mature myelination oligodendrocytes (Yawno et al., 2007). Allopregnanolone is metabolized by the rate limiting enzymes 5α-reductases type 1 and 2 (5αR1 and 2) from progesterone (**Figure 1**) (Martini et al., 1996; Mellon and Griffin, 2002). In addition to the allopregnanolone supplied by the placenta to the fetus, the fetal brain is also capable of metabolizing allopregnanolone from placentally derived precursors including progesterone and 5α-dihyroprogesterone (5α-pregnane-3,20-dione), thus there is also a high level of allopregnanolone locally produced and maintained within the fetal brain (Stoffel-Wagner, 2001; Nguyen et al., 2004). However, we have previously shown in the developmentally relevant guinea pig (Morrison et al., 2018), a precocial species with similar hormonal profile to humans throughout pregnancy, that following the loss of the placenta at birth both progesterone and allopregnanolone levels decline rapidly within 24 h, highlighting the necessity of the placenta for the supply of steroidogenic precursors (Kelleher et al., 2013). Both of the rate-limiting enzymes 5αR1 and 2 are expressed in the placenta, and sheep and rat studies show that the 5αR2 isoform is most strongly expressed on neurons and glia within the developing fetal brain in late gestation (Martini et al., 1996; Nguyen et al., 2003).

Birth-associated loss of gestational allopregnanolone concentrations occurs earlier than normal in neonates that are born preterm leading to a damaging increase in excitation. Recent studies by our group have shown there is a dramatic drop in brain allopregnanolone concentrations following term and preterm birth compared to fetal levels (Kelleher et al., 2011a, 2013). Furthermore, preterm delivered animals also had significantly decreased myelination (evidenced by reduced MBP expression) in the CA1 region of the hippocampus and adjacent subcortical white matter 24 h after delivery compared to animals delivered at term (Kelleher et al., 2013). We have also shown that preterm male and female neonates at term equivalence age exhibit deficits in MBP immunostaining of the CA1 region, subcortical white matter and posterior lobe of the cerebellum (Kelleher et al., 2013; Palliser et al., 2015), and that juvenile offspring present with lasting deficits in myelination of these regions in male and female guinea pigs (Shaw et al., 2016, 2017). Likewise, reduced allopregnanolone supply as a result of intrauterine growth restriction and also impairs myelin development of the CA1 in male fetuses (Cumberland et al., 2017b). We have found that the late developing cerebellum is particularly vulnerable to the insults associated with preterm delivery. In addition to the CA1 region of the hippocampus, reductions in myelination of the posterior lobe of the cerebellum were evident in preterm guinea pig neonates at PND1 (Shaw et al., 2015). Furthermore, at term equivalence age we have demonstrated that not only is the expression of the level of mature oligodendrocytes reduced, but also that reductions are present throughout the oligodendrocyte lineage thereby lessening the potential of catch-up growth to occur (Palliser et al., 2015). By juvenile age we further observed that there were

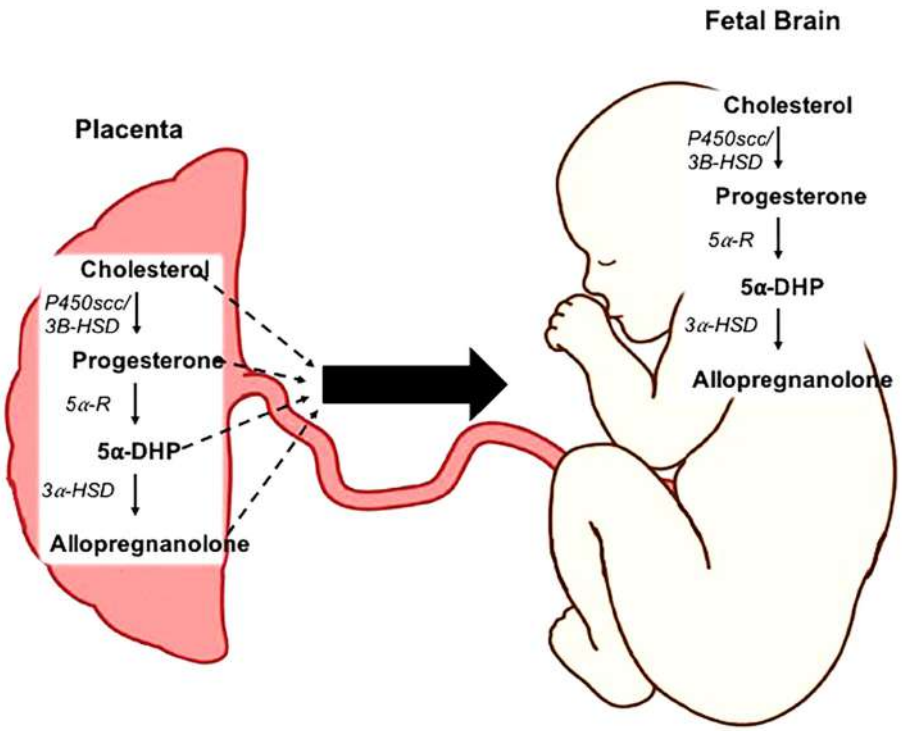


FIGURE 1 | Neurosteroidogenesis in the placenta and fetal brain. Cholesterol is metabolized into progesterone by the enzymes cholesterol side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD). The rate-limiting enzymes 5α-reductase type 1 and 2 (5α-R) facilitate the conversion of progesterone into 5α-dihydroprogesterone (5α-DHP). Allopregnanolone is then synthesized from this precursor by 3α-hydroxysteroid dehydrogenase (3α-HSD). This process can occur both within the placenta, and *de novo* within the fetal brain.

sex dependent alterations in myelination of the posterior lobe of the cerebellum as well as in components of the GABAergic pathway (Shaw et al., 2017). Functional imaging studies suggest that the posterior lobe of the cerebellum is particularly involved in cognition and emotion (Stoodley, 2012), as it is interconnected with the prefrontal cortex, association cortices, and the limbic system, which allows for its involvement in higher order executive functioning (Stoodley and Schmahmann, 2010). Therefore, the altered development of this area may be having a role in some of the neurobehavioral disorders that are more common following premature birth, such as ADHD and autism.

Our studies indicate that juvenile males show a hyperactive phenotype following preterm birth (Shaw et al., 2016). Additionally, they exhibit behavior similar to that observed in mouse models of ADHD where, as with our study, within open field test conditions the spontaneous distance traveled, and time spent mobile is markedly higher for the affected mice compared to the controls (Kim et al., 2014). This hyperactive behavior has parallels with clinical studies where ex-preterm male children show an increased incidence of hyperactivity disorders (Linnet et al., 2006; Lindstrom et al., 2011). Taken together, these data emphasize the importance of allopregnanolone for myelination and optimal development of the GABAergic system to occur in fetal and neonatal life. We therefore speculate that the changes in neurodevelopmental and behavioral function we see following preterm birth may be accounted for by the loss of allopregnanolone supply.

Pharmacological Reduction of the *in utero* Neurosteroid Environment

The deficits in myelination seen following preterm birth can be mimicked by the administration of a 5α-reductase inhibitor, finasteride, directly to the fetal circulation preventing the metabolism of progesterone to allopregnanolone within the fetal brain. This intervention results in an increase of damaging excitation in the brain of fetal sheep due to reduced suppression by allopregnanolone (Nicol et al., 2001). As a result of this excitation, cell death is increased in areas such as the hippocampus, cerebellum, and white matter tracts. In another study in fetal sheep, allopregnanolone synthesis was reduced through inhibition of progesterone production by trilostane (a 3β-hydroxysteroid dehydrogenase inhibitor). This resulted in reduced fetal sleep-like behavior but increased arousal-like activity (Crossley et al., 1997), resulting in increased brain excitability and damaging seizures (Mirmiran, 1995; Nicol et al., 1997). Furthermore, when progesterone was replaced by exogenous supplementation, the occurrence of sleep-like behavior returned to normal fetal patterns (Crossley et al., 1997). Exposure to finasteride has also been shown to increase apoptotic cells in the CA1 and CA3 regions of the hippocampus, and

the cerebellar molecular and granular layers in fetal sheep, as well as increasing the number of dead Purkinje cells in the cerebellum (Yawno et al., 2009). Importantly, co-infusion of finasteride and the allopregnanolone analog alfaxolone completely ameliorated the deleterious effects of finasteride treatment. Similarly allopregnanolone itself has also been shown to protect the fetal brain when insults occur, in a sheep model the introduction of brief asphyxia in the presence of finasteride induced cell death in the hippocampus, however, when allopregnanolone was present in normal concentrations this asphyxia- induced damage did not occur (Nicol et al., 2001). *In utero* administration of finasteride to guinea pigs has also highlighted the key role of allopregnanolone in myelination, as a reduction in myelination in the subcortical white matter was present following inhibition of allopregnanolone synthesis (Kelleher et al., 2011b). Interestingly, administration of the allopregnanolone precursor, progesterone, to *in vitro* rat cerebellum slices increased both the proliferation of myelinating oligodendrocytes and the rate of myelination (Ghoumari et al., 2003). Follow up studies then revealed that this effect was achieved by neurosteroids acting on the $GABA_A$ receptors (Ghoumari et al., 2005). Together these studies emphasize the important role of allopregnanolone in not just the development of the brain, but also for protection from hypoxia (**Figure 2**).

A reduction in allopregnanolone concentrations during pregnancy can also have long-lasting effects on the offspring. In guinea pigs, late gestation maternally administered finasteride resulted in an anxiety-like phenotype in female offspring, along with reductions in components of the GABAergic pathway within the amygdala (Cumberland et al., 2017a). Furthermore, there was also decreased expression of neurosteroid-sensitive $GABA_A$ receptors and increased astrocyte activation within the cerebellum of these animals (Cumberland et al., 2017c). In a similar study, finasteride treatment to pregnant rats during late gestation resulted in increased serum corticosterone concentrations in their juvenile offspring, decreased hippocampal allopregnanolone levels and impaired performance in memory tasks (Paris et al., 2011). Studies inhibiting the production of allopregnanolone in adult rats highlight the importance of allopregnanolone for the prevention of neurodevelopmental disorders throughout life as reductions in the concentration of allopregnanolone within the hippocampus (Frye and Walf, 2002) or the amygdala (Walf et al., 2006) increased anxiety-like behaviors in these animals. Furthermore, multiple neurological conditions are characterized by a reduced level of circulating allopregnanolone in adults, including post-traumatic stress disorder (Rasmusson et al., 2006), major depressive disorder (Strohle et al., 1999), and premenstrual dysphoric disorder (Monteleone et al., 2000; Lombardi et al., 2004).

Combined Effect of Reduced Neurosteroid Exposure and Increased Cortisol

An underlying factor involved in the development of hyperactivity and anxiety following preterm birth may be increased cortisol. In our studies we have observed increased circulating cortisol levels in preterm offspring at birth (Shaw et al., 2015), PND1 (Shaw et al., 2015), and juvenility (Shaw et al., 2016). In humans, one study has found that as birth weight and gestational age decreases, there is an increase in circulating cortisol (Kajantie et al., 2002), and early life stress has also been shown to negatively impact hippocampal development with long-term effects into adolescence (Hodel et al., 2015). Interestingly at juvenility, we found that male preterm offspring had increased baseline concentrations of circulating cortisol that were unaffected by exposure to foreign situations (in the form of behavioral testing). Meanwhile, juvenile females born preterm experienced a substantial rise in cortisol in response to foreign situations compared to term-born females, suggesting that they have an anxious phenotype and increased fear response (Shaw et al., 2016). These data highlight the sexually dimorphic effects that preterm birth has on programming of the hypothalamic pituitary axis, with a blunting of the stress response following preterm birth in males, but an increased response in females. Previous studies in guinea pigs suggest that prenatally increased cortisol may program adverse behavior in childhood, for example maternal stress exposure in pregnancy was shown to result in increased anxious behaviors in juvenile female offspring (Bennett et al., 2015). This is consistent with studies showing that prenatal stresses 'programs' the HPA axis (Kapoor and Matthews, 2005, 2008; Kapoor et al., 2006). This results in a greater postnatal sensitivity of the HPA axis to stressful stimuli, in turn contributing to behavioral disorders. The programming mechanism has been shown to be mediated by changes at the level of the hypothalamus (Kapoor and Matthews, 2005, 2008;

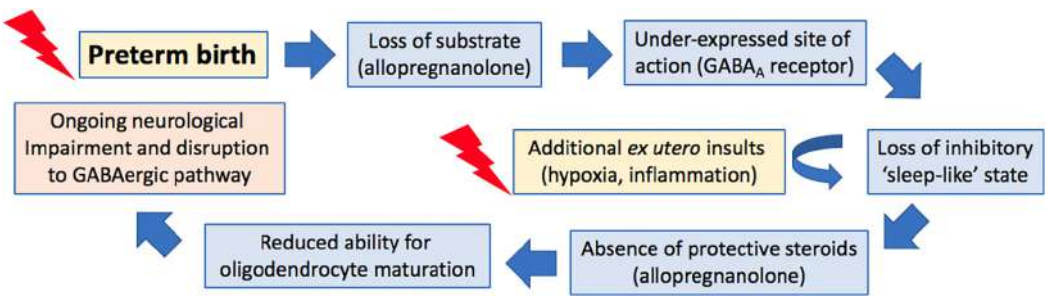


FIGURE 2 | Proposed cascade of events following preterm birth that lead to ongoing neurological impairments.

Kapoor et al., 2006). Therefore, even in the absence of a parallel change in postnatal cortisol concentrations, early exposure to increased cortisol concentration can program an altered behavioral response to stress-inducing situations.

These behavior-altering effects of cortisol may also involve interactions between cortisol and allopregnanolone. Glucocorticoids, such as cortisol, are known to adversely affect allopregnanolone production. Studies in guinea pigs have previously demonstrated that repeated administration of betamethasone (a synthetic glucocorticoid) to pregnant dams reduced the allopregnanolone synthesizing capacity of both the placenta and the fetal brain as demonstrated by a reduction in the expression of the rate-limiting enzyme 5α-reductase type 2 in both tissues (McKendry et al., 2009). Interestingly expression of this enzyme is also decreased in the brain of preterm guinea pig neonates (Kelleher et al., 2013), possibly as a result of exogenous glucocorticoid exposure, part of the gold standard treatment to reduce short-term morbidity and mortality following preterm birth. Our studies have also shown that both late gestation maternal stress and pharmacological inhibition of allopregnanolone synthesis by finasteride result in a reduction of allopregnanolone concentrations in the fetus, with development of an anxious phenotype in female juvenile offspring (Bennett et al., 2015; Cumberland et al., 2017a). In light of these data and the findings of the studies presented here we suggest that in addition to the lack of protection of allopregnanolone against excitotoxic damage, and the raised levels of cortisol present following early exposure to the *ex utero* environment, that cortisol hinders the synthesis and action of any offspring derived allopregnanolone in the preterm neonate and that this has lasting implications on neurodevelopment and behavior (**Figure 2**).

NEUROSTEROIDS AND THE EXTRA SYNAPTIC $GABA_A$ RECEPTOR

Inhibitory allopregnanolone exert effects throughout the brain to suppress excessive excitation. This suppression is achieved by increasing GABAergic inhibition (Herd et al., 2007). Allopregnanolone is an allosteric agonist of the $GABA_A$ receptors and specifically enhances $GABA_A$ receptor mediated inhibition, which results in anxiolytic, anti-convulsant, anesthetic, analgesic, and sedative effects (Harrison and Simmonds, 1984; Harrison et al., 1987; Lambert et al., 1987; Majewska, 1992; Paul and Purdy, 1992; Olsen and Sapp, 1995; Belelli and Lambert, 2005). These effects are achieved by activation of the extra synaptic receptors, which are known to be particularly sensitive to allopregnanolone. $GABA_A$ receptors form a gated chloride ionophore channel and specific binding sites for benzodiazepines, barbiturates, and anesthetics, however, neurosteroids are thought to bind to a separate allosteric steroid-binding site (Delaney and Sah, 1999; Macdonald and Botzolakis, 2009). $GABA_A$ receptors exhibit inhibitory effects in response to neurosteroid stimulation in adult animals and from mid gestation onward in the fetus, however, they are also capable of exhibiting excitatory actions in early gestation and these excitatory actions are known to stimulate glial cells and neuronal outgrowth (Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005). Whether the effect is inhibitory or excitatory is determined by the chloride gradient of the receptor-ionophore, determined by the intracellular chloride concentration. This in turn is primarily regulated by the K^+/Cl^- co-transporter-2 (KCC2) (Rivera et al., 1999, 2005). The expression and activity of this integral co-transporter is regulated by the phosphorylation of its Ser940 residue, with dephosphorylation resulting in downregulation of the co-transporter, increasing the intracellular chloride concentration, and switching to excitatory GABA actions (Lee et al., 2007; Lee et al., 2011).

$GABA_A$ receptors are involved in a broad range of functions including controlling the excitability of the brain, modulation of anxiety, as well as cognition, memory, and learning (Sieghart et al., 1999). In addition to neurons, extra synaptic neurosteroid sensitive receptors are highly expressed on glial cells including oligodendrocytes (Arellano et al., 2016) throughout the fetal brain from mid-gestation onward (Williamson et al., 1998; Crossley et al., 2000; Hirst et al., 2008). The expression of $GABA_A$ receptors in the fetal brain increases as gestation advances, reaching their highest levels of expression by full term gestation in most areas, such as the cerebral cortex and hypothalamus (Crossley et al., 2000, 2003; Nguyen et al., 2003). $GABA_A$ receptors exist in a pentameric formation of 5 subunits with a central selective chloride anion channel. The five subunits come from a pool of 19 different subunits, α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3 and subunit composition varies greatly depending on the function of the receptor (Barnard et al., 1998; Belelli et al., 2009). Synaptic receptors, which are responsible for fast transmission, usually feature the α1-3, β1-3, and γ2 subunits (Essrich et al., 1998), whilst the extra synaptic receptors that contribute to tonic inhibition (Belelli et al., 2009) possess the α4-6 and δ subunits (Burgard et al., 1996). Rather than produce an increase in amplitude of miniature inhibitory postsynaptic currents (mIPSCs), neurosteroids have been shown to increase the duration of the amplification by altering the kinetics of the $GABA_A$-gated ion channels (Lambert et al., 2003). This increase in duration is neuron specific, with different brain regions requiring different concentrations of neurosteroids to induce the same effect. Specifically, the CA1 neurons of the hippocampus, cerebellar granule cells, and Purkinje cells appear to be more sensitive to neurosteroids, only requiring low nanomolar concentrations to increase duration of amplification (Harney et al., 2003; Cooper et al., 2004), and this is primarily due to subunit composition.

Receptor subunit composition plays an important role in determining receptor affinity for various ligands. Benzodiazepines for example are known to be attracted to receptors containing a γ subunit, whilst those featuring α6 are unresponsive to benzodiazepines (Delaney and Sah, 1999). Whilst there is a specific binding site for 3α-hydroxy-neurosteroids such as allopregnanolone, the composition of subunits affects the sensitivity of the receptor to stimulation (Belelli et al., 2002; Hosie et al., 2007). Regional specificity also exists for these receptors, for example in a mouse knockout of

the δ subunit tonic conductance was significantly reduced in the cerebellum, however, in the CA1 region of the hippocampus there was no effect on conductance (Stell et al., 2003). This regional specificity is due to differences in expression of various subunits throughout the brain and whilst the α6 and δ subunits, which are co-expressed in many receptors, are highly expressed in the cerebellum, tonic conductance in the hippocampus is controlled primarily by receptors containing the α4 and α5 subunits, in addition to those containing the δ subunit.

The role of specific neurosteroid-sensitive subunits in behavior has been revealed in knockout mouse models. For example, global deletion of the δ subunit significantly reduces the anxiolytic and anti-convulsant effects induced by the allopregnanolone analog ganaxolone, confirming that neurosteroids bind to the δ subunit containing $GABA_A$ receptors to exert their inhibitory functions (Mihalek et al., 1999). Increased anxiety-like behavior was also present in a α4 subunit knockout mice as demonstrated by an increased preference for dark enclosed spaces in a T-maze (Loria et al., 2013). Seizure susceptibility has also been shown as increased following this knockout (Chandra et al., 2008). Similarly, it has been demonstrated that pro-epileptic behavior is increased in mice lacking the δ subunit (Mihalek et al., 1999; Spigelman et al., 2002, 2003). Taken together these data indicate the importance of configurations of the $GABA_A$ receptors and the necessity of the expression of key subunits for neurosteroid binding and for their effects on behavior.

Of particular importance to preterm-associated neurodevelopmental impairment is the ability of allopregnanolone to promote $GABA_A$ receptor-mediated maturation of oligodendrocytes. Administration of progesterone to rat cerebellar slice cultures increased the expression of the mature myelinating oligodendrocyte marker, myelin basic protein (MBP) (Ghoumari et al., 2003). The enhancement of myelination was achieved by allopregnanolone, the neuroactive metabolite of progesterone, acting via the $GABA_A$ receptors located on oligodendrocytes as a selective $GABA_A$ receptor antagonist inhibited this promyelinating effect.

$GABA_A$ RECEPTORS AND PRETERM BIRTH

In juvenile guinea pigs born preterm, altered GABAergic pathway development is evident at juvenility in the cerebellum. Intriguingly, despite reduced expression of both subunits in the preterm neonatal cerebellums (**Figure 3**) (Shaw et al., 2015) mRNA expression of allopregnanolone sensitive $GABA_A$ receptor subunits α6 and δ are not altered in these preterm-born animals at juvenility (Shaw et al., 2017). These observations suggest that sometime between birth and juvenile age in the guinea pig (PND28) there is either a 'catch-up' in these key $GABA_A$ receptor subunits expression, or conversely, that levels in the brain of term born animals have dropped to lower levels. Subunits of the $GABA_A$ receptor are reported to go through age-related changes in expression, with early development often a period of high expression, followed by down regulation in adulthood (Yu et al., 2006). This age-related change in expression follows the maturation profile of the brain and therefore if the neurosteroid-sensitive receptors in the preterm brain do not undergo any 'catch-up' between birth and juvenility this may contribute to preterm-associated changes in neurodevelopment. An additional vulnerability that has been reported for preterm neonates is an observed lack of a birth-related adaptive increase in the cerebellar expression of the α6 and δ $GABA_A$ receptor subunits after birth (**Figure 2**) (Shaw et al., 2015). This potentially reduces the effect of allopregnanolone postnatally, exposing the immature brain to damaging excitotoxicity. Knockout studies of the δ subunit, which is known to commonly group with the α6 subunit, have shown a link between a lack of these subunits with the manifestation of multiple neurodevelopmental phenotypes such as anxiety-like behavior and pro-epileptic behavior (Mihalek et al., 1999; Spigelman et al., 2002, 2003). Interestingly, receptor

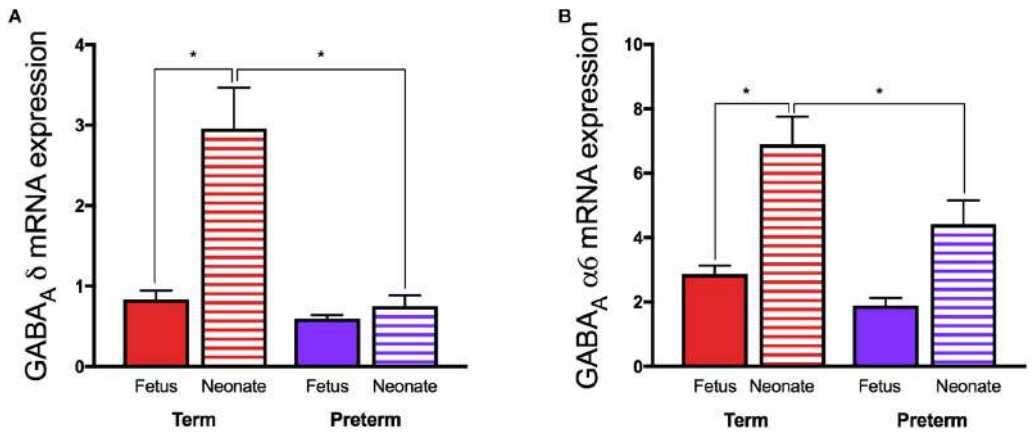


FIGURE 3 | Relative mRNA expression of the $GABA_A$ receptor **(A)** δ and **(B)** α6 subunits in guinea pig cerebellum. Fetal tissue was obtained at GA69 (term) and GA62 (preterm) ages, and neonatal tissue from 24 h after term or preterm birth. (*$p < 0.05$, $n = 11–16$). Adapted and reprinted with permission from Shaw et al. (2015).

changes are present in human brain tissue in disorders that primarily affect myelination, such as multiple sclerosis (Luchetti et al., 2011), and whilst their precise role in disease progression is unknown, the neurosteroid sensitive $GABA_A$ receptors present a common link between initial myelination, 'catch up' and remyelination processes, and behavioral state.

Conversely, the hippocampal $GABA_A$ neurosteroid sensitive receptor subunits appear to be largely unaffected by preterm delivery with the exception of a decrease in the expression of the α5 subunit mRNA at juvenility (Shaw et al., 2016). This particular subunit is known to mediate tonic inhibition in the CA1 of the hippocampus, is required for associative learning and furthermore is known to be reduced in response to increased levels of cortisol (Crestani et al., 2002; Verkuyl et al., 2004; Glykys and Mody, 2006). Thus, a reduction in α5 subunit expression in childhood may reduce tonic inhibition, thereby increasing excitation in the hippocampus, which in turn may contribute to the risk of hyperactivity-disorders in male children born preterm.

POTENTIAL OF NEUROSTEROIDS AS A PROTECTIVE THERAPY

Steroid hormones, including progesterone, allopregnanolone and potentially other neuroactive metabolites, can exert neuroprotective effects following damage to neurons and glia by preventing excitation, apoptosis, and inflammation, as well as by regenerative mechanisms (Schumacher et al., 2004). Studies in adult rats have demonstrated the therapeutic effect of progesterone injections on TBI where progesterone administration reduced neuronal loss (Roof et al., 1994, 1996; He et al., 2003). Similarly, allopregnanolone administration was shown to reduce memory deficits and loss of neurons in the frontal cortex of these rats following bilateral injury by stimulating trophic effects (He et al., 2003). Importantly, in rat astrocytes and oligodendroglial progenitor primary cell cultures, progesterone exposure upregulated expression of the promyelinating factor insulin-like growth factor 1

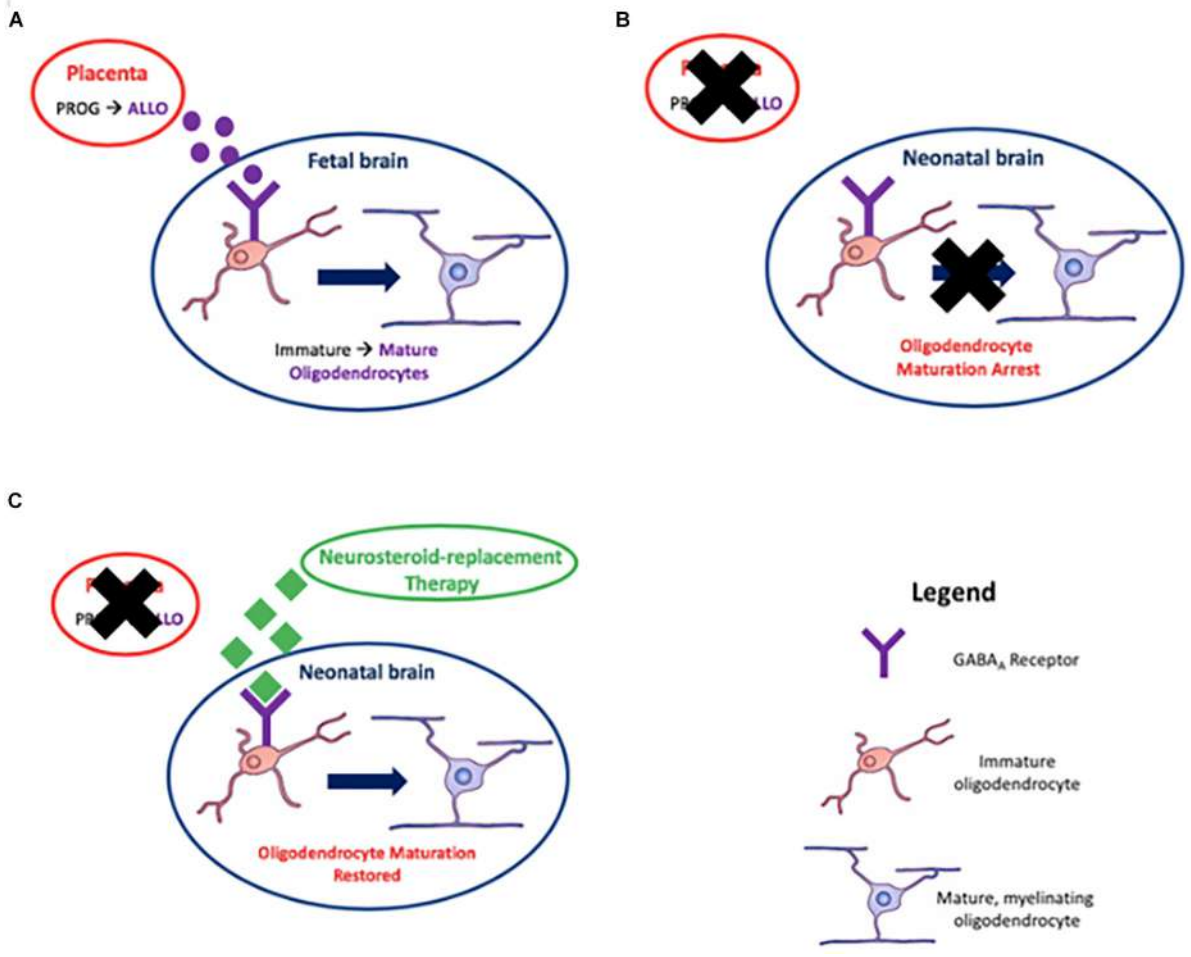


FIGURE 4 | Myelination, the process of surrounding nerve axons with a myelin sheath, is achieved by oligodendrocytes. Placentally derived allopregnanolone (ALLO), the neuroactive metabolite of progesterone (PROG), promotes maturation of oligodendrocytes *in utero* via action on $GABA_A$ receptors in **(A)**; premature loss of the placenta due to preterm birth results in an arrest in this process in **(B)**; and reinstating $GABA_A$ receptor signaling by neurosteroid-replacement therapy may restore oligodendrocyte maturation leading to correct myelination in neonates born preterm, thus improving neurological function in **(C)**.

(Chesik and De Keyser, 2010) and, in organotypic slice cultures of rat cerebellum, myelination was stimulated by progesterone following its metabolism into allopregnanolone and its' trophic actions mediated by actions on $GABA_A$ receptors (Ghoumari et al., 2003). Both progesterone and allopregnanolone have been shown to be effective at reducing the pro-apoptotic activity of caspase-3, reducing astrogliosis as evidenced by GFAP staining, and improving performance in both the spatial learning task and memory function in adult male rats (Djebaili et al., 2005). Furthermore, rat studies have identified reductions in inflammatory cytokines TNF-α and IL-1β following TBI and subsequent progesterone or allopregnanolone administration (He et al., 2004). Following the potential benefits of progesterone therapy observed in animal studies, a randomized phase III clinical trial of progesterone (ProTECT) for treatment of acute TBI in adults was performed. This showed that progesterone treatment resulted in a lower 30-day mortality risk, and that patients were more likely to have a moderate to good outcome than those receiving placebo (Wright et al., 2007). Likewise, a large clinical trial in China is showing similar therapeutic benefits following progesterone therapy (Xiao et al., 2008).

The role of progesterone as a precursor of allopregnanolone, and the number of positive studies relating to the use of progesterone, led to us examining the use of progesterone replacement therapy in preterm guinea pig neonates. In contrast to the earlier finding of effects on TBI in rats, we observed detrimental effects on postnatal neurodevelopment particularly in the male offspring. From this preliminary study, it appears that progesterone is metabolized differently by the male neonates and instead of producing allopregnanolone, much of the steroid is converted to cortisol. These males, with high plasma and salivary cortisol concentrations, also had reductions in myelination of the cerebellum and subcortical white matter, highlighting the vulnerability of these male neonates to increased cortisol as a result of increased postnatal progesterone (Palliser et al., 2015).

Previous studies have also investigated the potential use of allopregnanolone to restore neurosteroid deficits. Preliminary findings, however, suggested that allopregnanolone had limited effectiveness due to the very short half-life of allopregnanolone, or other possible metabolic conversion making therapeutic concentration difficult to achieve. To avoid both of these issues with allopregnanolone therapy, as well as potential conversion of allopregnanolone to its less active isomers, we explored a possible postnatal therapy with ganaxolone.

Ganaxolone

Ganaxolone is a 3β-methylated synthetic analog of allopregnanolone initially developed by Edward Monaghan at CoSensys in 1998, however, in 2004 Marinus Pharmaceuticals Inc., acquired the development and commercialization rights (Nohria and Giller, 2007). Marinus Pharmaceuticals then carried out a number of clinical trials using ganaxolone, some of which are still ongoing. Ganaxolone features a methyl group that prevents metabolism into other active steroids (Carter et al., 1997), and a half-life of 12–20 h in humans (Monaghan et al., 1997). Ganaxolone acts in a very similar manner to allopregnanolone and binds to the neurosteroid-binding site of $GABA_A$ receptors, producing similar anxiolytic and anti-seizure effects. The addition of the methyl group markedly improves oral pharmacokinetics and in addition ganaxolone is not readily metabolized to other steroids that may bind elsewhere and produce unwanted effects (Carter et al., 1997). Allopregnanolone can for example be metabolized into the 3β-isomer that is either inactive, or at higher doses, may block the steroid site on the $GABA_A$ receptor.

Animal pharmacokinetic studies demonstrate that ganaxolone has a large volume of distribution as administration of radioactively labeled ganaxolone has shown wide distribution, and due to its' lipophilic nature, it becomes concentrated in the brain with a brain-to-plasma concentration of between 5 and 10 (Nohria and Giller, 2007; Reddy and Rogawski, 2012). In addition to pharmacokinetic studies there have been a number of animal studies relating to the use of ganaxolone and behavioral disorders. In an adult mouse model of Angelman syndrome (which is characterized by severe developmental delay, motor impairments, and epilepsy) treatment with ganaxolone over a period of 4 weeks was shown to ameliorate behavioral abnormalities (Ciarlone et al., 2017). Other mouse models of neurodevelopmental disorders have highlighted the therapeutic benefits of ganaxolone, including an adult mouse model of autism where ganaxolone reversed the autistic phenotype (Kazdoba et al., 2016), and an adult post-traumatic stress mouse model where again ganaxolone therapy improved behavioral changes such as aggression and anxiety (Pinna and Rasmusson, 2014). Despite numerous animal models of behavioral disorders demonstrating the therapeutic potential of ganaxolone in ameliorating disease states, there is limited information regarding the effects on neurodevelopment or myelination in these models. There has been one model where administration of ganaxolone to Niemann-Pick Type C diseased adult mice identified protection against Purkinje cell death, which is similar to the previously reported protective mechanisms of allopregnanolone (Mellon et al., 2008). Furthermore, there has only been one neonatal animal study using ganaxolone therapy, in a rat model of infantile spasms where the onset, number, and duration of spasms were reduced by ganaxolone therapy (Yum et al., 2014). An additional study examining the neuroprotective effects of ganaxolone following neonatal seizures in sheep is ongoing but shows promise (Yawno et al., 2017).

A number of phase 2 clinical trials have examined the use of ganaxolone for epilepsy and infantile spasms, as well as for posttraumatic stress disorder, migraine, and the developmental problems associated with fragile X syndrome (Nohria and Giller, 2007; Reddy and Rogawski, 2012). Daily drug doses of up to 1,875 mg in adults and 54 mg/kg in children have been trialed, and it has been shown that a single oral dose of 1,600 mg can result in peak plasma concentrations of up to 460 ng/mL. Recently a randomized phase 2 trial for ganaxolone as an add-on therapy for severe seizure disorders took place in 147 adults (Sperling et al., 2017). The subjects received 1,500 mg/day spread over three doses for 8 weeks. The treatment resulted in an 18% decrease in mean weekly seizure frequency, compared to a 2% increase in the placebo group. The treatment was reported as safe and well tolerated with similar rates of discontinuation

due to adverse effects in the placebo and ganaxolone groups (ganaxolone 7.1% versus 6.1% for placebo). The most common side effects were classified as mild to moderate and included dizziness (16.3% versus 8.2% in placebo), fatigue (16.3% versus 8.2%), and somnolence (13.3% versus 2.0%).

In the context of preterm birth, we have recently reported that ganaxolone neurosteroid-replacement therapy given to preterm guinea pigs between birth and term 'due date' improved myelination of the CA1 region of the hippocampus and overlying subcortical white matter, in addition to reduction in hyperactive behavior (Shaw et al., 2018). This was the first study to show that neurosteroid-replacement therapy can replicate the *in utero* neurosteroid environment and that this restores neurodevelopment to a normal, term-born, trajectory (**Figure 4**). By combining our recent studies on pregnancy compromises in the developmentally relevant guinea pig (Morrison et al., 2018) and the impact of disturbances in allopregnanolone levels on the developing fetus, the preterm neonate, and the long-term effects on the juvenile, we now suggest that re-establishment of neurosteroid action in the period between birth and term equivalence is a prospective therapy for future clinical use. Whilst more studies required, particularly on optimal dosing and longer-term outcomes, we suggest this study provides the impetus and a path for future preclinical trials using neurosteroid-replacement therapy following preterm birth. Furthermore, this therapy may be useful following other pregnancy compromises discussed previously where a major contributing factor to deficits in neurodevelopment is a lack of allopregnanolone exposure.

CONCLUSION

Until recently, the risk of neurodevelopmental impairment in children born moderate-late preterm who required little to no clinical intervention, was thought to be minimal, however, data from large international cohorts clearly demonstrate that this is not the case. Albeit that the effect size is not as great as for those born at extremes of gestational age, the significantly larger number of children born at moderate-late preterm gestations means that this is an increasingly large public health issue, with important implications for the provision of educational and other resources throughout childhood. Currently, there are no targeted therapies available to prevent the development of these neurodevelopmental problems, and as such therapy is limited to symptom management for the most affected children.

Through use of studies in our model of preterm birth in the guinea pig we have begun to address these gaps in the knowledge of neurodevelopment following preterm birth. We suggest key pathways involved, targets for intervention, and a therapy for prevention of preterm-associated neurodevelopmental disorders. These studies are in their preliminary stages and whilst we have identified a target for improving outcomes, there are many aspects to this therapy that we are yet to investigate. Our pilot studies are primarily focused on identifying an optimal dose that promotes oligodendrocyte maturation but minimizes adverse side effects. Once we identify an ideal dose, we can then determine whether there are interactions with other therapies that the preterm neonate may be exposed to, such as synthetic glucocorticoids, and potentially in the future, for asphyxiated preterm infants, therapeutic hypothermia as a co-therapy.

AUTHOR CONTRIBUTIONS

JS: primary author. MB, RD, and GC: revisions and edits. JH and HP: co-senior authors, revisions and edits, and concept design.

FUNDING

This study was funded by the National Health and Medical Research Council (NHMRC) (Grant No. APP1003517) (Newcastle, Australia), by funds from the Department of Paediatrics and Child Health, University of Otago, Wellington (Wellington, New Zealand), and project grants awarded to MB from the University of Otago, The Neonatal Trust, and The Royal Australasian College of Physicians (Wellington, New Zealand). Financial support to JS was provided through an Australian Government Research Training Program Scholarship. RD was funded by a University of Otago Health Sciences Career Development Postdoctoral Fellowship.

REFERENCES

Ananth, C. V., and Vintzileos, A. M. (2006). Epidemiology of preterm birth and its clinical subtypes. *J. Matern. Fetal Neonatal Med.* 19, 773–782.

Antony, J., Van Marle, G., and Opii, W. (2004). Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. *Nat. Neurosci.* 7, 1088–1095. doi: 10.1038/nn1319

Arellano, R. O., Sanchez-Gomez, M. V., Alberdi, E., Canedo-Antelo, M., Chara, J. C., Palomino, A., et al. (2016). Axon-to-Glia interaction regulates GABAA receptor expression in oligodendrocytes. *Mol. Pharmacol.* 89, 63–74. doi: 10.1124/mol.115.100594

Arnold, S. E., Trojanowski, J. Q. (1996). Human fetal hippocampal development: I. Cytoarchitecture, myeloarchitecture, and neuronal morphologic features. *J. Comp. Neurol.* 367, 274–292.

Back, S. A., Han, B. H., Luo, N. L., Chricton, C. A., Xanthoudakis, S., Tam, J., et al. (2002). Selective vulnerability of late oligodendrocyte progenitors to hypoxia–ischemia. *J. Neurosci.* 22, 455–463. doi: 10.1523/jneurosci.22-02-00455.2002

Barnard, E., Skolnick, P., Olsen, R., Mohler, H., Sieghart, W., Biggio, G., et al. (1998). International Union of Pharmacology. XV. Subtypes of γ-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.* 50, 291–314.

Baron, I. S., Litman, F. R., Ahronovich, M. D., and Baker, R. (2012). Late preterm birth: a review of medical and neuropsychological childhood outcomes. *Neuropsychol. Rev.* 22, 438–450. doi: 10.1007/s11065-012-9210-5

Belelli, D., Casula, A., Ling, A., and Lambert, J. J. (2002). The influence of subunit composition on the interaction of neurosteroids with $GABA_A$ receptors. *Neuropharmacology* 43, 651–661. doi: 10.1016/s0028-3908(02)00172-7

Belelli, D., Harrison, N. L., Maguire, J., Macdonald, R. L., Walker, M. C., and Cope, D. W. (2009). Extrasynaptic GABAA receptors: form, pharmacology,

and function. *J. Neurosci.* 29, 12757–12763. doi: 10.1523/JNEUROSCI.3340-09.2009

Belelli, D., and Lambert, J. J. (2005). Neurosteroids: endogenous regulators of the GABAA receptor. *Nat. Rev. Neurosci.* 6, 565–575. doi: 10.1038/nrn1703

Bennett, G. A., Palliser, H. K., Shaw, J. C., Walker, D., and Hirst, J. J. (2015). Prenatal stress alters hippocampal neuroglia and increases anxiety in childhood. *Dev. Neurosci.* 37, 533–545. doi: 10.1159/000437302

Berry, M. J., Foster, T., Rowe, K., Robertson, O., Robson, B., and Pierse, N. (2018). Gestational age, health, and educational outcomes in adolescents. *Pediatrics* 74, 200–201. doi: 10.1097/01.ogx.0000554436.92583.c7

Blasko, I., Humpel, C., and Grubeck-Loebenstein, B. (2009). *Astrocytes and Oligodendrocytes During Normal Brain Ageing*. Oxford: Academic Press.

Burgard, E. C., Tietz, E. I., Neelands, T. R., and Macdonald, R. L. (1996). Properties of recombinant gamma-aminobutyric acid A receptor isoforms containing the alpha 5 subunit subtype. *Mol. Pharmacol.* 50, 119–127.

Caldinelli, C., Froudist-Walsh, S., Karolis, V., Tseng, C. E., Allin, M. P., Walshe, M., et al. (2017). White matter alterations to cingulum and fornix following very preterm birth and their relationship with cognitive functions. *Neuroimage* 150, 373–382. doi: 10.1016/j.neuroimage.2017.02.026

Carter, R., Wood, P. J., and Wieland, S. (1997). Characterization of the anticonvulsant properties of Ganaxolone (CCD 1042; 3α-Hydroxy-3β-methyl-5α-pregnan-20-one), a selective, high-affinity, steroid modulator of the γ-aminobutyric acida receptor. *J. Pharmacol. Exp. Ther.* 280, 1284–1295.

Chandra, D., Werner, D. F., Liang, J., Suryanarayanan, A., Harrison, N. L., Spigelman, I., et al. (2008). Normal acute behavioral responses to moderate/high dose ethanol in GABAA receptor alpha 4 subunit knockout mice. *Alcohol Clin. Exp. Res.* 32, 10–18. doi: 10.1111/j.1530-0277.2007.00563.x

Cheatham, C., Bauer, P., and Georgieff, M. (2006). Predicting individual differences in recall by infants born preterm and full term. *Infancy* 10, 17–42. doi: 10.1207/s15327078in1001_2

Cheong, J. L., and Doyle, L. W. (2012). Increasing rates of prematurity and epidemiology of late preterm birth. *J. Paediatr. Child Health* 48, 784–788. doi: 10.1111/j.1440-1754.2012.02536.x

Chesik, D., and De Keyser, J. (2010). Progesterone and dexamethasone differentially regulate the IGF-system in glial cells. *Neurosci. Lett.* 468, 178–182. doi: 10.1016/j.neulet.2009.10.051

Chyi, L. J., Lee, H. C., Hintz, S. R., Gould, J. B., and Sutcliffe, T. L. (2008). School outcomes of late preterm infants: special needs and challenges for infants born at 32 to 36 weeks gestation. *J. Pediatr.* 153, 25–31. doi: 10.1016/j.jpeds.2008.01.027

Ciarlone, S. L., Wang, X., Rogawski, M. A., and Weeber, E. J. (2017). Effects of the synthetic neurosteroid ganaxolone on seizure activity and behavioral deficits in an Angelman syndrome mouse model. *Neuropharmacology* 116, 142–150. doi: 10.1016/j.neuropharm.2016.12.009

Colella, M., Biran, V., and Baud, O. (2016). Melatonin and the newborn brain. *Early Hum. Dev.* 102, 1–3. doi: 10.1016/j.earlhumdev.2016.09.001

Committee, on Fetus and Newborn, Papile, L. A., Baley, J. E., Benitz, W., Cummings, J., Carlo, W. A., et al. (2014). Hypothermia and neonatal encephalopathy. *Pediatrics* 133, 1146–1150. doi: 10.1542/peds.2014-0899

Cooper, E. J., Johnston, G. A., and Edwards, F. A. (2004). Effects of a naturally occurring neurosteroid on GABAA IPSCs during development in rat hippocampal or cerebellar slices. *J. Physiol.* 521, 437–449. doi: 10.1111/j.1469-7793.1999.00437.x

Counsell, S., Rutherford, M., Cowan, F., and Edwards, A. (2003). Magnetic resonance imaging of preterm brain injury. *Arch. Dis. Child. Fetal Neonatal Ed.* 88, F269–F274.

Crestani, F., Keist, R., Fritschy, J. M., Benke, D., Vogt, K., Prut, L., et al. (2002). Trace fear conditioning involves hippocampal alpha5 GABA(A) receptors. *Proc. Natl. Acad. Sci. U.S.A.* 99, 8980–8985. doi: 10.1073/pnas.142288699

Crossley, K. J., Nicol, M. B., Hirst, J. J., Walker, D. W., and Thorburn, G. D. (1997). Suppression of arousal by progesterone in fetal sheep. *Reprod. Fertil. Dev.* 9, 767–773.

Crossley, K. J., Nitsos, I., Walker, D. W., Lawrence, A. J., Beart, P. M., and Hirst, J. J. (2003). Steroid-sensitive GABAA receptors in the fetal sheep brain. *Neuropharmacology* 45, 461–472. doi: 10.1016/s0028-3908(03)00196-5

Crossley, K. J., Walker, D. W., Beart, P. M., and Hirst, J. J. (2000). Characterisation of GABAA receptors in fetal, neonatal and adult ovine brain: region and age related changes and the effects of allopregnanolone. *Neuropharmacology* 39, 1514–1522. doi: 10.1016/s0028-3908(99)00222-1

Cumberland, A. L., Palliser, H. K., Crombie, G. K., Walker, D. W., and Hirst, J. J. (2017a). Increased anxiety-like phenotype in female guinea pigs following reduced neurosteroid exposure in utero. *Int. J. Dev. Neurosci.* 58, 50–58. doi: 10.1016/j.ijdevneu.2017.02.001

Cumberland, A. L., Palliser, H. K., Rani, P., Walker, D. W., and Hirst, J. J. (2017b). Effects of combined IUGR and prenatal stress on the development of the hippocampus in a fetal guinea pig model. *J. Dev. Orig. Health. Dis.* 8, 584–596. doi: 10.1017/S2040174417000307

Cumberland, A. L., Palliser, H. K., Walker, D. W., and Hirst, J. J. (2017c). Cerebellar changes in guinea pig offspring following suppression of neurosteroid synthesis during late gestation. *Cerebellum* 16, 306–313. doi: 10.1007/s12311-016-0802-0

Darlow, B., Horwood, L., Wynn-Williams, M., Mogridge, N., and Austin, N. (2009). Admissions of all gestations to a regional neonatal unit versus controls; 2 year outcome. *J. Paediatr. Child Health* 45, 187–193. doi: 10.1111/j.1440-1754.2008.01457.x

Delaney, A. J., and Sah, P. (1999). GABA receptors inhibited by benzodiazepines mediate fast inhibitory transmission in the central amygdala. *J. Neurosci.* 19, 9698–9704. doi: 10.1523/jneurosci.19-22-09698.1999

Djebaili, M., Guo, Q., Pettus, E., Hoffman, S., and Stein, D. (2005). The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and funcitonal deficits after traumatic brain injury in rats. *J. Neurotrauma* 22, 106–118. doi: 10.1089/neu.2005.22.106

Doyle, L. W., Crowther, C. A., Middleton, P., Marret, S., and Rouse, D. (2009). Magnesium sulphate for women at risk of preterm birth for neuroprotection of the fetus. *Cochrane Database Syst. Rev.* 1:CD004661. doi: 10.1089/neu.2005.22.106

Dutta, R., Chang, A., Doud, M. K., Kidd, G. J., Ribaudo, M. V., Young, E. A., et al. (2011). Demyelination causes synaptic alterations in hippocampi from multiple sclerosis patients. *Ann. Neurol.* 69, 445–454. doi: 10.1002/ana.22337

Elgen, S., Sommerfelt, K., Leversen, K., and Markestad, T. (2014). Minor neurodevelopmental impairments are associated with increased occurrence of ADHD symptoms in children born extremely preterm. *Eur. Child Adolesc. Psychiatry* 24, 463–470. doi: 10.1007/s00787-014-0597-9

Essrich, C., Lorez, M., Benson, J. A., Fritschy, J. M., and Luscher, B. (1998). Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin. *Nat. Neurosci.* 1, 563–571. doi: 10.1038/2798

Frey, H. A., and Klebanoff, M. A. (2016). The epidemiology, etiology, and costs of preterm birth. *Semin. Fetal Neonatal Med.* 21, 68–73. doi: 10.1016/j.siny.2015.12.011

Frye, C. A., and Walf, A. A. (2002). Changes in progesterone metabolites in the hippocampus can modulate open field and forced swim test behavior of proestrous rats. *Horm. Behav.* 41, 306–315. doi: 10.1006/hbeh.2002.1763

Galinsky, R., Draghi, V., Wassink, G., Davidson, J. O., Drury, P. P., Lear, C. A., et al. (2017). Magnesium sulfate reduces EEG activity but is not neuroprotective after asphyxia in preterm fetal sheep. *J. Cereb. Blood Flow Metab.* 37, 1362–1373. doi: 10.1177/0271678X16655548

Ghoumari, A. M., Baulieu, E. E., and Schumacher, M. (2005). Progesterone increases oligodendroglial cell proliferation in rat cerebellar slice cultures. *Neuroscience* 135, 47–58. doi: 10.1016/j.neuroscience.2005.05.023

Ghoumari, A. M., Ibanez, C., El-Etr, M., Leclerc, P., Eychenne, B., O'Malley, B. W., et al. (2003). Progesterone and its metabolites increase myelin basic protein expression in organotypic slice cultures of rat cerebellum. *J. Neurochem.* 86, 848–859. doi: 10.1046/j.1471-4159.2003.01881.x

Glykys, J., and Mody, I. (2006). Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABA A receptor alpha5 subunit-deficient mice. *J. Neurophysiol.* 95, 2796–2807. doi: 10.1152/jn.01122.2005

Goldenberg, R. L., Culhane, J., Iams, J., and Romero, R. (2008). Epidemiology and causes of preterm birth. *Lancet* 371, 75–84.

Gurka, M., Locasale-crouch, J., and Blackman, J. (2010). Long-term cognition, acheivment, socioemotional, and behavioural development of health late-preterm infants. *Arch. Pediatr. Adolesc. Med.* 164, 525–532. doi: 10.1001/archpediatrics.2010.83

Harney, S. C., Frenguelli, B. G., and Lambert, J. J. (2003). Phosphorylation influences neurosteroid modulation of synaptic $GABA_A$ receptors in rat CA1 and dentate gyrus neurones. *Neuropharmacology* 45, 873–883. doi: 10.1016/s0028-3908(03)00251-x

Harrison, N. L., Majewska, M. D., Harrington, J. W., and Barker, J. L. (1987). Structure-activity relationships for steroid interaction with the gamma-aminobutyric acidA receptor complex. *J. Pharmacol. Exp. Ther.* 241, 346–353.

Harrison, N. L., and Simmonds, M. A. (1984). Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res.* 323, 287–292. doi: 10.1016/0006-8993(84)90299-3

He, J., Evans, C., Hoffman, S., Oyesiku, N., and Stein, D. (2004). Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. *Exp. Neurol.* 189, 404–412. doi: 10.1016/j.expneurol.2004.06.008

He, J., Hoffman, S., and Stein, D. (2003). Allopregnanolone, a progesterone metabolite, enhances behavioural recovery and decreases neuronal loss after traumatic brain injury. *Restor. Neurol. Neurosci.* 22, 19–31.

Herd, M. B., Belelli, D., and Lambert, J. J. (2007). Neurosteroid modulation of synaptic and extrasynaptic GABA(A) receptors. *Pharmacol. Ther.* 116, 20–34. doi: 10.1016/j.pharmthera.2007.03.007

Herrera, T. I., Edwards, L., Malcolm, W. F., Smith, P. B., Fisher, K. A., Pizoli, C., et al. (2018). Outcomes of preterm infants treated with hypothermia for hypoxic-ischemic encephalopathy. *Early Hum. Dev.* 125, 1–7. doi: 10.1016/j.earlhumdev.2018.08.003

Hirst, J. J., Palliser, H. K., Yates, D. M., Yawno, T., and Walker, D. W. (2008). Neurosteroids in the fetus and neonate: potential protective role in compromised pregnancies. *Neurochem. Int.* 52, 602–610. doi: 10.1016/j.neuint.2007.07.018

Hodel, A. S., Hunt, R. H., Cowell, R. A., Van Den Heuvel, S. E., Gunnar, M. R., and Thomas, K. M. (2015). Duration of early adversity and structural brain development in post-institutionalized adolescents. *Neuroimage* 105, 112–119. doi: 10.1016/j.neuroimage.2014.10.020

Hosie, A. M., Wilkins, M. E., and Smart, T. G. (2007). Neurosteroid binding sites on $GABA_A$ receptors. *Pharmacol. Ther.* 116, 7–19.

Huddy, C., Johnson, A., and Hope, P. (2001). Educational and behavioural problems in babies of 32-35 weeks gestation. *Arch. Dis. Child.* 85, F23–F28.

Jacobs, S. E., Berg, M., Hunt, R., Tarnow-Mordi, W. O., Inder, T. E., and Davis, P. G. (2013). Cooling for newborns with hypoxic ischaemic encephalopathy. *Cochrane Database Syst. Rev.* 1:CD003311.

Kajantie, E., Phillips, D. I., Andersson, S., Barker, D. J., Dunkel, L., Forsén, T., et al. (2002). Size at birth, gestational age and cortisol secretion in adult life: foetal programming of both hyper- and hypocortisolism? *Clin. Endocrinol.* 57, 635–641. doi: 10.1046/j.1365-2265.2002.01659.x

Kamyar, M., Manuck, T. A., Stoddard, G. J., Varner, M. W., and Clark, E. (2016). Magnesium sulfate, chorioamnionitis, and neurodevelopment after preterm birth. *BJOG* 123, 1161–1166. doi: 10.1111/1471-0528.13460

Kapoor, A., Dunn, E., Kostaki, A., Andrews, M. H., and Matthews, S. G. (2006). Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. *J. Physiol.* 572, 31–44. doi: 10.1113/jphysiol.2006.105254

Kapoor, A., and Matthews, S. G. (2005). Short periods of prenatal stress affect growth, behaviour and hypothalamo-pituitary-adrenal axis activity in male guinea pig offspring. *J. Physiol.* 566, 967–977. doi: 10.1113/jphysiol.2005.090191

Kapoor, A., and Matthews, S. G. (2008). Prenatal stress modifies behavior and hypothalamic-pituitary-adrenal function in female guinea pig offspring: effects of timing of prenatal stress and stage of reproductive cycle. *Endocrinology* 149, 6406–6415. doi: 10.1210/en.2008-0347

Kazdoba, T. M., Hagerman, R. J., Zolkowska, D., Rogawski, M. A., and Crawley, J. N. (2016). Evaluation of the neuroactive steroid ganaxolone on social and repetitive behaviors in the BTBR mouse model of autism. *Psychopharmacology* 233, 309–323. doi: 10.1007/s00213-015-4115-7

Kelleher, M. A., Hirst, J. J., and Palliser, H. K. (2013). Changes in neuroactive steroid concentrations after preterm delivery in the Guinea pig. *Reprod. Sci.* 20, 1365–1375. doi: 10.1177/1933719113485295

Kelleher, M. A., Palliser, H. K., and Hirst, J. J. (2011a). "Neurosteroid replacement therapy in the preterm neonate," in *Proceedings of the 38th annual meeting of the Fetal and Neonatal Physiological Society*, (Palm Cove, QLD).

Kelleher, M. A., Palliser, H. K., Walker, D. W., and Hirst, J. J. (2011b). Sex-dependent effect of a low neurosteroid environment and intrauterine growth restriction on foetal guinea pig brain development. *J. Endocrinol.* 208, 301–309. doi: 10.1677/JOE-10-0248

Kim, P., Choi, C. S., Park, J. H., Joo, S. H., Kim, S. Y., Ko, H. M., et al. (2014). Chronic exposure to ethanol of male mice before mating produces attention deficit hyperactivity disorder-like phenotype along with epigenetic dysregulation of dopamine transporter expression in mouse offspring. *J. Neurosci. Res.* 92, 658–670. doi: 10.1002/jnr.23275

Kirkegaard, I., Obel, C., Hedegaard, M., and Henriksen, T. (2006). Gestational age and birth weight in relation to school performance of 10 year old children: a follow-up study of childrenborn after 32 completed weeks. *Pediatrics* 118, 1600–1606. doi: 10.1542/peds.2005-2700

Koning, G., Leverin, A. L., Nair, S., Schwendimann, L., Ek, J., Carlsson, Y., et al. (2017). Magnesium induces preconditioning of the neonatal brain via profound mitochondrial protection. *J. Cereb. Blood Flow Metab.* [Epub ahead of print],

Lambert, J., Peters, J., and Cottrell, G. (1987). Actions of synthetic and endogenous steroids on the $GABA_A$ receptor. *Trends Pharmacol. Sci.* 8, 224–227.

Lambert, J. J., Belelli, D., Peden, D. R., Vardy, A. W., and Peters, J. A. (2003). Neurosteroid modulation of $GABA_A$ receptors. *Prog. Neurobiol.* 71, 67–80.

Laptook, A. R. (2017). Therapeutic hypothermia for preterm infants with hypoxic-ischemic encephalopathy: how do we move forward? *J. Pediatr.* 183, 8–9. doi: 10.1016/j.jpeds.2016.12.074

Lee, H. H., Deeb, T. Z., Walker, J. A., Davies, P. A., and Moss, S. J. (2011). NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor-mediated currents. *Nat. Neurosci.* 14, 736–743. doi: 10.1038/nn.2806

Lee, H. H., Walker, J. A., Williams, J. R., Goodier, R. J., Payne, J. A., and Moss, S. J. (2007). Direct protein kinase C-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter KCC2. *J. Biol. Chem.* 282, 29777–29784. doi: 10.1074/jbc.m705053200

Lekic, T., Manaenko, A., Rolland, W., Virbel, K., Hartman, R., Tang, J., et al. (2011). Neuroprotection by melatonin after germinal matrix hemorrhage in neonatal rats. *Acta Neurochir. Suppl.* 111, 201–206. doi: 10.1007/978-3-7091-0693-8_34

Liao, G., Cheung, S., Galeano, J., Ji, A. X., Qin, Q., and Bi, X. (2009). Allopregnanolone treatment delays cholesterol accumulation and reduces autophagic/lysosomal dysfunction and inflammation in Npc1-/- mouse brain. *Brain Res.* 1270, 140–151. doi: 10.1016/j.brainres.2009.03.027

Lindahl, J., Kjellsen, B., Tigert, J., and Miskimins, R. (2008). In utero PCP exposure alters oligodendrocyte differentiation and myelination in developing rat frontal cortex. *Brain Res.* 1234, 137–147. doi: 10.1016/j.brainres.2008.06.126

Lindstrom, K., Lindblad, F., and Hjern, A. (2011). Preterm birth and attention-deficit hyperactivity disorder in schoolchildren. *Pediatrics* 127, 858–865. doi: 10.1542/peds.2010-1279

Linnet, K., Wisborg, K., Agerbo, E., Secher, N., Thomsen, P., and Henriksen, T. (2006). Gestational age, birth weight, and the risk of hyperkinetic disorder. *Arch. Dis. Child* 91, 655–660. doi: 10.1136/adc.2005.088872

Loe, I. M., Lee, E. S., Luna, B., and Feldman, H. M. (2011). Behavior problems of 9-16 year old preterm children: biological, sociodemographic, and intellectual contributions. *Early Hum. Dev.* 87, 247–252. doi: 10.1016/j.earlhumdev.2011.01.023

Lombardi, I., Luisi, S., Quirici, B., Monteleone, P., Bernardi, F., Liut, M., et al. (2004). Adrenal response to adrenocorticotropic hormone stimulation in patients with premenstrual syndrome. *Gynecol. Endocrinol.* 18, 79–87. doi: 10.1080/09513590310001652955

Loria, C. J., Stevens, A. M., Crummy, E., Casadesus, G., Jacono, F. J., Dick, T. E., et al. (2013). Respiratory and behavioral dysfunction following loss of the GABAA receptor alpha4 subunit. *Brain Behav.* 3, 104–113. doi: 10.1002/brb3.122

Luchetti, S., Huitinga, I., and Swaab, D. F. (2011). Neurosteroid and GABA-A receptor alterations in Alzheimer's disease, Parkinson's disease and multiple sclerosis. *Neuroscience* 191, 6–21. doi: 10.1016/j.neuroscience.2011.04.010

Macdonald, R. L., and Botzolakis, E. (2009). "GABAA receptor channels," in *Physiology and Pathology of Chloride Transporters and Channels in the Nervous*

System: From Molecules to Diseases, eds F. Javier Alvarez-Leefmans and E. Delpire (Amsterdam: Elsevier Science).

Majewska, M. D. (1992). Neurosteroids: endogenous bimodal modulators of the GABAA receptor. *Mechanism of action and physiological significance. Prog. Neurobiol.* 38, 379–395.

Marret, S., Ancel, P., and Marpeau, L. (2007). Neonatal and 5 year outcomes after birth at 30-34 weeks gestation. *Obstet Gynecol* 110, 72–80. doi: 10.1097/01.aog.0000267498.95402.bd

Martini, L., Celotti, F., and Melcangi, R. (1996). Testosterone and progesterone metabolism in the central nervous system: cellular localization and mechanism of control of the enzymes involved. *Cell. Mol. Neurobiol.* 16, 271–282. doi: 10.1007/bf02088095

Mathews, T., Menacker, F., and MacDorman, M. F. (2002). Infant mortality statistics from the period linked birth/infant death data set. *Natl. Vital Stat. Rep.* 2004, 1–32.

Matsusue, Y., Horii-Hayashi, N., Kirita, T., and Nishi, M. (2014). Distribution of corticosteroid receptors in mature oligodendrocytes and oligodendrocyte progenitors of the adult mouse brain. *J. Histochem. Cytochem.* 62, 211–226. doi: 10.1369/0022155413517700

McKendry, A., Palliser, H., Yates, D., Walker, D., and Hirst, J. (2009). The effect of betamethasone treatment on neuroactive steroid synthesis in a foetal guinea pig model of growth restriction. *J. Neuroendocrinol.* 22, 166–174. doi: 10.1111/j.1365-2826.2009.01949.x

Mellon, S. H., Gong, W., and Schonemann, M. D. (2008). Endogenous and synthetic neurosteroids in treatment of Niemann-Pick Type C disease. *Brain Res. Rev.* 57, 410–420. doi: 10.1016/j.brainresrev.2007.05.012

Mellon, S. H., and Griffin, L. D. (2002). Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol. Metab.* 13, 35–43. doi: 10.1016/s1043-2760(01)00503-3

Mihalek, R. M., Banerjee, P. K., Korpi, E. R., Korpi, E. R., Quinlan, J. J., Firestone, L. L., et al. (1999). Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor delta subunit knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 12905–12910. doi: 10.1073/pnas.96.22.12905

Mirmiran, M. (1995). The function of fetal/neonatal rapid eye movement sleep. *Behav. Brain Res.* 69, 13–22. doi: 10.1016/0166-4328(95)00019-p

Monaghan, E. P., Navalta, L. A., Shum, L., Ashbrook, D. W., and Lee, D. A. (1997). Initial human experience with ganaxolone, a neuroactive steroid with antiepileptic activity. *Epilepsia* 38, 1026–1031. doi: 10.1111/j.1528-1157.1997.tb01486.x

Monteleone, P., Luisi, S., Tonetti, A., Bernardi, F., Genazzani, A. D., Luisi, M., et al. (2000). Allopregnanolone concentrations and premenstrual syndrome. *Eur. J. Endocrinol.* 142, 269–273. doi: 10.1530/eje.0.1420269

Morrison, J. L., Botting, K. J., Darby, J. R. T., Dyson, R. M., Gatford, K. L., Gray, C., et al. (2018). Invited review: guinea pig models for translation of DOHAD into the clinic. *J. Physiol.* 596, 5535–5569. doi: 10.1113/JP274948

Morse, S., Zheng, H., Tang, Y., and Roth, J. (2009). Early school-age outcomes of late preterm infants. *Pediatrics* 123, 622–629.

Moster, D., Lie, R. T., and Markestad, T. (2008). Long-term medical and social consequences of preterm birth. *N. Engl. J. Med.* 359, 262–273. doi: 10.1056/NEJMoa0706475

Nguyen, P. N., Billiards, S. S., Walker, D. W., and Hirst, J. J. (2003). Changes in 5 alpha-pregnane steroids and neurosteroidogenic enzyme expression in fetal sheep with umbilicoplacental embolization. *Pediatr. Res.* 54, 840–847. doi: 10.1203/01.pdr.0000088066.47755.36

Nguyen, P. N., Yan, E. B., Castillo-Melendez, M., Walker, D. W., and Hirst, J. J. (2004). Increased allopregnanolone levels in the fetal sheep brain following umbilical cord occlusion. *J. Physiol.* 560, 593–602. doi: 10.1113/jphysiol.2004.069336

Nicol, M., Hirst, J., and Walker, D. (1998). Effect of pregnane steroids on electrocortical activity and somatosensory evoked potentials in fetal sheep. *Neurosci. Lett.* 253, 111–114. doi: 10.1016/s0304-3940(98)00627-2

Nicol, M., Hirst, J., Walker, D., and Thorburn, G. (1997). Effect of alteration of maternal plasma progesterone concentrations on fetal behavioural state during late gestation. *J. Endocrinol.* 152, 379–386. doi: 10.1677/joe.0.1520379

Nicol, M. B., Hirst, J. J., and Walker, D. W. (2001). Effect of finasteride on behavioural arousal and somatosensory evoked potentials in fetal sheep. *Neurosci. Lett.* 306, 13–16. doi: 10.1016/s0304-3940(01)01861-4

Nohria, V., and Giller, E. (2007). Ganaxolone. *Neurotherapeutics* 4, 102–105. doi: 10.1016/j.nurt.2006.11.003

Olsen, R., and Sapp, D. (1995). Neuroactive steroid modulation of GABAA receptors. *Adv. Biochem. Psychopharmacol.* 48, 57–74.

Owens, D. F., and Kriegstein, A. R. (2002). Is there more to GABA than synaptic inhibition? *Nat. Rev. Neurosci.* 3, 715–727. doi: 10.1038/nrn919

Palliser, H. K., Kelleher, M. A., Tolcos, M., Walker, D. W., and Hirst, J. J. (2015). Effect of postnatal progesterone therapy following preterm birth on neurosteroid concentrations and cerebellar myelination in guinea pigs. *J. Dev. Orig. Health. Dis.* 6, 350–361. doi: 10.1017/S2040174415001075

Paris, J. J., Brunton, P. J., Russell, J. A., Walf, A. A., and Frye, C. A. (2011). Inhibition of 5alpha-reductase activity in late pregnancy decreases gestational length and fecundity and impairs object memory and central progestogen milieu of juvenile rat offspring. *J. Neuroendocrinol.* 23, 1079–1090. doi: 10.1111/j.1365-2826.2011.02219.x

Paul, S. M., and Purdy, R. (1992). Neuroactive steroids. *FASEB J.* 6, 2311–2322.

Petrini, J., Dias, T., McCormick, M., Massolo, M., Green, N., and Escobar, G. (2009). Increased risk of adverse neurological development for late preterm infants. *J. Pediatr.* 154, 169–176. doi: 10.1016/j.jpeds.2008.08.020

Pinna, G., and Rasmusson, A. M. (2014). Ganaxolone improves behavioral deficits in a mouse model of post-traumatic stress disorder. *Front. Cell Neurosci.* 8:256. doi: 10.3389/fncel.2014.00256

Potijk, M. R., de Winter, A. F., Bos, A. F., Kerstjens, J. M., and Reijneveld, S. A. (2012). Higher rates of behavioural and emotional problems at preschool age in children born moderately preterm. *Arch. Dis. Child.* 97, 112–117. doi: 10.1136/adc.2011.300131

Rao, R., Trivedi, S., Vesoulis, Z., Liao, S. M., Smyser, C. D., and Mathur, A. M. (2017). Safety and short-term outcomes of therapeutic hypothermia in preterm neonates 34-35 weeks gestational age with hypoxic-ischemic encephalopathy. *J. Pediatr.* 183, 37–42. doi: 10.1016/j.jpeds.2016.11.019

Rasmusson, A. M., Pinna, G., Paliwal, P., Weisman, D., Gottschalk, C., Charney, D., et al. (2006). Decreased cerebrospinal fluid allopregnanolone levels in women with posttraumatic stress disorder. *Biol. Psychiatry* 60, 704–713. doi: 10.1016/j.biopsych.2006.03.026

Reddy, D. S., and Rogawski, M. A. (2012). "Neurosteroids - endogenous regulators of seizure susceptibility and role in the treatment of epilepsy," in *Jasper's Basic Mechanisms of the Epilepsies*, 4th Edn, eds J. L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen, and A. V. Delgado-Escueta (Rockville, MD: Bethesda).

Rees, S., Harding, R., and Walker, D. (2008). An adverse intrauterine environment: implications for injury and altered development of the brain. *Int. J. Dev. Neurosci.* 26, 3–11. doi: 10.1016/j.ijdevneu.2007.08.020

Rees, S., and Inder, T. (2005). Fetal and neonatal origins of altered brain development. *Early Hum. Dev.* 81, 753–761. doi: 10.1016/j.earlhumdev.2005.07.004

Represa, A., and Ben-Ari, Y. (2005). Trophic actions of GABA on neuronal development. *Trends Neurosci.* 28, 278–283. doi: 10.1016/j.tins.2005.03.010

Rivera, C., Voipio, J., and Kaila, K. (2005). Two developmental switches in GABAergic signalling: the K+-Cl- cotransporter KCC2 and carbonic anhydrase CAVII. *J. Physiol.* 562, 27–36. doi: 10.1113/jphysiol.2004.077495

Rivera, C., Voipio, J., Payne, J. A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., et al. (1999). The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397, 251–255. doi: 10.1038/16697

Rivkin, M. J. (1997). Hypoxic-ischemic brain injury in the term newborn. Neuropathology, clinical aspects, and neuroimaging. *Clin. Perinatol.* 24, 607–625. doi: 10.1016/s0095-5108(18)30161-1

Romeo, D., Di Stefano, A., and Conversano, M. (2010). Neurodevelopmental outcome at 12 and 18 months in late preterm infants. *Eur. J. Paediatr. Neurol.* 14, 503–507. doi: 10.1016/j.ejpn.2010.02.002

Roof, R., Duvdevani, R., Braswell, L., and Stein, D. (1994). Progesterone facilitates cognitive recovery and reduces secondary neuronal loss caused by cortical contusion injury in male rats. *Exp. Neurol.* 129, 64–69. doi: 10.1006/exnr.1994.1147

Roof, R., Duvdevani, R., Heyburn, J., and Stein, D. (1996). Progesterone rapidly decreases brain edema: treatment delayed up to 24 hours is still effective. *Exp. Neurol.* 138, 246–251. doi: 10.1006/exnr.1996.0063

Schendel, D., and Bhasin, T. (2008). Birth weight and gestational age characteristics of children with autism, including a comparison with other developmental disabilities. *Pediatrics* 121, 1155–1164. doi: 10.1542/peds.2007-1049

Schermann, L., and Sedin, G. (2004). Cognitive function at 10 years of age in children who have required neonatal intensive care. *Acta Paediatr.* 93, 1619–1629. doi: 10.1111/j.1651-2227.2004.tb00853.x

Schumacher, M., Guennoun, R., Robert, F., Carelli, C., Gago, N., Ghoumari, A., et al. (2004). Local synthesis and dual actions of progesterone in the nervous system: neuroprotection and myelination. *Growth Horm. IGF Res.* 14(Suppl. A), S18–S33.

Shaw, J. C., Dyson, R. M., Palliser, H. K., Gray, C., Berry, M. J., and Hirst, J. J. (2018). Neurosteroid replacement therapy using the allopregnanolone-analogue ganaxolone following preterm birth in male guinea pigs. *Pediatr. Res.* 85, 86–96. doi: 10.1038/s41390-018-0185-7

Shaw, J. C., Palliser, H. K., Dyson, R. M., Berry, M. J., and Hirst, J. J. (2017). Disruptions to the cerebellar GABAergic system in juvenile guinea pigs following preterm birth. *Int. J. Dev. Neurosci.* 65, 1–10. doi: 10.1016/j.ijdevneu.2017.10.002

Shaw, J. C., Palliser, H. K., Dyson, R. M., Hirst, J. J., and Berry, M. J. (2016). Long-term effects of preterm birth on behavior and neurosteroid sensitivity in the guinea pig. *Pediatr. Res.* 80, 275–283. doi: 10.1038/pr.2016.63

Shaw, J. C., Palliser, H. K., Walker, D. W., and Hirst, J. (2015). Preterm birth affects GABAA receptor subunit mRNA levels during the foetal-to-neonatal transition in guinea pigs. *J. Dev. Orig. Health Dis.* 6, 250–260. doi: 10.1017/S2040174415000069

Sieghart, W., Fuchs, K., Tretter, V., Ebert, V., Jechlinger, M., Höger, H., et al. (1999). Structure and subunit composition of $GABA_A$ receptors. *Neurochem. Int.* 34, 379–385.

Simmons, L. E., Rubens, C. E., Darmstadt, G. L., and Gravett, M. G. (2010). Preventing preterm birth and neonatal mortality: exploring the epidemiology, causes, and interventions. *Semin. Perinatol.* 34, 408–415. doi: 10.1053/j.semperi.2010.09.005

Singh, G., Kenney, M., Ghandour, R., Kogan, M., and Lu, M. (2013). Mental health outcomes in US children and adolescents born prematurely or with low birthwight. *Dep. Res. Treat.* 2013:570743. doi: 10.1155/2013/570743

Sperling, M. R., Klein, P., and Tsai, J. (2017). Randomized, double-blind, placebo-controlled phase 2 study of ganaxolone as add-on therapy in adults with uncontrolled partial-onset seizures. *Epilepsia* 58, 558–564. doi: 10.1111/epi.13705

Spigelman, I., Li, Z., Banerjee, P. K., Mihalek, R. M., Homanics, G. E., and Olsen, R. W. (2002). Behavior and physiology of mice lacking the GABAA-receptor delta subunit. *Epilepsia* 43(Suppl. 5), 3–8. doi: 10.1046/j.1528-1157.43.s.5.8.x

Spigelman, I., Li, Z., Liang, J., Samzadeh, S., Mihalek, R. M., Homanics, G. E., et al. (2003). Reduced inhibition and sensitivity to neurosteroids in hippocampus of mice lacking the GABA(A) receptor delta subunit. *J. Neurophysiol.* 90, 903–910. doi: 10.1152/jn.01022.2002

Stell, B. M., Brickley, S. G., Tang, C., and Farrant, M. (2003). Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing GABAA receptors. *Proc. Natl. Acad. Sci.* 100, 14439–14444. doi: 10.1073/pnas.2435457100

Stoffel-Wagner, B. (2001). Neurosteroid metabolism in the human brain. *Eur. J. Endocrinol.* 145, 669–679. doi: 10.1530/eje.0.1450669

Stoodley, C. J. (2012). The cerebellum and cognition: evidence from functional imaging studies. *Cerebellum* 11, 352–365. doi: 10.1007/s12311-011-0260-7

Stoodley, C. J., and Schmahmann, J. D. (2010). Evidence for topographic organization in the cerebellum of motor control versus cognitive and affective processing. *Cortex* 46, 831–844. doi: 10.1016/j.cortex.2009.11.008

Strohle, A., Romeo, E., Hermann, B., Pasini, A., Spalletta, G., di Michele, F., et al. (1999). Concentrations of 3 alpha-reduced neuroactive steroids and their precursors in plasma of patients with major depression and after clinical recovery. *Biol. Psychiatry* 45, 274–277. doi: 10.1016/s0006-3223(98)00328-x

Talge, N., Holzman, C., Wang, J., Lucia, V., Gardiner, J., and Breslau, N. (2010). Late-preterm birth and its association with cognitive and socioemotional outcomes at 6 years of age. *Pediatrics* 126, 1124–1131. doi: 10.1542/peds.2010-1536

van Baar, A. L., Vermaas, J., Knots, E., de Kleine, M. J., and Soons, P. (2009). Functioning at school age of moderately preterm children born at 32 to 36 weeks' gestational age. *Pediatrics* 124, 251–257. doi: 10.1542/peds.2008-2315

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., et al. (2016). Impaired oligodendrocyte maturation in preterm infants: potential therapeutic targets. *Prog. Neurobiol.* 136, 28–49. doi: 10.1016/j.pneurobio.2015.11.002

Verkuyl, J. M., Hemby, S. E., and Joels, M. (2004). Chronic stress attenuates GABAergic inhibition and alters gene expression of parvocellular neurons in rat hypothalamus. *Eur. J. Neurosci.* 20, 1665–1673. doi: 10.1111/j.1460-9568.2004.03568.x

Vicari, S., Caravale, B., Carlesimo, G. A., Casadei, A. M., and Allemand, F. (2004). Spatial working memory deficits in children at ages 3-4 who were low birth weight, preterm infants. *Neuropsychology* 18, 673–678. doi: 10.1037/0894-4105.18.4.673

Villapol, S., Fau, S., Renolleau, S., Biran, V., Charriaut-Marlangue, C., and Baud, O. (2011). Melatonin promotes myelination by decreasing white matter inflammation after neonatal stroke. *Pediatr. Res.* 69, 51–55. doi: 10.1203/PDR.0b013e3181fcb40b

Vollmer, B., Lundequist, A., Martensson, G., Nagy, Z., Lagercrantz, H., Smedler, A. C., et al. (2017). Correlation between white matter microstructure and executive functions suggests early developmental influence on long fibre tracts in preterm born adolescents. *PLoS One* 12:e0178893. doi: 10.1371/journal.pone.0178893

Volpe, J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 8, 110–124. doi: 10.1016/S1474-4422(08)70294-1

Volpe, J. J. (2001). Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr. Res.* 50, 553–562. doi: 10.1203/00006450-200111000-00003

Volpe, J. J. (2003). Cerebral white matter injury of the premature infant-more common than you think. *Pediatrics* 112, 176–180. doi: 10.1542/peds.112.1.176

Volpe, J. J. (2008). *Neurology of the Newborn*. Amsterdam: Elsevier Health Sciences.

Walf, A. A., Sumida, K., and Frye, C. A. (2006). Inhibiting 5alpha-reductase in the amygdala attenuates antianxiety and antidepressive behavior of naturally receptive and hormone-primed ovariectomized rats. *Psychopharmacology* 186, 302–311. doi: 10.1007/s00213-005-0100-x

Wilkinson, D., Shepherd, E., and Wallace, E. M. (2016). Melatonin for women in pregnancy for neuroprotection of the fetus. *Cochrane Database Syst. Rev.* 3:CD010527. doi: 10.1002/14651858.CD010527.pub2

Williamson, A., Mellor, J., Grant, A., and Randall, A. (1998). Properties of $GABA_A$ receptors in cultured rat oligodendrocyte progenitor cells. *Neuropharmacology* 37, 859–873. doi: 10.1016/s0028-3908(98)00016-1

Woythaler, M., McCormick, M., and Smith, V. (2011). Late preterm infants have worse 24 month neurodevelopmental outcomes than term infants. *Pediatrics* 127, 622–629.

Wright, D. W., Kellermann, A. L., Hertzberg, V. S., Clark, P. L., Frankel, M., Goldstein, F. C., et al. (2007). ProTECT: a randomized clinical trial of progesterone for acute traumatic brain injury. *Ann. Emerg. Med.* 49, 391–402.

Xiao, G., Wei, J., Yan, W., Wang, W., and Lu, Z. (2008). Improved outcomes from the administration of progesterone for patients with acute severe traumatic brain injury: a randomized controlled trial. *Crit. Care* 12:R61. doi: 10.1186/cc6887

Yawno, T., Hirst, J. J., Castillo-Melendez, M., and Walker, D. W. (2009). Role of neurosteroids in regulating cell death and proliferation in the late gestation fetal brain. *Neuroscience* 163, 838–847. doi: 10.1016/j.neuroscience.2009.07.009

Yawno, T., Miller, S. L., Bennet, L., Wong, F., Hirst, J. J., Fahey, M., et al. (2017). Ganaxolone: a new treatment for neonatal seizures. *Front. Cell Neurosci.* 11:246. doi: 10.3389/fncel.2017.00246

Yawno, T., Yan, E., Walker, D., and Hirst, J. (2007). Inhibition of neurosteroid synthesis increases asphyxia-induced brain injury in the late gestation fetal sheep. *Neuroscience* 146, 1726–1733. doi: 10.1016/j.neuroscience.2007.03.023

Yawno, T., Yan, E. B., Hirst, J. J., and Walker, D. W. (2011). Neuroactive steroids induce changes in fetal sheep behavior during normoxic and asphyxic states. *Stress* 14, 13–22. doi: 10.3109/10253890.2010.504789

Yu, Z. Y., Wang, W., Fritschy, J. M., Witte, O. W., and Redecker, C. (2006). Changes in neocortical and hippocampal GABAA receptor subunit distribution during brain maturation and aging. *Brain Res.* 1099, 73–81. doi: 10.1016/j.brainres.2006.04.118

Yum, M. S., Lee, M., Ko, T. S., and Velisek, L. (2014). A potential effect of ganaxolone in an animal model of infantile spasms. *Epilepsy Res.* 108, 1492–1500. doi: 10.1016/j.eplepsyres.2014.08.015

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Shaw, Berry, Dyson, Crombie, Hirst and Palliser. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 22 May 2019
doi: 10.3389/fphys.2019.00563

Dysmaturation of Somatostatin Interneurons Following Umbilical Cord Occlusion in Preterm Fetal Sheep

Maryam Ardalan[1], Pernilla Svedin[1], Ana A. Baburamani[2], Veena G. Supramaniam[2], Joakim Ek[1], Henrik Hagberg[2,3] and Carina Mallard[1]*

[1] Centre for Perinatal Medicine and Health, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [2] Centre for the Developing Brain, Department of Perinatal Imaging and Health, School of Biomedical Engineering and Imaging Sciences, King's College London, London, United Kingdom, [3] Centre for Perinatal Medicine and Health, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

OPEN ACCESS

Edited by:
Charles Evans Wood, University of Florida, United States

Reviewed by:
Mhoyra Fraser, The University of Auckland, New Zealand
Dean A. Myers, The University of Oklahoma Health Sciences Center, United States

****Correspondence:***
Maryam Ardalan
maryam.ardalan@gu.se;
maryamardalan@gmail.com

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *15 February 2019*
Accepted: *24 April 2019*
Published: *22 May 2019*

Citation:
Ardalan M, Svedin P, Baburamani AA, Supramaniam VG, Ek J, Hagberg H and Mallard C (2019) Dysmaturation of Somatostatin Interneurons Following Umbilical Cord Occlusion in Preterm Fetal Sheep. Front. Physiol. 10:563. doi: 10.3389/fphys.2019.00563

Introduction: Cerebral white matter injury is the most common neuropathology observed in preterm infants. However, there is increasing evidence that gray matter development also contributes to neurodevelopmental abnormalities. Fetal cerebral ischemia can lead to both neuronal and non-neuronal structural-functional abnormalities, but less is known about the specific effects on interneurons.

Objective: In this study we used a well-established animal model of fetal asphyxia in preterm fetal sheep to study neuropathological outcome. We used comprehensive stereological methods to investigate the total number of oligodendrocytes, neurons and somatostatin (STT) positive interneurons as well as 3D morphological analysis of STT cells 14 days following umbilical cord occlusion (UCO) in fetal sheep.

Materials and Methods: Induction of asphyxia was performed by 25 min of complete UCO in five preterm fetal sheep (98–100 days gestational age). Seven, non-occluded twins served as controls. Quantification of the number of neurons (NeuN), STT interneurons and oligodendrocytes (Olig2, CNPase) was performed on fetal brain regions by applying optical fractionator method. A 3D morphological analysis of STT interneurons was performed using IMARIS software.

Results: The number of Olig2, NeuN, and STT positive cells were reduced in IGWM, caudate and putamen in UCO animals compared to controls. There were also fewer STT interneurons in the ventral part of the hippocampus, the subiculum and the entorhinal cortex in UCO group, while other parts of cortex were virtually unaffected ($p > 0.05$). Morphologically, STT positive interneurons showed a markedly immature structure, with shorter dendritic length and fewer dendritic branches in cortex, caudate, putamen, and subiculum in the UCO group compared with control group ($p < 0.05$).

Conclusion: The significant reduction in the total number of neurons and oligodendrocytes in several brain regions confirm previous studies showing susceptibility of both neuronal and non-neuronal cells following fetal asphyxia. However, in

the cerebral cortex significant dysmaturation of STT positive neurons occurred in the absence of cell loss. This suggests an abnormal maturation pattern of GABAergic interneurons in the cerebral cortex, which might contribute to neurodevelopmental impairment in preterm infants and could implicate a novel target for neuroprotective therapies.

Keywords: GABA, interneurons, somatostatin, stereology, preterm

INTRODUCTION

Preterm birth and its associated complications are among the most serious global health issues that modern society faces (Blencowe et al., 2013). Particularly, extreme prematurity (<28 weeks gestation) is associated with poor neurodevelopmental outcome with increased prevalence of cognitive and motor delays (Pascal et al., 2018). The etiology of preterm brain injury is likely to be multifactorial, but circulatory disturbances and inflammation are the critical contributing factors in the pathophysiology of impairment of brain development (Hagberg et al., 2015). Neurodevelopmental disability in infants and children born preterm, so called encephalopathy of prematurity, is associated with impaired cerebral maturation, including white and gray matter volumes, cortical folding, and gyral complexity (Boardman et al., 2006; Volpe, 2009). There is also evidence of delayed cellular maturation, reduced dendritic arborization, impaired synaptogenesis, and connectivity (Tau and Peterson, 2010; Ball et al., 2013), but limited knowledge on specific cell populations.

Depending on the brain region, GABAergic cortical interneurons represent about 10–20% of the neurons within the neocortex (Le Magueresse and Monyer, 2013). These cells control the excitation/inhibition balance, which is crucial for normal brain development and cortical plasticity (Scheyltjens and Arckens, 2016; Fowke et al., 2018). Thus, injury to the immature brain has the potential to affect GABAergic circuitry and cortical function with the consequence of several neurologic disorders (Powell et al., 2003; Butt et al., 2017). Somatostatin (STT)-positive GABAergic neurons are one of the most prevalent populations of early born interneurons with a crucial role in early cortical circuit formation (Rudy et al., 2011). STT is not only a marker of specific types of interneuron but also an inhibitory neuropeptide released from GABAergic neurons (Ludwig and Pittman, 2003; Yavorska and Wehr, 2016). Cortical interneuron subtypes are specified during the fetal period, followed by migration and then differentiation when reaching their cortical destination (Wonders and Anderson, 2006). The timing of migration of STT progenitor cells indicate that they are the first interneurons that migrate to deep layers of the cortical plate during brain development (Miyoshi and Fishell, 2011). Early-born STT neurons localize mainly in cortical layer 5/6 and they persist throughout development in deep layers of the cortex (Rudy et al., 2011). Due to their special characteristics and connectivity, STT interneurons regulate brain plasticity by mediating the maturation of deep layer cortical circuits (Liguz-Lecznar et al., 2016; Tuncdemir et al., 2016). Experimental evidence in neonatal rats indicates that perinatal asphyxia can cause motor deficits related to the loss of GABAergic neurons including calbindin- and parvalbumin-positive interneurons in striatum (Van de Berg et al., 2003). A recent study showed that parvalbumin-positive neurons are reduced following cerebral ischemia in late gestation fetal sheep (Fowke et al., 2018). Post-mortem studies in preterm infants born at 25–32 weeks gestation, demonstrate that in addition to oligodendrocyte loss and axonal disruption, the number of GABAergic interneurons is significantly decreased in brains with white matter lesions (Robinson et al., 2006). More importantly, disruption of the early (but not late) STT interneuron network resulted in impairment of synaptic maturation of thalamocortical inputs onto parvalbumin interneurons (Tuncdemir et al., 2016). However, the effect of perinatal asphyxia at mid-gestation on number and maturation of STT interneurons is not known. In this study we aimed to examine the morphology and distribution of pathological changes of STT-positive GABAergic interneurons following transient *in utero* asphyxia in fetal sheep. *In utero* asphyxia in preterm fetal sheep is a suitable animal model to study complex pathophysiological processes that contribute to brain injury in the preterm infant (Bennet et al., 2012). Specifically, with respect to neuropathology, sheep have similar proportions of gray and white matter as the human (Mallard et al., 2003; Koehler et al., 2018). In these studies, we used a well-established animal model in preterm fetal sheep where asphyxia is induced by transient umbilical cord occlusion (UCO) at midgestation, which is equivalent to 25–30 weeks gestation in the human with respect to brain development (Mallard et al., 1994).

MATERIALS AND METHODS

Fetal Surgery and Umbilical Cord Occlusion

Animal experiments were approved by the local Animal Ethics Committee of Gothenburg (No. 166/13) and performed according to the guidelines for animal experimentation by the Swedish Department of Agriculture. Eight time-mated pregnant ewes were fasted overnight and then underwent aseptic surgery at 95–96 days gestation as previously described (Mallard et al., 2003). Prior to anesthesia induction, the ewe was given Stesolid (Diazepam, 0.1–0.2 mg/kg, i.v.). Anesthesia was induced by sodium pentothal (13 mg/kg, i.v.), followed by intubation and isoflurane (1.5%) and the ewe was also given one injection of Temgesic (Buprenorphine, 0.005–0.02 mg/kg, i.v.) and Garamycin (Gentamicin, 5 mg/kg, i.m). The uterine horn was exposed through a midline incision and a small

hysterectomy incision was made over the fetal head through the uterine wall, parallel to any vessels. An inflatable silastic cuff was placed around the umbilical cord (OCHD16, DocXS Biomedical Products, Ukiah, CA, United States). Polyvinyl catheters (i.d. 1 mm, Smiths Medical and tip 0.4 mm, Agnthos, Sweden) were inserted into each brachial/axillary artery and brachial vein. An amnion catheter (i.d. 2.0 mm, Portex, Smiths Medical, Minneapolis, MN, United States) was secured to the ear. In case of twins, only one fetus was instrumented. At the end of the operation, catheters were filled with 50 IU/ml heparinised saline. The uterus was closed in two layers and catheters exteriorized via a trocar. One catheter was placed in the tarsal vein of the ewe. Sheep were allowed to recover for 3–5 days following surgery before experiments began. During this period Gentaject (Gentamicin; 5 mg/kg, i.v.) was administered to the ewe daily.

Induction of asphyxia was performed by 25 min of complete UCO at 98–100 days gestation as previously described (Mallard et al., 2003).

Tissue Processing and Immunohistochemistry

A total of five fetuses with UCO and seven non-occluded twins were included in the study. Ewes were euthanized by a maternal intravenous injection of sodium pentobarbitone 2 weeks after UCO (at 112–114 days gestation). The fetuses were immediately removed, perfusion fixed *in situ* through the carotid arteries with saline (0.9%) followed by 4% paraformaldehyde (PFA) and then brains were removed and further immersion-fixed in 4% PFA until processing (at least 1 month). Brains were separated into left and right hemisphere. The right hemisphere was cut into four coronal blocks (A–D) at a thickness of 5 mm (**Figure 1**), blocks were separately paraffin embedded and then sectioned on a microtome (Thermo Scientific^{TM} HM 355S Automatic Microtome) based on a systematic sampling principle. Following deparaffinization and rehydration, immunohistochemistry was carried out on paraffin sections by boiling in citrate buffer for antigen retrieval and blocking for endogenous peroxidase (3% H_2O_2 in PBS) for 10 min. Non-specific binding was blocked by 4% serum for 30 min in room temperature, followed by incubation with primary antibodies to quantify the number of neurons [monoclonal mouse anti-NeuN (Millipore MAB377) 1:250]; transcription factor expressed in all oligodendrocytes throughout their lineage (1:100 poly-clonal rabbit anti-Olig2, Chemicon, AB9610); immature and mature oligodendrocytes (CNPase, monoclonal mouse, 1:200; Sigma-Aldrich, C5922) and STT (1:100 monoclonal rat anti-STT, IgG2b, Clone YC7, Abcam ab150348) in PBS overnight at 4°C. The next day, sections were incubated with appropriate secondary antibodies [Horse-anti-mouse biotinylated, Goat-anti-rabbit biotinylated, Goat-anti-rat biotinylated (Vector)] (1:250) in PBS for 1 h in room temperature, followed by addition of ABC solution [VECTASTAIN Elite ABC HRP Kit (Peroxidase, Standard, PK-6100)] for 1 h. Visualization of stained cells was performed by 3,3′-diaminobenzidine (DAB) for 10 min, after which slides were coverslipped.

Estimation of the Total Number of Cells in Brain Regions

Unbiased estimation of the total number of neurons, oligodendrocytes (mature and immature), and STT interneurons was performed by applying the optical fractionator method (Gundersen, 1986). The newCAST software (Visiopharm, Hørsholm, Denmark) was used with a light microscope (Leica DM6000 B, Germany) modified for stereology with a digital camera (Leica DFC 295, Germany) and a motorized microscope stage (Ludl MAC 5000, United States) and 63X oil-immersed lens. Cells were analyzed once the soma of the cells were in focus and inside the unbiased counting frame. For analysis, a 10-μm height disector was applied to each section. Delineation of the area of interest was done using a 5× objective lens. The calculation for counting the number of cells was done according to the applied method in Ardalan et al. (2016) paper.

$$N = \frac{1}{SSF} \cdot \frac{1}{ASF} \cdot \frac{1}{HSF} \cdot \Sigma Q^{-}$$

N is the total number of cells per brain region; ΣQ^{-} is the number of counted cells; *SSF* is the section sampling fraction; *ASF* is an area sampling fraction; and *HSF* is the height sampling fraction (1/10).

Acquisition of Images and Morphological Analysis of Somatostatin Interneurons

A systematic set of Z-stacks of the brain regions including cortex, putamen, caudate, subiculum, and entorhinal cortex (EC) on the STT stained sections were captured by using a 63X oil-immersed lens on a light microscope modified for stereology. Application of this method of image acquisition, made it possible to Z-stack capture of more than one interneuron in one image. The height of the Z stacks was 16 μm equal to the thickness of sections, and the acquisition of images was performed in steps of 1 μm.

The captured images were analyzed by using Filament Tracers algorithm in Imaris software (Version 8.4, Bitplane A.G., Zurich, Switzerland), and the morphological parameters of STT interneurons including the number of dendrites, the total length of the dendrites, and the sholl intersections were quantified. Sholl analysis was performed to explore the complexity of cellular branches (Anzabi et al., 2018). By selecting the center of STT soma as a reference point, the length of the dendrites was measured based on the radial distance from the reference point (soma) in 20 μm. The sum of the number of branching intersections for all circles, for each cell, and the mean of the results from 15 STT cells per region per animal was quantified. The reliability of the STT reconstruction was considered by following sampling/selection criteria; (1) the STT cell soma had to be in the middle of the section thickness with the clear border; (2) the STT cell had to have intact clear dendrites; and (3) the dendrites of the selected cell should not be obscured with the branches of nearby cells or the background staining.

All measurements were performed by a researcher blinded to the groups of animals.

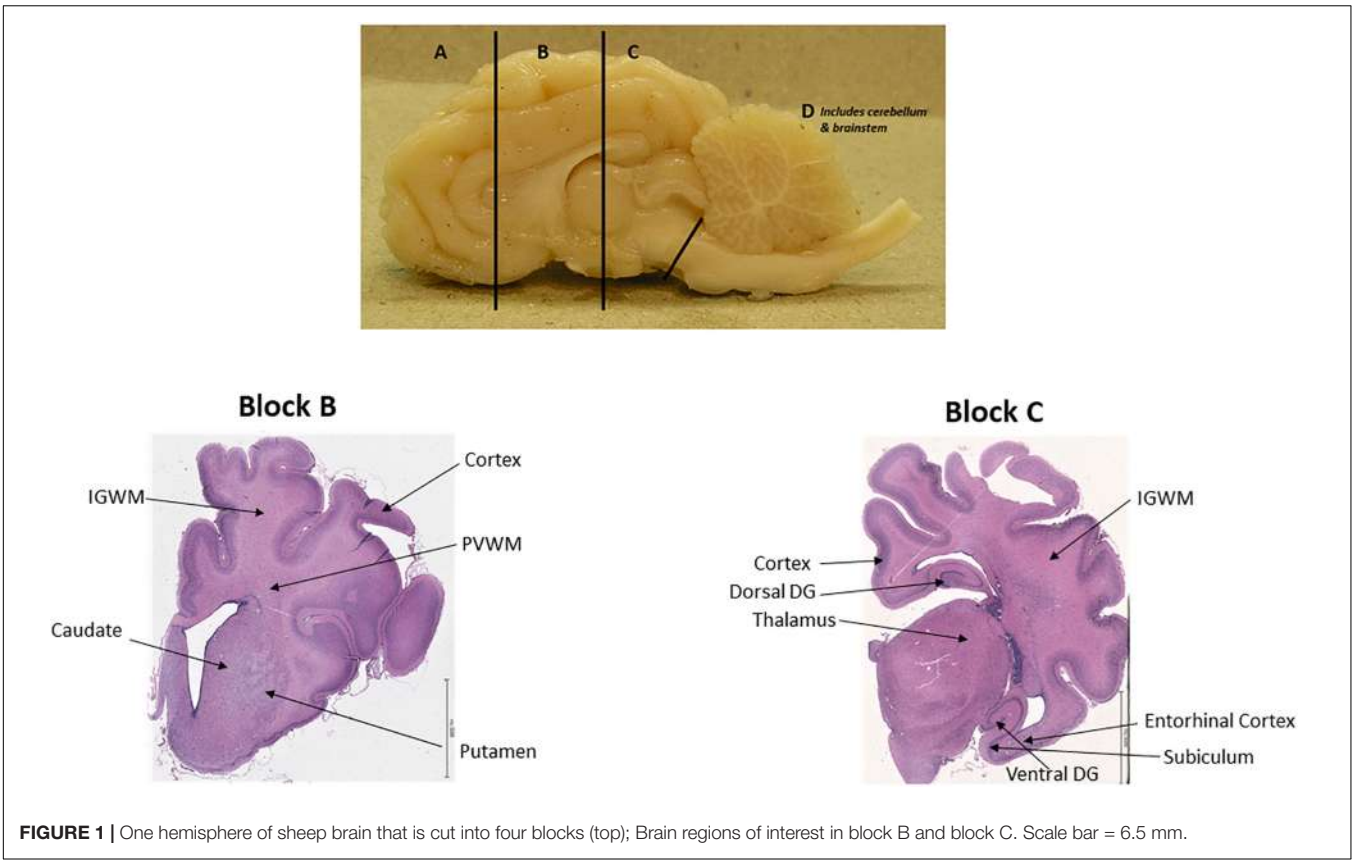


FIGURE 1 | One hemisphere of sheep brain that is cut into four blocks (top); Brain regions of interest in block B and block C. Scale bar = 6.5 mm.

Neuropathology Assessment

Two anatomical levels of the brain at the level of lateral ventricle/basal ganglia and at the level of the thalamus and hippocampus, corresponding to section 720 and section 1120, respectively, in the Sheep Ovis Aries atlas[1] were used for analysis (**Figure 1**). Brain sections was deparaffinised and stained with acid fuchsin/thionin for structural analysis (Bennet et al., 2012). Briefly, sections were stained with thionin for 4 min, followed with rinsing in distilled water, acid fuchsin staining for 20 s and dehydrated through a graded series of alcohol, cleared in xylene and coverslipped.

Acid-fuchsin/thionin-stained sections were examined for gross structural damage, including areas of pallor, neuronal eosinophilia, karyopyknosis, cavitation, necrosis, and infarction. The cortex, subcortical and periventricular white matter (PVWM), striatum/basal ganglia and hippocampus were all assessed. A neuropathology score was assigned to each brain region and section according to criteria described in **Table 1**. All neuropathology scoring was performed by an assessor blinded to the identity of the animal group.

Statistical Analysis

All data was analyzed by using IBM Corp. Released 2013 (IBM SPSS Statistics for Windows, Version 22.0. IBM Corp., Armonk, NY, United States) and graphs were created by using Sigmaplot 12.5 (SYSTAT, San Jose, CA, United States). Prior to statistical tests, normal distribution of data was tested by making a Q–Q plot of the data. The variance homogeneity of data was tested by Levene's test. In the cases that the distribution of data was not normal and/or data variance was different, a logarithmic transformation was performed before statistical testing was employed. The collected data from the two groups (UCO and control) was compared using the independent *t*-test. In all cases, the

TABLE 1 | Scoring template for neuropathology based upon tissue damage in acid-fuchsin/thionin stained sections.

Neuropathology scoring	
Acid-fuschsin/thionin	
0	Absent
1	Neuronal eosinophilia, karyopyknosis; small patches of necrotic cells, small areas of pallor
2	Areas with increased cellularity; moderate patches of necrotic cells, moderate areas of pallor
3	PVL/IVH; severe tissue loss; large infarcts/cavitation

Adapted from Fraser et al. (2007) and Dean et al. (2011). PVL, periventricular leukomalacia; IVH, intraventricular hemorrhage.

[1]https://msu.edu/user/brains/brains/sheep/scans/0720/image1.html

TABLE 2 | Descriptive information of neuropathological scores in different brain regions for each UCO animal.

	Sheep # 4A	Sheep # 5A	Sheep # 12A	Sheep # 14	Sheep # 15A
	AF/T* score	AF/T score	AF/T score	AF/T score	AF/T score
Periventricular white matter	1	1	1	0	0
Subcortical white matter	2	2	2	0	0
Cortex/gray matter	0	0	0	0	0
Basal ganglia/striatum	0	0	0	0	0
Hippocampus	0	0	0	0	0

AF/T, acid-fuchsin/thionin.

significance level was set at $p < 0.05$. Data are presented as mean ± SD.

RESULTS

Gross Neuropathology

There were no large areas of infarcts or PVL/IVH in any of the UCO animals, however, 3/5 animals displayed areas of pallor and small patches of necrotic cells in the subcortical and PVWM (**Table 2**). There was no visible neuropathological abnormality, based on the gross neuropathological score, in the cortex, basal ganglia, and hippocampus (**Table 2**).

Effect of UCO on Number of STT Interneurons

The number of STT interneurons was counted in different brain regions in block B: cortex, intragyral white matter (IGWM), PVWM, caudate and putamen (**Figure 2**) and block C: EC, subiculum, and dentate gyrus (DG) subregion of dorsal and ventral hippocampi (**Figure 3**). The UCO group showed a significant reduction in the number of STT positive interneurons in the caudate, putamen and IGWM compared to the control group ($p = 0.04$; $p = 0.000$; $p = 0.030$; **Figure 2** and **Supplementary Table 1**). In the DG area of hippocampus, a significant effect of UCO was observed on the number of STT interneurons in the ventral part of the DG compared with control group ($p = 0.05$), however, there was no significant change in the number of STT cells in the dorsal DG in UCO group ($p = 0.21$). Moreover, our results indicated significant reduction in the number of STT cells in the subiculum of UCO animals compared to the control group ($p = 0.02$). The number of STT-positive interneurons in the EC was significantly lower in UCO group vs. control group ($p = 0.03$; **Figure 3** and **Supplementary Table 1**), while the other parts of cortex, were virtually unaffected by the UCO ($p > 0.05$).

Effect of UCO on the STT Interneuron Morphology

The morphological analysis of STT positive cells showed that the total length of dendrites was significantly shorter in layer six of the cortex, caudate, and putamen in UCO group compared to control group, respectively ($p = 0.005$; $p = 0.01$; $p = 0.01$) (**Figure 4**). Moreover, UCO resulted in shorter length of STT dendrites in subiculum and EC ($p < 0.001$; $p < 0.001$) (**Figure 5**).

The number of dendrites of STT cells in the cortex, caudate, and putamen was remarkably higher in control group in comparison with UCO group ($p = 0.006$; $p = 0.02$; $p = 0.04$) (**Figure 4**). The number of STT dendrites in subiculum was significantly lower in UCO group compared with control group ($p = 0.002$), while there was no significant change in numbers of STT dendrites in the EC following UCO compared to control group ($p = 0.38$) (**Figure 5**). Regarding the effect of UCO on the complexity of STT dendrites in brain regions, sholl analysis showed significant reduction of the STT arborization in cortex, caudate and putamen in UCO group compared to the control group by showing significantly lower number of branching intersections from the cell soma at distances of 20–120 μm ($p < 0.05$) (**Figure 6**). In the subiculum region, significant disturbance in the STT cells arborization in the UCO group was observed by showing a significant effect of UCO on the number of STT dendritic intersections at 40–100 μm from the soma compared to control ($p < 0.001$). In the EC, there were significant differences in STT dendritic complexity at 20–80 μm from the soma in UCO compared with the control group ($p < 0.001$) (**Figure 6**).

Effect of UCO on the Oligodendrocyte Number

Umbilical cord occlusion was associated with a significant loss of Olig2 cells in IGWM, thalamus, putamen and caudate compared to control group ($p < 0.05$; **Figure 7** and **Supplementary Table 2**), however, no significant difference in the number of Olig2 cells was observed in cortex and PVWM compared to the control group ($p > 0.05$). Counting the number of CNPase positive cells was performed in IGWM area and the Levene's test showed significant difference in variance homogeneity and therefore a logarithmic transformation before applying independent *t*-test was done. There was a significantly reduced number of CNPase positive cells in UCO animals compared to the control group ($p = 0.01$) (**Figure 8**).

Effect of UCO on the Neuronal Number

Quantification of the number of mature (NeuN positive) neurons was done in cortex, IGWM, PVWM, putamen and caudate. The results indicated a significant negative effect of UCO on the number of NeuN positive cells in all these areas ($p = 0.003$; $p = 0.004$; $p = 0.04$; $p = 0.01$; $p = 0.05$) (**Figure 9** and **Supplementary Table 3**).

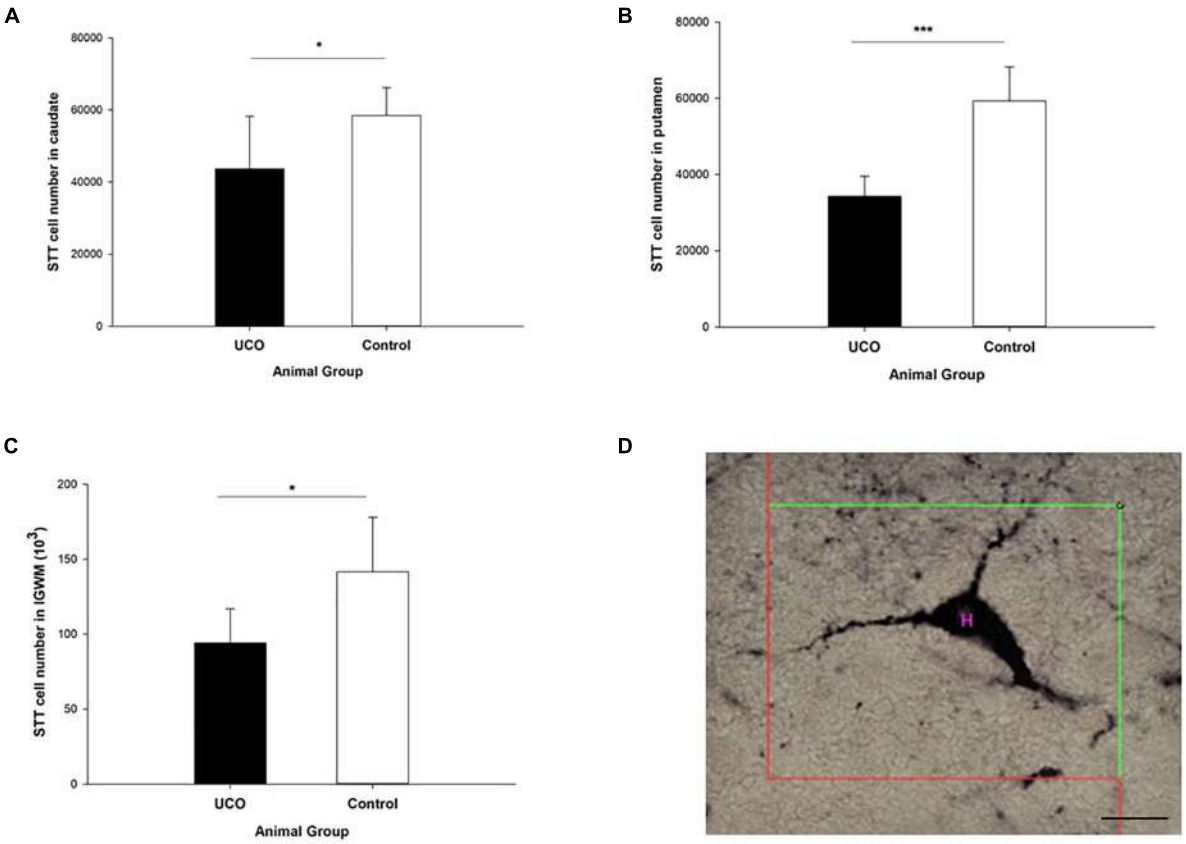


FIGURE 2 | The effect of umbilical cord occlusion (UCO) on the total number of somatostatin (STT) interneurons in block B regions of interest: **(A)** caudate; **(B)** putamen; **(C)** intragyral white matter (IGWM) (*$p < 0.05$, ***$p < 0.001$). **(D)** Illustration of counting the number of STT interneuron inside the unbiased counting frame on the light microscope connected to newCAST software with a 63X oil immersion lens. Mean ± SD. Scale bar = 15 μm.

DISCUSSION

To study the effect of prenatal asphyxia on the population of STT cells, we examined the number and morphology of STT cells in several brain regions 14 days following UCO in preterm fetal sheep. The animal model is well-established and regarded as suitable to examine the contribution of fetal asphyxia to neuropathological changes observed in infants born preterm (Koehler et al., 2018). Consistent with previous reports using the UCO model in preterm sheep, we found loss of NeuN positive neurons, particularly in subcortical gray matter areas, and reduced number of oligodendrocytes (Mallard et al., 2003; Bennet et al., 2007; van den Heuij et al., 2017). We provide new evidence demonstrating considerable dysmaturation of STT interneurons in different brain regions by showing a significant decrease in dendritic arborization of STT cells. While UCO resulted in a significant reduction in the number of STT interneurons in some brain regions, in the cerebral cortex, maturational delay occurred in the absence of loss of STT-positive cells. Further, while UCO resulted in a significant impairment of the morphology of STT cells in various brain regions, gross assessment of the brain injury did not concur with these alterations. Therefore, our results suggest high specific susceptibility of STT interneuron maturation to an asphyxial insult. Speculatively, this might impact on the balance between inhibitory and excitatory networks and could affect brain plasticity (Markram et al., 2004). A weakness of the study is that due to the size of the experimental groups, sex-dependent effects were not possible to determine. Further, the study would have been strengthened by addressing the role of neuroinflammation, such as effects on microglia and astrogliosis (GFAP).

About 20–30% of all neurons within the cerebral cortex are GABAergic interneurons and there are various subtypes of GABAergic interneurons, which have different spatial and temporal origins (Riedemann et al., 2016). GABAergic STT positive interneurons are the earliest born interneurons that migrate to the deep layers of the cortical plate during brain development (Miyoshi et al., 2007). It has been suggested that STT interneurons exhibit transient early synaptogenesis that is essential for the establishment of pavalbumin-dependent thalamocortical inhibition (Tuncdemir et al., 2016). A post-mortem study in infants born preterm (25–32 weeks gestation) showed that GABAergic interneurons are particularly vulnerable when also white matter injury is present (Liguz-Lecznar et al., 2016). It was also found that neuropeptide Y-positive

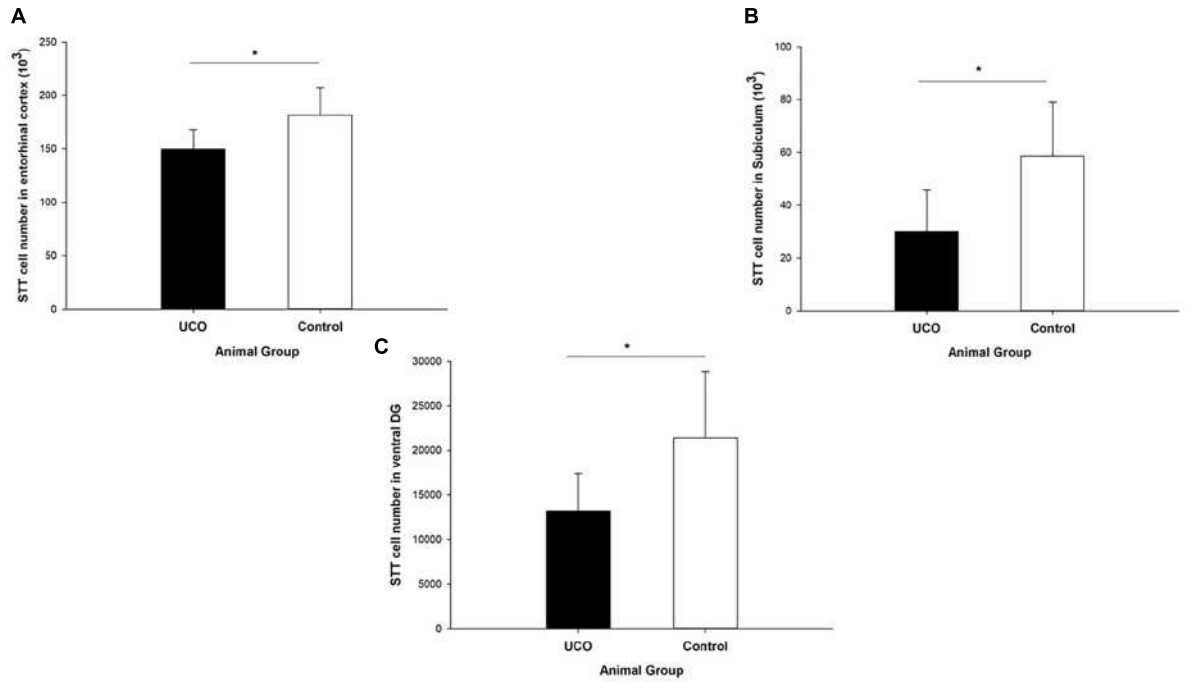


FIGURE 3 | The effect of UCO on the total number of STT interneurons in block C regions of interest: **(A)** entorhinal cortex, **(B)** subiculum, and **(C)** ventral DG. Mean ± SD. *$p < 0.05$. DG, dentate gyrus.

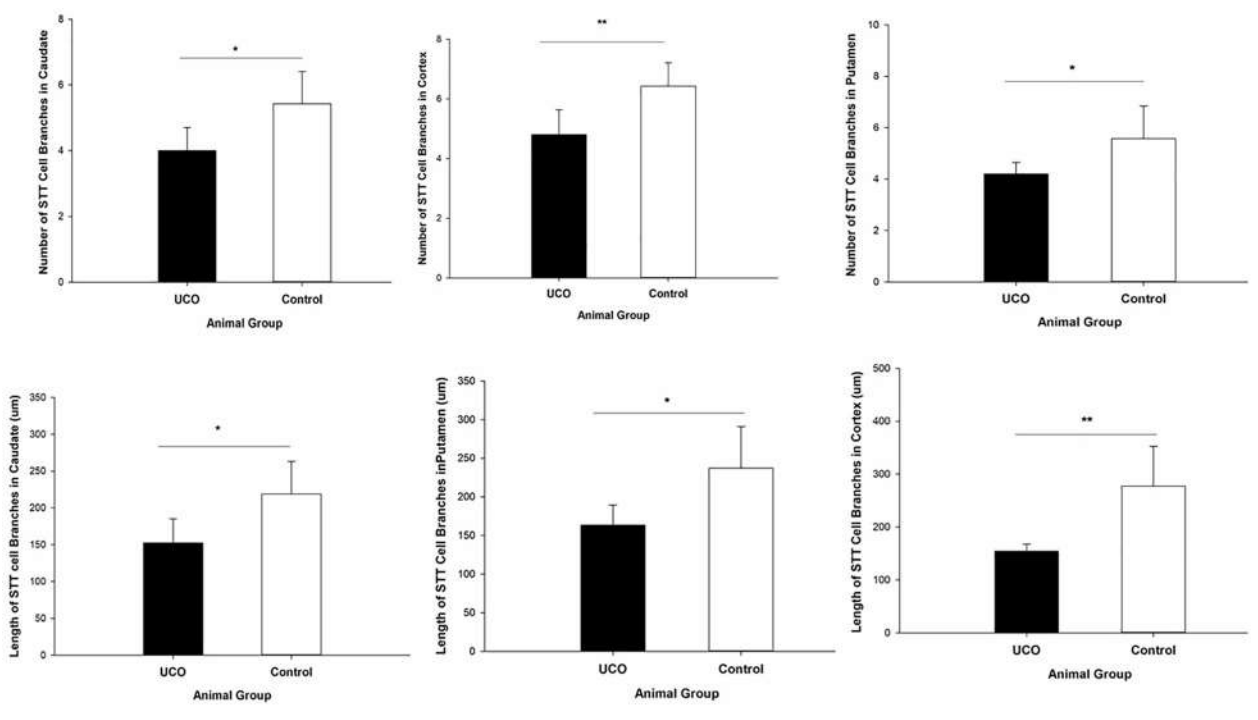


FIGURE 4 | Morphological analysis of STT interneurons showed a significant effect of UCO on the length and number of the STT dendrites in cortex, caudate, and putamen. Mean ± SD. *$p < 0.05$, **$p < 0.01$.

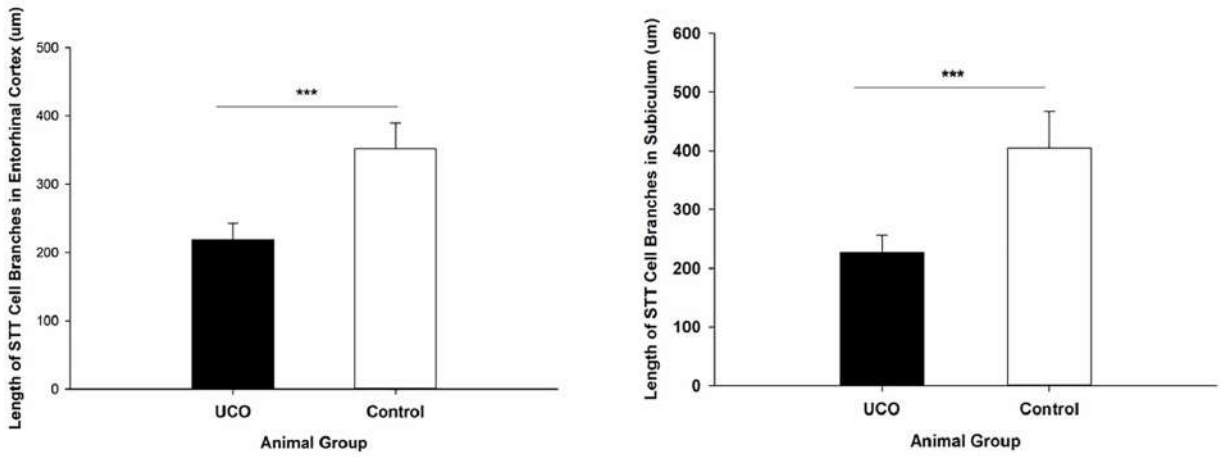


FIGURE 5 | Morphological analysis of STT interneurons showed a significant effect of UCO on the length of the STT dendrites in subiculum and EC. Mean ± SD. ***$p < 0.001$.

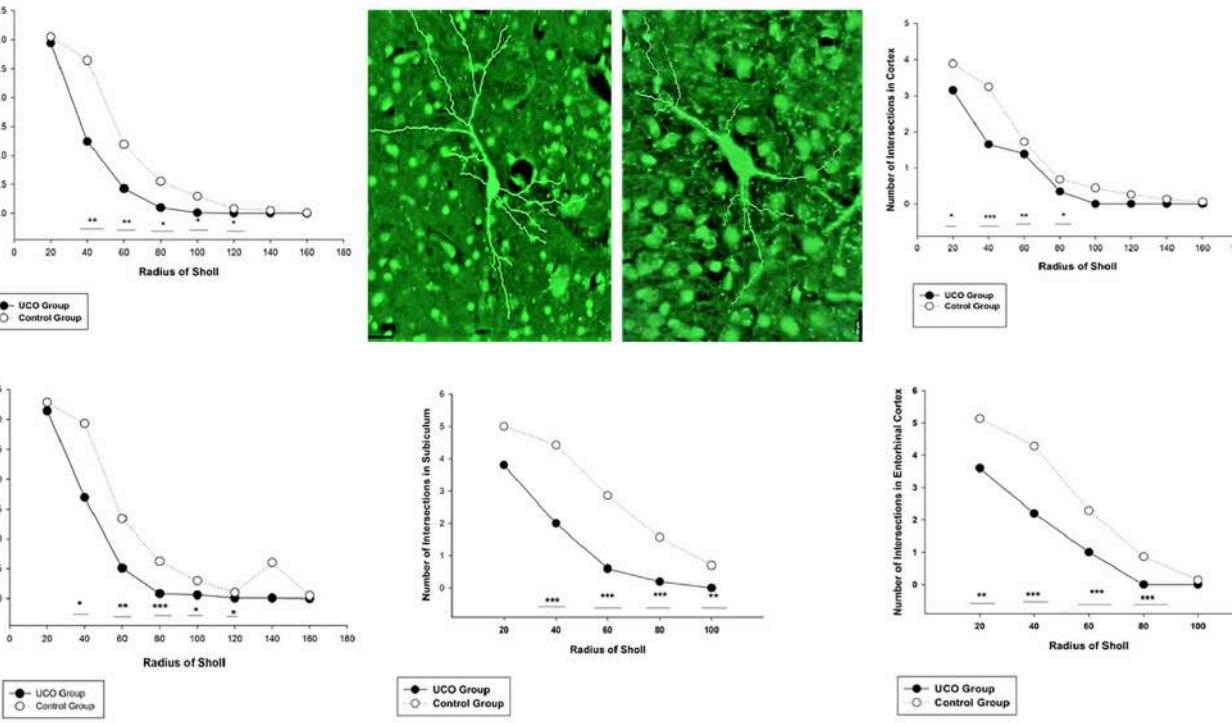


FIGURE 6 | Branching pattern alterations of STT interneurons in cortex, caudate, putamen, subiculum, and EC using Sholl analysis in UCO and control groups. The number of branching intersections at various distances away from the cell soma was significantly lower in the UCO group vs. control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Example of morphological analysis of STT interneuron in the putamen area of brain from control (left) and UCO (right) animals. Scale bar = 20 μm.

interneurons had shorter neurite lengths and there was a significantly lower number of calretinin-positive cells in telencephalon in these infants, which was suggested to contribute to impairment of cortical development (Robinson et al., 2006). Another clinical study found significant lower parvalbumin-positive neuronal density in 10 of 11 patients with severe periventricular leukomalacia (PVL), which correlated with developmental impairment of thalamocortical connections (Iai and Takashima, 1999). In an experimental *in utero* injury model with cortical dysplasia in mice, significant reduction in the total number of neurons and GABAergic interneurons in the neocortex was evident in early post-natal life (Deukmedjian et al., 2004). A recent study showed that global cerebral ischemia in near term fetal sheep causes

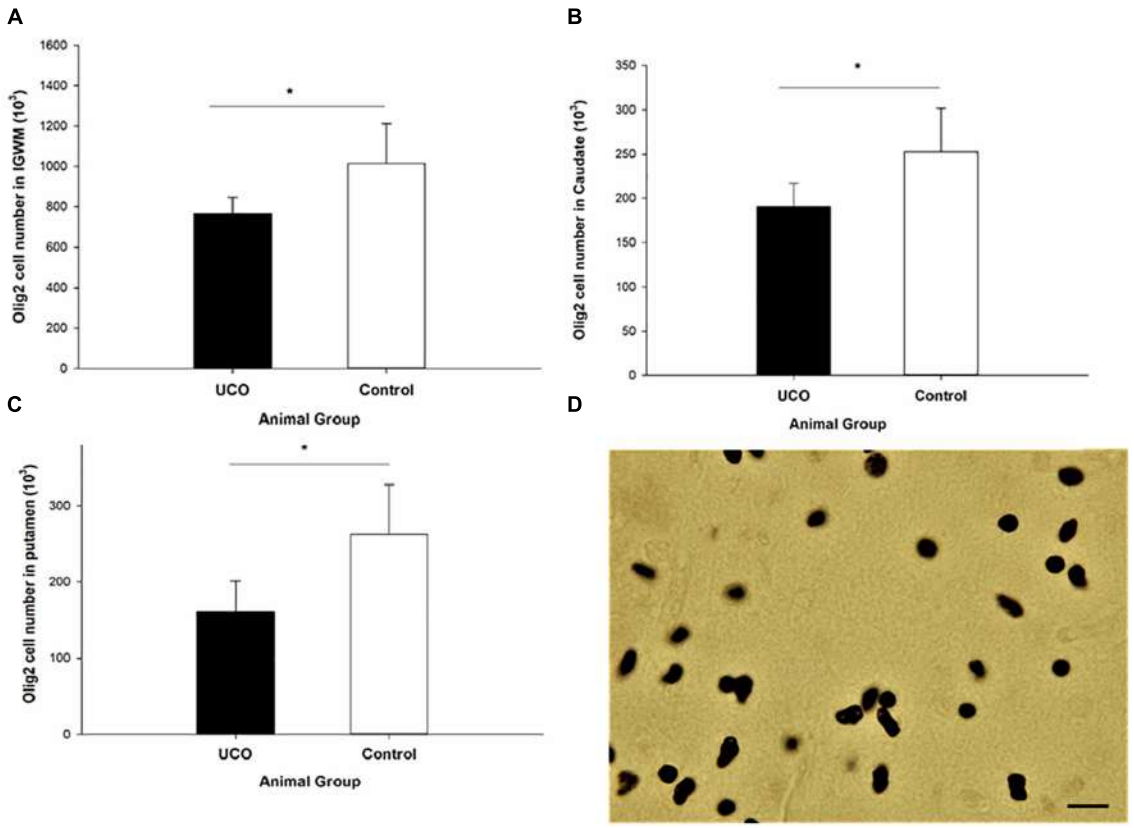


FIGURE 7 | The number of Olig-2 cells was significantly lower in **(A)** IGWM, **(B)** caudate, and **(C)** putamen areas in UCO group compared to the control group. $^*p < 0.05$. **(D)** Olig-2 positive cells with dark brown soma. Scale bar = 10 μm.

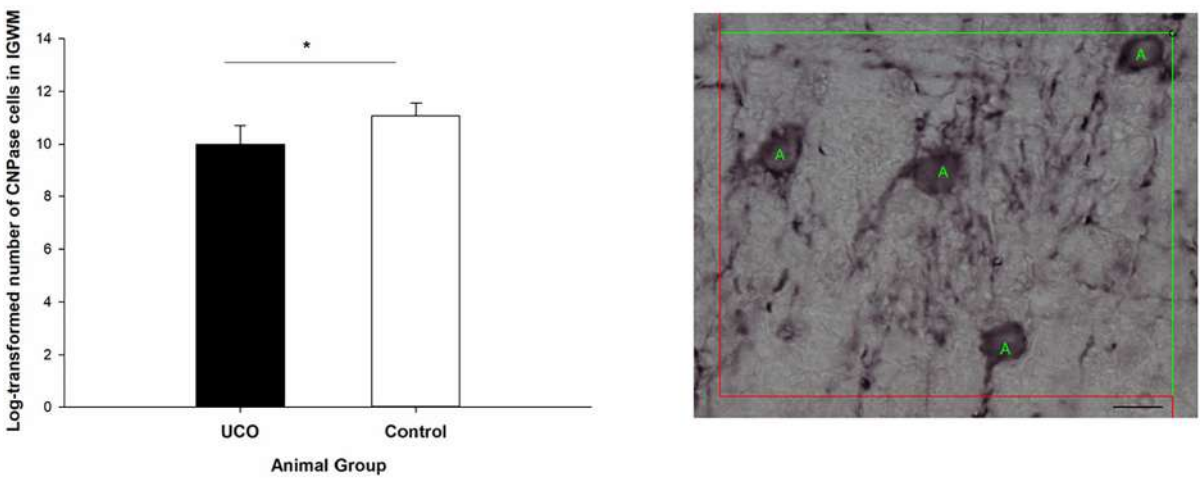


FIGURE 8 | Log transformed number of CNPase positive cells in the IGWM area of the UCO and the control groups. IGWM, intragyral white matter. $^*p < 0.05$ (left). Illustration of quantification of the number of CNPase cells in IGWM area of preterm fetal sheep (right). Scale bar = 10μm.

significant loss of GABAergic interneurons throughout the parasagittal cortex more specifically in cortical layer 6 (GAD+: by ∼88%; PV+: by ∼86%) at 1 week of recovery. It was suggested that the underlying mechanism of these changes was downregulation of interneuron markers on surviving neurons rather than cell death (Fowke et al., 2018). However, the previous studies did not examine the effect of perinatal brain damage on the number or maturation of the STT

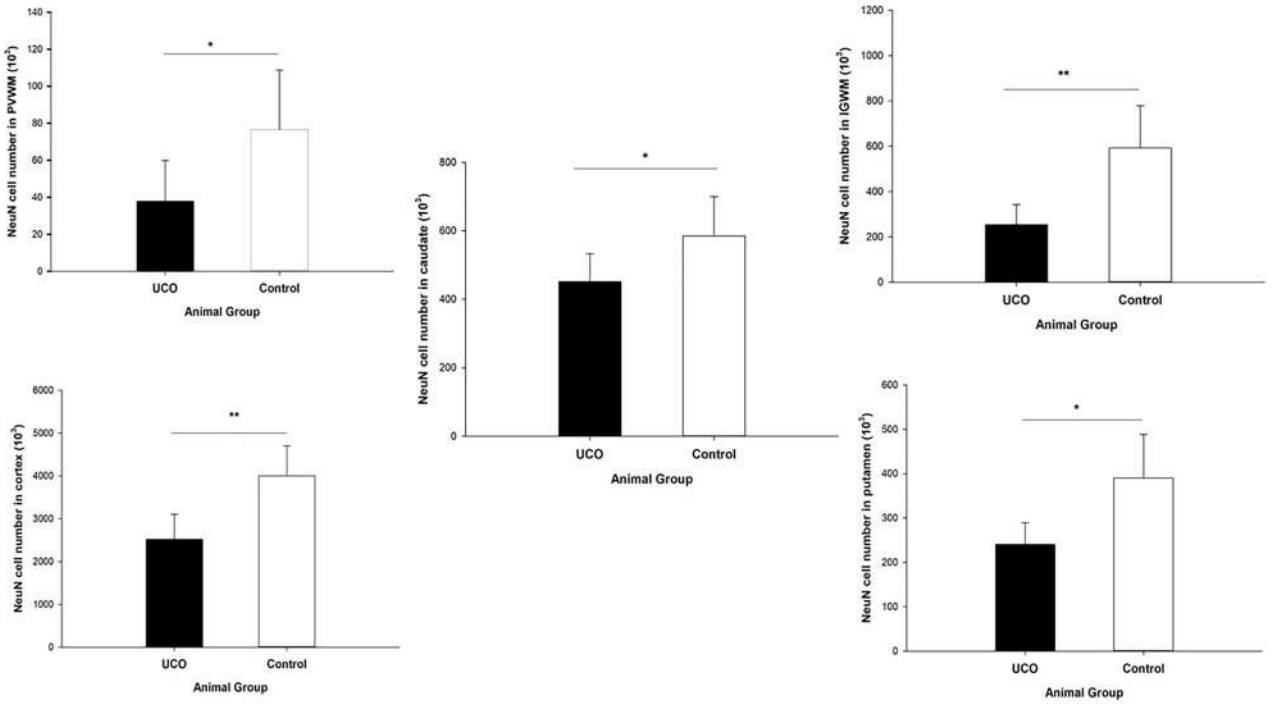


FIGURE 9 | Effect of UCO on the number of NeuN positive neurons in IGWM, cortex, PVWM, putamen, and caudate. *$p < 0.05$, **$p < 0.01$.

interneuron population. In the present study, we demonstrate that the number of STT interneurons is reduced in several subcortical regions including caudate, putamen, subiculum and ventral hippocampal DG, as well as EC, after UCO and the remaining STT neurons in these regions display altered morphological features. These regions are known to be sensitive to UCO resulting in neuronal loss as previously shown (Mallard et al., 2003; Bennet et al., 2007; van den Heuij et al., 2017). However, in the parasagittal cortex of UCO animals, morphological analysis of STT interneurons showed reduction of dendritic arborization and complexity of the dendritic branching pattern, which was not associated with an overall reduction in the number of STT positive cells. This finding indicates specifically perturbed maturation of STT cells rather than changes in interneuron genesis or apoptosis in parasagittal cortex following UCO. Interestingly, we could also detect a reduction of STT positive cells in the IGWM. These cells have been described in humans and primates as interstitial neurons that represent a specific population which is distinct from subplate neurons and neurons in adjacent structures (Judas et al., 2010). In fact, interstitial neurons may be future GABAergic neurons that tangentially migrate from the ganglionic eminence to the cortex in development (Yang et al., 2011). Overall our data draws the attention to maturational defects in the STT neuronal population after in utero asphyxia. Recently, two subtypes of STT interneurons, with distinct electrophysiological and morphological characteristics, were described (Riedemann et al., 2018). The neurochemical and functional diversity of STT interneurons may correlate with their vulnerability to utero asphyxia, which future studies should address.

It has been indicated that preterm birth results in activation of mechanisms such as dysynchrony of neurodevelopmental processing (Curristin et al., 2002) that can lead to maturational delay of both neurons and non-neuronal cells with the consequences of abnormality in circuit formation and function (Penn et al., 2016). Investigation of neuronal morphological development in premature infants suggests retardation of neuronal maturation (Takashima et al., 1982). In our study, the morphologic abnormalities in STT interneurons seen in different brain regions in the UCO group suggests delayed or arrested dendritic development of STT cells that may correspond to dysmaturation of cells. Vinall et al. (2013) demonstrated that the microstructural maturation rate of gray matter is lower in preterm infants in comparison with term infants due to multiple mechanisms including neuronal and non-neuronal cell death (Kinney et al., 2012) and disturbances in cellular arborization which may contribute to retarded maturation and underlie cognitive and learning disabilities in survivors of prenatal cerebral ischemia (Dean et al., 2013). Importantly, it has been shown that significant reduction of dendritic arborization and spine density in cortical and caudate projection neurons occurs without significant neuronal loss and therefore, it was concluded that reduction in dendritic arborization resulted from disrupted maturation, rather than from degeneration. In our study, we did find significant reduction in the number of NeuN positive cells in the cortex without significant changes in the number

of STT cells. This finding may be related to the higher expression of hypoxia-inducible factor-1 (HIF-1) demonstrated in cortical interneurons following hypoxic-ischemic insults (Ramamoorthy and Shi, 2014).

It is known that the morphology of neuropeptide Y-positive neurons with regard to the average length of the longest neurite is significantly shorter in infants with perinatal brain injury and this may contribute to the predisposition to epilepsy in premature infants (Robinson et al., 2006). It has been shown that 30 min UCO in preterm sheep is associated with abnormal EEG epileptiform activity (George et al., 2004) and loss of GABAergic interneurons was shown to be associated with epilepsy in mice (Asada et al., 1996; Schuler et al., 2001). However, a recent study did not find a relationship between loss of interneurons and seizure burden following cerebral ischemia in the near term fetal sheep (Fowke et al., 2018). Instead it was suggested that degradation of perineuronal nets may lead to increased interneuron excitability and seizure-like activity (Vedunova et al., 2013; Fowke et al., 2018). Accumulating clinical and experimental evidence also suggest an important role of subiculum in epilepsy and epileptogenesis by indicating that physiology and distribution of neuronal and interneuronal cells in subiculum can contribute to the pattern of epileptiform firing (Stafstrom, 2005). According to this hypothesis, the reduction of STT interneurons with morphological disturbances in the subiculum of UCO animals observed in the present study may explain the seizure activity previously reported in this model (Iai and Takashima, 1999).

Our results indicated a significant reduction in the number of STT cells in the ventral hippocampal DG but not in the dorsal DG. Previous studies in rodents indicate that STT interneurons are more abundant with a higher percentage of total GABA neurons in the ventral than in the dorsal DG (Kosaka et al., 1988; Jinno and Kosaka, 2003). STT neurons are known to be vulnerable to ischemia (Johansen et al., 1987; Esclapez and Houser, 1995) and the DG is a brain region with high rate of neurogenesis. Therefore, significant alteration of interneuron genesis or apoptosis in the ventral hippocampal DG may have contributed to the significant decrease in the number of STT cells in this area following UCO.

Regarding the impact of UCO on the number of oligodendrocytes, we found fewer Olig-2 and CNPase positive cells in IGWM in the UCO group compared with control group. This is consistent with previous studies in preterm fetal sheep that have shown a decline in both of these cell populations following UCO (Mallard et al., 2003; Bennet et al., 2007), but in contrast to others that reported a reduction in CNPase positive cells in IGWM, PVWM, without a change in number of Olig-2 positive oligodendrocytes (Drury et al., 2014; van den Heuij et al., 2017). Disturbances of oligodendrocyte development is dependent on the timing and location (Marin-Padilla, 1997). In brains of infants with severe focal lesions in the white matter (PVL), there is a notably effect on the development of oligodendrocytes (Iida et al., 1995; Robinson et al., 2006) and studies have shown an increased Olig-2 cell population in proximity to necrotic white matter lesions (Billiards et al., 2008). A preserved Olig-2 cell density is believed to be due to robust proliferation of pre-OLs, but with subsequent failure to terminally differentiate, resulting in myelin deficiency (Back and Rosenberg, 2014). However, in our study, we found significant loss of both CNPase positive and Olig-2 positive oligodendrocytes in the white matter which may correspond to cell death as well as maturational arrest of oligodendrocytes.

CONCLUSION

On the basis of our findings showing an abnormal maturation pattern in a population of GABAergic interneurons, STT interneurons, after UCO in preterm fetal sheep, we speculate that these changes could contribute to neurodevelopmental delay in preterm infants and impact on the balance between inhibitory and excitatory networks and may implicate a novel target for neuroprotective therapies.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

Animal experiments were approved by the local Animal Ethics Committee of Gothenburg (No. 166/13) and performed according to the guidelines for animal experimentation by the Swedish Department of Agriculture.

AUTHOR CONTRIBUTIONS

CM, HH, AB, VS, and MA designed the study. MA, PS, AB, VS, and JE performed the experiments, data collection, and data analysis. CM and MA interpreted the results and wrote the manuscript. All authors provided the conceptual advice, commented on the manuscript, and approved the final version of the manuscript for submission.

FUNDING

This work was supported by the Medical Research Council (MR/K006355/1), the Wellcome/EPSRC Centre for Medical Engineering at King's College London (WT 203148/Z/16/Z), and the National Institute for Health Research (NIHR), Biomedical Research Centre based at Guy's and St. Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health. We thank Wellcome Trust Programme grant (WT094823), Swedish Brain Foundation (FO2013-095, FO2014-008, FO2015-0094, and FO2017-0032), Swedish Medical Research Council (VR 2015-02493; 2017

-01409), ALFGBG-718591, ALFGBG-722491, ERA-net (EU; VR 529-2014-7551), Lilla Barnets Foundation (SLL 2017-0863), and Åhlén-Stiftelsen and Torsten Söderberg Stiftelsen (M98/15). GlaxoSmithKline provided financial funding for this study in the form of research service contract.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00563/full#supplementary-material

REFERENCES

Anzabi, M., Ardalan, M., Iversen, N. K., Rafati, A. H., Hansen, B., and Ostergaard, L. (2018). Hippocampal atrophy following subarachnoid hemorrhage correlates with disruption of astrocyte morphology and capillary coverage by AQP4. *Front. Cell. Neurosci.* 12:19. doi: 10.3389/fncel.2018.00019

Ardalan, M., Wegener, G., Polsinelli, B., Madsen, T. M., and Nyengaard, J. R. (2016). Neurovascular plasticity of the hippocampus one week after a single dose of ketamine in genetic rat model of depression. *Hippocampus* 26, 1414–1423. doi: 10.1002/hipo.22617

Asada, H., Kawamura, Y., Maruyama, K., Kume, H., Ding, R., Ji, F. Y., et al. (1996). Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures. *Biochem. Biophys. Res. Commun.* 229, 891–895. doi: 10.1006/bbrc.1996.1898

Back, S. A., and Rosenberg, P. A. (2014). Pathophysiology of glia in perinatal white matter injury. *Glia* 62, 1790–1815. doi: 10.1002/glia.22658

Ball, G., Boardman, J. P., Aljabar, P., Pandit, A., Arichi, T., Merchant, N., et al. (2013). The influence of preterm birth on the developing thalamocortical connectome. *Cortex* 49, 1711–1721. doi: 10.1016/j.cortex.2012.07.006

Bennet, L., Roelfsema, V., George, S., Dean, J. M., Emerald, B. S., and Gunn, A. J. (2007). The effect of cerebral hypothermia on white and grey matter injury induced by severe hypoxia in preterm fetal sheep. *J. Physiol.* 578(Pt 2), 491–506. doi: 10.1113/jphysiol.2006.119602

Bennet, L., Tan, S., Van den Heuij, L., Derrick, M., Groenendaal, F., van Bel, F., et al. (2012). Cell therapy for neonatal hypoxia-ischemia and cerebral palsy. *Ann. Neurol.* 71, 589–600. doi: 10.1002/ana.22670

Billiards, S. S., Haynes, R. L., Folkerth, R. D., Borenstein, N. S., Trachtenberg, F. L., Rowitch, D. H., et al. (2008). Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia. *Brain Pathol.* 18, 153–163. doi: 10.1111/j.1750-3639.2007.00107.x

Blencowe, H., Cousens, S., Chou, D., Oestergaard, M., Say, L., Moller, A. B., et al. (2013). Born too soon: the global epidemiology of 15 million preterm births. *Reprod. Health* 10(Suppl. 1):S2. doi: 10.1186/1742-4755-10-S1-S2

Boardman, J. P., Counsell, S. J., Rueckert, D., Kapellou, O., Bhatia, K. K., Aljabar, P., et al. (2006). Abnormal deep grey matter development following preterm birth detected using deformation-based morphometry. *Neuroimage* 32, 70–78. doi: 10.1016/j.neuroimage.2006.03.029

Butt, S. J., Stacey, J. A., Teramoto, Y., and Vagnoni, C. (2017). A role for GABAergic interneuron diversity in circuit development and plasticity of the neonatal cerebral cortex. *Curr. Opin. Neurobiol.* 43, 149–155. doi: 10.1016/j.conb.2017.03.011

Curristin, S. M., Cao, A., Stewart, W. B., Zhang, H., Madri, J. A., Morrow, J. S., et al. (2002). Disrupted synaptic development in the hypoxic newborn brain. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15729–15734. doi: 10.1073/pnas.232568799

Dean, J. M., McClendon, E., Hansen, K., Azimi-Zonooz, A., Chen, K., Riddle, A., et al. (2013). Prenatal cerebral ischemia disrupts MRI-defined cortical microstructure through disturbances in neuronal arborization. *Sci. Transl. Med.* 5:168ra7. doi: 10.1126/scitranslmed.3004669

Dean, J. M., van de Looij, Y., Sizonenko, S. V., Lodygensky, G. A., Lazeyras, F., Bolouri, H., et al. (2011). Delayed cortical impairment following lipopolysaccharide exposure in preterm fetal sheep. *Ann. Neurol.* 70, 846–856. doi: 10.1002/ana.22480

Deukmedjian, A. J., King, M. A., Cuda, C., and Roper, S. N. (2004). The GABAergic system of the developing neocortex has a reduced capacity to recover from in utero injury in experimental cortical dysplasia. *J. Neuropathol. Exp. Neurol.* 63, 1265–1273. doi: 10.1093/jnen/63.12.1265

Drury, P. P., Davidson, J. O., Bennet, L., Booth, L. C., Tan, S., Fraser, M., et al. (2014). Partial neural protection with prophylactic low-dose melatonin after asphyxia in preterm fetal sheep. *J. Cereb. Blood Flow Metab.* 34, 126–135. doi: 10.1038/jcbfm.2013.174

Esclapez, M., and Houser, C. R. (1995). Somatostatin neurons are a subpopulation of GABA neurons in the rat dentate gyrus: evidence from colocalization of pre-prosomatostatin and glutamate decarboxylase messenger RNAs. *Neuroscience* 64, 339–355. doi: 10.1016/0306-4522(94)00406-u

Fowke, T. M., Galinsky, R., Davidson, J. O., Wassink, G., Karunasinghe, R. N., Prasad, J. D., et al. (2018). Loss of interneurons and disruption of perineuronal nets in the cerebral cortex following hypoxia-ischaemia in near-term fetal sheep. *Sci. Rep.* 8:17686. doi: 10.1038/s41598-018-36083-y

Fraser, M., Bennet, L., Helliwell, R., Wells, S., Williams, C., Gluckman, P., et al. (2007). Regional specificity of magnetic resonance imaging and histopathology following cerebral ischemia in preterm fetal sheep. *Reprod. Sci.* 14, 182–191. doi: 10.1177/1933719107299612

George, S., Gunn, A. J., Westgate, J. A., Brabyn, C., Guan, J., and Bennet, L. (2004). Fetal heart rate variability and brain stem injury after asphyxia in preterm fetal sheep. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 287, R925–R933.

Gundersen, H. J. (1986). Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William, R. Thompson. *J. Microsc.* 143(Pt 1), 3–45. doi: 10.1111/j.1365-2818.1986.tb02764.x

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Iai, M., and Takashima, S. (1999). Thalamocortical development of parvalbumin neurons in normal and periventricular leukomalacia brains. *Neuropediatrics* 30, 14–18. doi: 10.1055/s-2007-973450

Iida, K., Takashima, S., and Ueda, K. (1995). Immunohistochemical study of myelination and oligodendrocyte in infants with periventricular leukomalacia. *Pediatr. Neurol.* 13, 296–304. doi: 10.1016/0887-8994(95)00192-1

Jinno, S., and Kosaka, T. (2003). Patterns of expression of neuropeptides in GABAergic nonprincipal neurons in the mouse hippocampus: quantitative analysis with optical disector. *J. Comp. Neurol.* 461, 333–349. doi: 10.1002/cne.10700

Johansen, F. F., Zimmer, J., and Diemer, N. H. (1987). Early loss of somatostatin neurons in dentate hilus after cerebral ischemia in the rat precedes CA-1 pyramidal cell loss. *Acta Neuropathol.* 73, 110–114. doi: 10.1007/bf00693775

Judas, M., Sedmak, G., Pletikos, M., and Jovanov-Milosevic, N. (2010). Populations of subplate and interstitial neurons in fetal and adult human telencephalon. *J. Anat.* 217, 381–399. doi: 10.1111/j.1469-7580.2010.01284.x

Kinney, H. C., Haynes, R. L., Xu, G., Andiman, S. E., Folkerth, R. D., Sleeper, L. A., et al. (2012). Neuron deficit in the white matter and subplate in periventricular leukomalacia. *Ann. Neurol.* 71, 397–406. doi: 10.1002/ana.22612

Koehler, R. C., Yang, Z. J., Lee, J. K., and Martin, L. J. (2018). Perinatal hypoxic-ischemic brain injury in large animal models: relevance to human neonatal encephalopathy. *J. Cereb. Blood Flow Metab.* 38, 2092–2111. doi: 10.1177/0271678X18797328

Kosaka, T., Wu, J. Y., and Benoit, R. (1988). GABAergic neurons containing somatostatin-like immunoreactivity in the rat hippocampus and dentate gyrus. *Exp. Brain Res.* 71, 388–398.

Le Magueresse, C., and Monyer, H. (2013). GABAergic interneurons shape the functional maturation of the cortex. *Neuron* 77, 388–405. doi: 10.1016/j.neuron.2013.01.011

Liguz-Lecznar, M., Urban-Ciecko, J., and Kossut, M. (2016). Somatostatin and somatostatin-containing neurons in shaping neuronal activity and plasticity. *Front. Neural Circuits* 10:48. doi: 10.3389/fncir.2016.00048

Ludwig, M., and Pittman, Q. J. (2003). Talking back: dendritic neurotransmitter release. *Trends Neurosci.* 26, 255–261. doi: 10.1016/s0166-2236(03)00072-9

Mallard, C., Welin, A. K., Peebles, D., Hagberg, H., and Kjellmer, I. (2003). White matter injury following systemic endotoxemia or asphyxia in the fetal sheep. *Neurochem. Res.* 28, 215–223. doi: 10.1023/a:1022368915400

Mallard, E. C., Williams, C. E., Johnston, B. M., and Gluckman, P. D. (1994). Increased vulnerability to neuronal damage after umbilical cord occlusion in fetal sheep with advancing gestation. *Am. J. Obstet. Gynecol.* 170(1 Pt 1), 206–214. doi: 10.1016/s0002-9378(94)70409-0

Marin-Padilla, M. (1997). Developmental neuropathology and impact of perinatal brain damage. II: white matter lesions of the neocortex. *J. Neuropathol. Exp. Neurol.* 56, 219–235. doi: 10.1097/00005072-199703000-00001

Markram, H., Toledo-Rodriguez, M., Wang, Y., Gupta, A., Silberberg, G., and Wu, C. (2004). Interneurons of the neocortical inhibitory system. *Nat. Rev. Neurosci.* 5, 793–807. doi: 10.1038/nrn1519

Miyoshi, G., Butt, S. J., Takebayashi, H., and Fishell, G. (2007). Physiologically distinct temporal cohorts of cortical interneurons arise from telencephalic Olig2-expressing precursors. *J. Neurosci.* 27, 7786–7798. doi: 10.1523/jneurosci.1807-07.2007

Miyoshi, G., and Fishell, G. (2011). GABAergic interneuron lineages selectively sort into specific cortical layers during early postnatal development. *Cereb. Cortex* 21, 845–852. doi: 10.1093/cercor/bhq155

Pascal, A., Govaert, P., Oostra, A., Naulaers, G., Ortibus, E., and Van den Broeck, C. (2018). Neurodevelopmental outcome in very preterm and very-low-birthweight infants born over the past decade: a meta-analytic review. *Dev. Med. Child Neurol.* 60, 342–355. doi: 10.1111/dmcn.13675

Penn, A. A., Gressens, P., Fleiss, B., Back, S. A., and Gallo, V. (2016). Controversies in preterm brain injury. *Neurobiol. Dis.* 92(Pt A), 90–101. doi: 10.1016/j.nbd.2015.10.012

Powell, E. M., Campbell, D. B., Stanwood, G. D., Davis, C., Noebels, J. L., and Levitt, P. (2003). Genetic disruption of cortical interneuron development causes region- and GABA cell type-specific deficits, epilepsy, and behavioral dysfunction. *J. Neurosci.* 23, 622–631. doi: 10.1523/jneurosci.23-02-00622.2003

Ramamoorthy, P., and Shi, H. (2014). Ischemia induces different levels of hypoxia inducible factor-1alpha protein expression in interneurons and pyramidal neurons. *Acta Neuropathol. Commun.* 2:51. doi: 10.1186/2051-5960-2-51

Riedemann, T., Schmitz, C., and Sutor, B. (2016). Immunocytochemical heterogeneity of somatostatin-expressing GABAergic interneurons in layers II and III of the mouse cingulate cortex: a combined immunofluorescence/design-based stereologic study. *J. Comp. Neurol.* 524, 2281–2299. doi: 10.1002/cne.23948

Riedemann, T., Straub, T., and Sutor, B. (2018). Two types of somatostatin-expressing GABAergic interneurons in the superficial layers of the mouse cingulate cortex. *PLoS One* 13:e0200567. doi: 10.1371/journal.pone.0200567

Robinson, S., Li, Q., Dechant, A., and Cohen, M. L. (2006). Neonatal loss of gamma-aminobutyric acid pathway expression after human perinatal brain injury. *J. Neurosurg.* 104(6 Suppl.), 396–408. doi: 10.3171/ped.2006.104.6.396

Rudy, B., Fishell, G., Lee, S., and Hjerling-Leffler, J. (2011). Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. *Dev. Neurobiol.* 71, 45–61. doi: 10.1002/dneu.20853

Scheyltjens, I., and Arckens, L. (2016). The current status of somatostatin-interneurons in inhibitory control of brain function and plasticity. *Neural Plast.* 2016:8723623. doi: 10.1155/2016/8723623

Schuler, V., Luscher, C., Blanchet, C., Klix, N., Sansig, G., Klebs, K., et al. (2001). Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B(1)). *Neuron* 31, 47–58. doi: 10.1016/s0896-6273(01)00345-2

Stafstrom, C. E. (2005). The role of the subiculum in epilepsy and epileptogenesis. *Epilepsy Curr.* 5, 121–129. doi: 10.1111/j.1535-7511.2005.00049.x

Takashima, S., Becker, L. E., and Chan, F. W. (1982). Retardation of neuronal maturation in premature infants compared with term infants of the same postconceptional age. *Pediatrics* 69, 33–39.

Tau, G. Z., and Peterson, B. S. (2010). Normal development of brain circuits. *Neuropsychopharmacology* 35, 147–168. doi: 10.1038/npp.2009.115

Tuncdemir, S. N., Wamsley, B., Stam, F. J., Osakada, F., Goulding, M., Callaway, E. M., et al. (2016). Early somatostatin interneuron connectivity mediates the maturation of deep layer cortical circuits. *Neuron* 89, 521–535. doi: 10.1016/j.neuron.2015.11.020

Van de Berg, W. D., Kwaijtaal, M., de Louw, A. J., Lissone, N. P., Schmitz, C., Faull, R. L., et al. (2003). Impact of perinatal asphyxia on the GABAergic and locomotor system. *Neuroscience* 117, 83–96. doi: 10.1016/s0306-4522(02)00787-x

van den Heuij, L. G., Fraser, M., Miller, S. L., Jenkin, G., Wallace, E. M., Davidson, J. O., et al. (2017). Delayed intranasal infusion of human amnion epithelial cells improves white matter maturation after asphyxia in preterm fetal sheep. *J. Cereb. Blood Flow Metab.* 39, 223–239. doi: 10.1177/0271678X17729954

Vedunova, M., Sakharnova, T., Mitroshina, E., Perminova, M., Pimashkin, A., Zakharov, Y., et al. (2013). Seizure-like activity in hyaluronidase-treated dissociated hippocampal cultures. *Front. Cell. Neurosci.* 7:149. doi: 10.3389/fncel.2013.00149

Vinall, J., Grunau, R. E., Brant, R., Chau, V., Poskitt, K. J., Synnes, A. R., et al. (2013). Slower postnatal growth is associated with delayed cerebral cortical maturation in preterm newborns. *Sci. Transl. Med.* 5:168ra8. doi: 10.1126/scitranslmed.3004666

Volpe, J. J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 8, 110–124. doi: 10.1016/S1474-4422(08)70294-1

Wonders, C. P., and Anderson, S. A. (2006). The origin and specification of cortical interneurons. *Nat. Rev. Neurosci.* 7, 687–696. doi: 10.1038/nrn1954

Yang, Y., Fung, S. J., Rothwell, A., Tianmei, S., and Weickert, C. S. (2011). Increased interstitial white matter neuron density in the dorsolateral prefrontal cortex of people with schizophrenia. *Biol. Psychiatry* 69, 63–70. doi: 10.1016/j.biopsych.2010.08.020

Yavorska, I., and Wehr, M. (2016). Somatostatin-expressing inhibitory interneurons in cortical circuits. *Front. Neural Circuits* 10:76. doi: 10.3389/fncir.2016.00076

Conflict of Interest Statement: The authors declare that this study received funding from GlaxoSmithKline. The funder had some involvement in the study design, but no role in data collection and analysis, decision to publish, or preparation of the manuscript. All authors declare no conflict of interest.

Copyright © 2019 Ardalan, Svedin, Baburamani, Supramaniam, Ek, Hagberg and Mallard. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

CASE REPORT
published: 24 May 2019
doi: 10.3389/fphys.2019.00623

Post-mortem Characterisation of a Case With an *ACTG1* Variant, Agenesis of the Corpus Callosum and Neuronal Heterotopia

Regina Vontell[1,2], Veena G. Supramaniam[1], Alice Davidson[1], Claire Thornton[1,3], Andreas Marnerides[4], Muriel Holder-Espinasse[5], Suzanne Lillis[5], Shu Yau[5], Mattias Jansson[5], Henrik E. Hagberg[1,6] and Mary A. Rutherford[1]**

[1] Centre for the Developing Brain, Division of Imaging Sciences and Biomedical Engineering, King's College London, St Thomas' Hospital, London, United Kingdom, [2] Department of Neurology, University of Miami Miller School of Medicine, Miami, FL, United States, [3] Department of Comparative Biomedical Sciences, Royal Veterinary College, London, United Kingdom, [4] Department of Cellular Pathology, Guy's and St Thomas' NHS Foundation Trust, St Thomas' Hospital, London, United Kingdom, [5] Department of Clinical Genetics, Guy's and St Thomas' NHS Foundation Trust, Guy's Hospital, London, United Kingdom, [6] Perinatal Center, Department of Physiology and Neuroscience – Department of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

OPEN ACCESS

Edited by:
Mary Tolcos,
RMIT University, Australia

Reviewed by:
Gavin John Clowry,
Newcastle University, United Kingdom
Fiona Francis,
INSERM U839 Institut du Fer à Moulin, France

***Correspondence:**
Regina Vontell
rvontell@miami.edu
Mary A. Rutherford
mary.rutherford@kcl.ac.uk

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: 12 October 2018
Accepted: 02 May 2019
Published: 24 May 2019

Citation:
Vontell R, Supramaniam VG, Davidson A, Thornton C, Marnerides A, Holder-Espinasse M, Lillis S, Yau S, Jansson M, Hagberg HE and Rutherford MA (2019) Post-mortem Characterisation of a Case With an ACTG1 Variant, Agenesis of the Corpus Callosum and Neuronal Heterotopia. Front. Physiol. 10:623. doi: 10.3389/fphys.2019.00623

Cytoplasmic Actin Gamma 1 (*ACTG1*) gene variant are autosomal dominant and can cause CNS anomalies (Baraitser Winter Malformation Syndrome; BWMS). *ACTG1* anomalies in offspring include agenesis of the corpus callosum (ACC) and neuronal heterotopia which are ectopic nodules of nerve cells that failed to migrate appropriately. Subcortical and periventricular neuronal heterotopia have been described previously in association with ACC. In this case report, we investigated a neonatal brain with an *ACTG1* gene variant and a phenotype of ACC, and neuronal heterotopia (ACC-H) which was diagnosed on antenatal MR imaging and was consistent with band heterotopia seen on post-mortem brain images. Histologically clusters of neurons were seen in both the subcortical and periventricular white matter (PVWM) brain region that coincided with impaired abnormalities in glial formation. Immunohistochemistry was performed on paraffin-embedded brain tissue blocks from this case with *ACTG1* variant and an age-matched control. Using tissue sections from the frontal lobe, we examined the distribution of neuronal cells (HuC/HuD, calretinin, and parvalbumin), growth cone (drebrin), and synaptic proteins (synaptophysin and SNAP-25). Additionally, we investigated how the *ACTG1* variant altered astroglia (nestin, GFAP, vimentin); oligodendroglia (OLIG2) and microglia (Iba-1) in the corpus callosum, cortex, caudal ganglionic eminence, and PVWM. As predicted in the *ACTG1* variant case, we found a lack of midline radial glia and glutamatergic fibers. We also found disturbances in the cortical region, in glial cells and a lack of extracellular matrix components in the *ACTG1* variant. The caudal ganglionic eminence and the PVWM regions in the *ACTG1* variant lacked several cellular components that were identified in a control case. Within the neuronal heterotopia, we found evidence of glutamatergic and GABAergic neurons with

apparent synaptic connections. The data presented from this case study with BWMS with variants in the *ACTG1* gene provides insight as to the composition of neuronal heterotopia, and how disturbances of important migratory signals may dramatically affect ongoing brain development.

Keywords: heterotopia, radial glia, corpus callosum, growth cone, synaptic proteins

INTRODUCTION

Baraitser Winter malformation syndrome (BWMS) is associated with variants in the Cytoplasmic Actin Gamma 1 genes *ACTB* or *ACTG1*, which encodes β- and γ-actins (Verloes et al., 2014). Besides cerebrofrontofacial dysmorphisms, common CNS anomalies are pachygyria, subcortical band heterotopia and agenesis of the corpus callosum (ACC) (Verloes et al., 2014). The corpus callosum consists of over 200 million glutamatergic axonal fibers that connect the two cerebral hemispheres. Its formation requires intricate orchestration of numerous processes involving neuronal migration, synapse formation, and axonal guidance. The corpus callosal structure is formed by 20 weeks gestation and expands during antenatal development as its fibers are progressively premyelinated (Paul, 2011). ACC represents one of the most common antenatally diagnosed malformations of the brain and may be isolated or associated with other cerebral or non-cerebral abnormalities. Neuronal heterotopia has been reported in association with ACC, which may be clinically detected with MR imaging or only diagnosed histologically at postmortem. It is possible that neuronal migration disturbances are a common and unrecognized accompaniment of apparently isolated ACC. Neuronal migration is a well-orchestrated event. Neuroepithelial cells produce early radial progenitor cells in the ventricular zone and provide important cues for migrating neurons (Kadhim et al., 1993). The failure of neuronal migration due to genetic variants that interfere with glial structure and function are associated with the presence of neuronal heterotopias (Marcorelles et al., 2010; Kato, 2015; Ibanez and Andressoo, 2016). The presence of even small focal heterotopias in a cortical region can affect distant brain regions and give rise to behavioral abnormalities (Edwards et al., 2014). Neurodevelopmental impairments in isolated ACC are variable but are more severe in the presence of additional anomalies, and the occurrence of overt neuronal heterotopia is associated with poorer outcomes (Edwards et al., 2014; Ishii et al., 2015).

In the human embryo, callosal axons are first identified at 74 days post-conception, with adult morphology achieved around 115 post-conception days (Achiron and Achiron, 2001). In the frontal brain, the forceps minor fiber bundle, also known as the anterior forceps, connects the lateral and medial surfaces of the frontal lobes and crosses the midline via the genu of the corpus callosum. During development, the leading edge of the axonal fibers transverse into the genu using chemoattractant cues expressed by radial glia. The combination of neurons and glia are referred to as the indusium griseum glia (Sturrock, 1978). The indusium griseum glia forms the wide-spanning fibrous region of corpus callosum creating the callosal glial sling (Shu and Richards, 2001; Paul, 2011). In ACC, there may be a spectrum of developmental abnormalities from the entire absence of the corpus callosum to a thinning of callosal fibers that can be seen in the forceps minor.

In the developing brain, the cells of the subependymal germinative zone, a remnant of the subventricular zone, maintain high expression of antigen Ki-67 or MIB-1 for cellular proliferation (Moss et al., 2016) to support both radial glia and neuronal production. In later stages, 15–24 weeks gestation (Kostoviæ et al., 2015) of development, radial glia and phenotypically related astroglia are highly positive for vimentin or glial fibrillary acid protein (GFAP) (Bramanti et al., 2010; Moss et al., 2016). These radial glia will continue from 17 weeks gestation to guide the neuronal migration and the commissural axons of the corpus callosum (Edwards et al., 2014) by extension of the radial processes.

Neurons migrate to the cortex in a succession of waves using radial glia but follow two basic migration paths, either radial or tangential (Volpe, 2008). Radial neuronal migration is used by glutamatergic neurons (Lagercrantz, 2010) which, from 17–30 weeks gestation, will subsequently create axons that express a synaptosome-associated protein of 25 kDa (SNAP-25). SNAP-25 protein plays an important role in synaptic development and also maintains life-long callosal connections (Condliffe and Matteoli, 2011; Antonucci et al., 2013; Yang et al., 2017; Pozzi et al., 2018). The tangentially migrating neurons originate from the lateral, medial and caudal ganglionic eminence and translocate tangentially across the radial glial fibers also in a succession of migration waves (Erzurumlu et al., 2006; Sultan et al., 2014) to reach the cortex or into the central gray matter structures (Brazel et al., 2003; Wonders and Anderson, 2006). Neurons originating in the ganglionic eminence are mainly gamma-aminobutyric acid (GABA) ergic (Marin and Rubenstein, 2001; Kolasinski et al., 2013). GABAergic interneurons follow guidance cues from the extracellular matrix (ECM) and express distinct proteins that are specific to the region of origin. For instance, medial ganglionic eminence neurons are positive for parvalbumin, and somatostatin (Clowry, 2014; Sultan et al., 2014) whilst caudal ganglionic eminence (CGE) neurons express calretinin and neuropeptide Y (Wonders and Anderson, 2006).

Migrating neurons seek out environmental cues using a leading edge or growth cone structure (Leviton and Gressens, 2007; Volpe, 2008). The growth cone

protrudes from the neuronal cell body as it leaves its site of origin (e.g., the ganglionic eminence) and elongates as it travels through the periventricular white matter (PVWM), using the radial glia as guideposts. The extended growth cone facilitates the neuron's ability to migrate through the hyaluronic acid-rich ECM environment to reach its final destination (e.g., cortex) (Bruckner et al., 1993; De Luca and Papa, 2016). During migration or on contact with other neurons, the growth cone tip maintains its splay formation by using microtubule bundles and actin filaments (F-actin) and recruiting of another set of ligands, end binding 3 (EB3) and drebrin proteins (Geraldo et al., 2008; Conde and Caceres, 2009; Geraldo and Gordon-Weeks, 2009; Sonego et al., 2015). The effect of an *ACTG1* variant on growth cone structure and function is not well described.

This case study uses immunohistochemical analysis to investigate the corpus callosum (forceps minor and the genu) and the frontal cortex in a child subsequently diagnosed with an *ACTG1* variant and with clinical signs consistent with BWMS. To determine the pathophysiology associated with the *ACTG1* variant, we analyzed the underlying perturbation of migratory cues in the CGE by visualizing proliferation, neuronal and growth cone proteins. Additionally, we sought to find if the CGE of this case of with *ACTG*1 variant differed from the control in protein expression of radial glia and other types of glial cell (e.g., oligodendroglia and microglia). Finally, we completed a molecular dissection of the neuronal heterotopia seen within the PVWM, both on *in vivo* imaging and at histology, here, we sought to detect growth cone proteins, synaptic proteins and define neuronal composition (i.e., glutamatergic and GABAergic).

METHODS

Both written and informed parental consent was obtained from the participants of this study, acquired for post-mortem examination and post-mortem research according to National Health Service United Kingdom and Human Tissue Authority guidelines. Research study ethics was obtained from the National Research Ethics Service (West London), United Kingdom [ethics number, 07/H0707/139; Post-mortem Magnetic Resonance Imaging (MRI) Study of the Developing Brain].

MRI

Magnetic Resonance Imaging of the fetal brains were acquired on a 1.5 Tesla Philips Ingenia scanner using single shot T2 weighted sequences acquired in three orthogonal planes using our standard clinical protocols. The case with the *ACTG1* variant was refrigerated (2–4°C) before post-mortem examination which was performed within 2 days of death. The whole post-mortem brain was fixed in 4% formalin for 5–6 weeks. Toward the end of the fixation period, MRI was performed on the fixed whole *ACTG1* brain at 3 Tesla (Philips), 21 days post-death.

Tissue Preparation

This study assessed histological findings in the post-mortem brain of our *ACTG1* variant case where the pregnancy was terminated at 35.71 weeks. Findings were compared with an age-matched brain from a preterm infant who died (oligohydramnios and immature lungs) at 32.71 weeks gestational (GA); (age-matched control). The control infant brain showed no significant pathology on gross and microscopic examination and was, therefore, felt appropriate to be used as a non-neuropathological age-matched control (control case).

For both cases, the whole brain was sliced by a pathologist (A.M.), and the tissue blocks were processed on a Leica Tissue Processor (Leica Biosystems, Newcastle, United Kingdom). The paraffin-embedded tissue blocks were sectioned at 6 μm using a Leica RM2245 microtome (Leica Microsystems Ltd., Newcastle, United Kingdom). Paraffin-embedded tissue sections taken from the caudal frontal lobes (at the level of the posterior Ammon's horn) were used for immunohistochemistry and histochemistry.

Immunohistochemistry

Standard immunohistochemistry procedures for the brain sections have been described previously (Vontell et al., 2013, 2015) with the addition of methyl green counterstain being substituted for hematoxylin. Primary antibodies, catalog numbers, species and concentrates along with the secondary antibodies are listed in **Supplementary Data**.

Histochemistry

The standard tissue paraffin block was sectioned at 6 μm and the slides were allowed to dry and then heated at 60°C for 30 min. Prior to staining, sections were deparaffinized in three changes of xylene and rehydrated through graded concentrations of ethanol. The histological stain, hematoxylin, and eosin (H&E), used to evaluate the general morphology of the tissue and orientation of the brain regions were described in Gill et al. (1974). The procedures for visualizing the extracellular matrix, the perineuronal nets, and hyaluronic acid content have been previously described (Bruckner et al., 1993) with the addition of Curtis Nuclear Stain (Leach, 1946).

Microscopic Analyses

Unbiased images were obtained using the CM1 and CM2 modules for virtual tissue scan (MicroBrightfield Inc., Colchester, VT, United States) using stereology software (Stereo Investigator version 8.27; MicroBrightfield Inc.). The average area of each contour was encompassed by a 1.0–1.5-mm^2 region, determined on the microscope using a 5× objective, to provide an average of 40 high-power field images per scan collected, using a 40× objective (0.0625 mm^2) or a 63× objective (0.0594 mm^2).

Whole Exome Sequencing and Analysis

Whole exome sequencing (WES) capture was performed using Agilent SureSelect XT Clinical Research Exome (CRE; SureSelectXT Human All Exon V5; Santa Clara, CA, United States) baited with clinically relevant genes. The enriched exome libraries were sequenced using paired-end, 125 cycle chemistry on an Illumina NextSeq 550 (Cambridge, United Kingdom). An integrated laboratory and bioinformatics

platform was used in which Agilent CRE libraries are sequenced with automatic data transfer to DNAnexus for alignment and variant calling using BWA and GATK. The quality threshold was set at 95% at 20X coverage. The generated VCF files were uploaded into QIAGEN Ingenuity Variant Analysis (IVA; Manchester, United Kingdom) for assessment. The use of in-house designed "virtual panels" or 100K Genomes "PanelApp" gene panels restricts the analysis to only clinically relevant genes. Assessment of pathogenicity was performed using Performed using QIAGEN IVA (CADD, CentoMD, EVS, Allele Frequency Community, JASPAR, Ingenuity Knowledge Base, Vista Enhancer, OMIM, gnomAD, Clinical Trials, BSIFT, TCGA, PolyPhen-2, 1000 Genome Frequency, Clinvar, DGV, COSMIC, ExAC, HGMD, PhyloP, DbSNP, TargetScan, SIFT4G), and Alamut for splice site analysis (Rouen, France; SpliceSiteFinder-like, MaxEntScan, NNSplice, GeneSplicer). Variant classifications were performed according to ACMG guidelines (Richards et al., 2015). The case was then presented at a mixed disciplinary team meeting where the genetic findings were discussed in light of the clinical findings and the phenotype of the patient. A diagnostic report was issued, alongside a list of all genes analyzed within the panel, detailing the coverage at 20X.

MRI FINDINGS

In this case study, we investigated a post-mortem brain 35.71 gestational age (GA; weeks). The mother (gravida 2 para 1) presented with a female fetus demonstrating both fetal growth restriction and signs consistent with ACC on an ultrasound conducted at 33 GA weeks. A fetal MRI conducted at 35.30 GA weeks confirmed the ultrasound diagnosis of ACC (**Figure 1B**) and associated colpocephaly, bilateral posterior dilatation of the lateral ventricles. The anterior commissure was thicker than usual, whereas the posterior commissure was only just visualized. In addition, there was incomplete operculisation of the Sylvian fissures. The MRI confirmed a small head circumference (<1st centile) and also detected a small cerebellar vermis (height < 1st centile), which was rotated away from the brainstem in the midline sagittal views, consistent with cerebellar hypoplasia (**Figure 1C**). The cerebellar hemispheres and brain stem appeared normal, but both the transcerebellar diameter and pontine brainstem diameter were < 1st centile. The frontal aspect of the brain appeared narrowed, and the frontal white matter demonstrated bilateral regions of abnormal low signal intensity on T2 weighted images (**Figure 1B**) which are seen in typical development (**Figure 1A**). The latter could not be more formally assessed as image quality was suboptimal. The parents decided to terminate the pregnancy at 35.71 GA.

The post-mortem MRI of the brain confirmed the antenatal findings of microcephaly, absent ACC and incomplete operculisation of the Sylvian fissure. In addition, there were extensive low signal intensity bands in the subcortical white matter on the T2 weighted images, consistent with band heterotopia (**Figure 1D**) and more marked than appreciated on the antenatal MRI.

The microscopic examination of the brain confirmed that this infant had evidence of abnormal neuronal migration with subcortical and PVWM neuronal heterotopia (**Figures 2A**, **6C**) and findings also confirmed ACC-H. The clinical post-mortem examination also detected a severely hypoplastic left kidney. The heterozygous *ACTG1 gene* variant [ACTG1 NM_001614.3 c.608C > T p.(Thr203Met)] was subsequently confirmed as a Pathogenic Class 4 (PS3) (Richards et al., 2015) based on the interpretation of the genetic and clinical findings and the phenotype of the patient.

RESULTS

These experiments were designed to assess the pathophysiological effect of the *ACTG1* variant by demonstrating differences between the gene variant and an age-matched control in the regions of the corpus callosum, frontal cortex, intermediate zone, CGE and in the PVWM. We also sought to examine the composition of the neuronal heterotopia (35.71 GA weeks) and the surrounding support structures (i.e., the germinal matrix and the radial glia).

Abnormal Midline Radial Glia and SNAP-25 Expression Is Seen in ACTG1 Variant

During development of the corpus callosum, the leading edge of the commissural axonal fibers expresses the SNAP-25 protein. The axons transverse into the genu by chemoattractant cues in the vimentin-positive radial glia of the early corpus callosum referred to as the callosal glial sling between 17 and 30 weeks gestation (Shu and Richards, 2001).

In ACC, there is a spectrum of developmental abnormalities ranging from the entire absence of the corpus callosum to conditions where the callosal fibers may have started to grow, but when unable to cross between the hemispheres, they grow toward the back of the same hemisphere where they began termed Probst bundles (Hetts et al., 2006).

We investigated the axonal fibers and the glial sling in two respective regions of the corpus callosum, the forceps minor connecting lateral and medial surfaces of the frontal lobes (**Figures 1E,G,I,K**) and the genu (**Figures 1F,H,J,L**) in both the control (**Figures 1E–H**) and the case with an *ACTG1* variant, aged 35 weeks (**Figures 1I–L**).

In the control case, the forceps minor had numerous SNAP-25 positive fibers (**Figure 1E**) that stretched across the hemisphere and into the genu (**Figure 1F**). The radial glia in the forceps minor were visualized using anti-vimentin (**Figure 1G**). Remnants of vimentin indusium griseum glia were still present in the genu (**Figure 1H**) which is considered normal in the third trimester of fetal development (Marcorelles et al., 2010).

The anti-SNAP-25 axonal fibers in the *ACTG1* variant were thicker and more sparse with perpendicular pointing fibers (**Figure 1I**) in the forceps minor but showed a more coiled shape in the genu (**Figure 1J**). The vimentin immunoreactivity showed different morphology in the *ACTG1* variant from that of the control. In the forceps minor

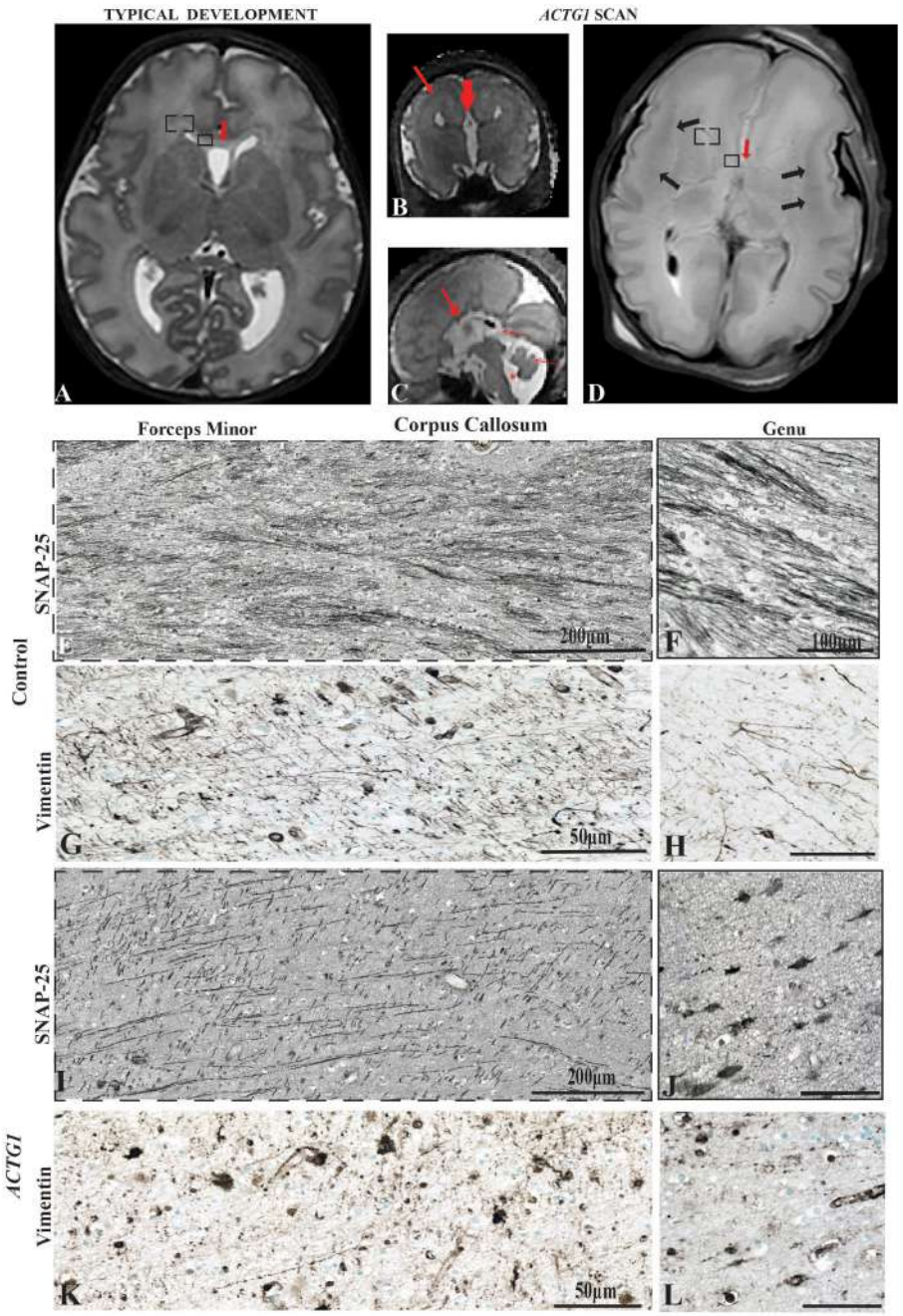


FIGURE 1 | Magnetic Resonance Imaging axial plane using T2 weighted images. In the transverse orientation, the scans show normal anatomical characteristics of a fetal brain at 35 GA wks **(A)**. Coronal T2 weighted image of the case with a variant in the *ACTG1* gene and agenesis of the corpus callosum with neuronal heterotopia (ACC-H) at 35.30 weeks showing the absence of the corpus callosum (thick red arrow) and prominent low signal intensity band in the subcortical white matter (thin red arrow; **B**). The sagittal T2 weighted image of the *ACTG1* variant, shows the absence of the corpus callosum (thick red arrow) and shortened cerebellar vermis, rotated away from the brainstem (thin red arrow) giving an enlarged fourth ventricle (asterisk; **C**). The genu of the corpus callosum is seen in panel (**A**; red arrow) but is absent in the case with agenesis of the corpus callosum (*ACTG1* variant, **D**, red arrow). On post-mortem imaging, the brain in the fetal case with *ACTG1* variant **(D)** showed an absence of the corpus callosum and decreased cortical folding frontally. There is extensive bilateral abnormal low signal intensity within the subcortical white matter (black arrows) on the T2 weighted MRI. Immunostaining of SNAP-25 **(E,F,I,J)** and vimentin **(G,H,K,L)** from the forceps minor **(E,G,I,K)** and the genu **(F,H,J,L)** of the corpus callosum of the frontal lobe (represented as a box-in region in images **A,D**). Image E shows normal axonal fibers in the control, whereas, in the photomicrograph I the axons are not as numerous and have unusual tangential fibers seen in *ACTG1* variant. Images **(F,J)**, show SNAP-25 positive fibers in the genu of the corpus callosum from the control case **(F)** which are severely reduced in ACC-H **(J)**. The callosal fibers rely on the midline glial structures to serve as guidance mechanisms. Image **(G)** shows normal vimentin positive indusium griseum glia (IGG) that guide the callosal axons of forceps minor. The horizontal IGGs are punctate in the *ACTG1* variant **(K)**. Callosal fibers cross the hemisphere by following tracts laid out by the glial wedge as seen in the control **(H)** which are absent in the *ACTG1* variant **(L)**. Scale bar in images **(H,J,L)** = 100 μm.

and the genu of the control there were fibrous elongated radial glia (**Figures 1G,H**), but in the *ACTG1* variant, there were cuboidal-shape astroglia (**Figures 1K,L**). These findings suggest that the indusium griseum glia of the corpus callosum failed to develop and lacked the SNAP-25 positive axonal fibers which are normally guided into the forceps minor.

Heterotopia Are Seen in the Subcortical Regions of the ACTG1 Variant

The MRI showed extensive bilateral low signal dense bands consistent with neuronal heterotopia in the case with *ACTG1* variant within the frontal-parietal cortical regions: these were absent in the control cases. We examined the cellular composition of the subcortical region at the level of the posterior Ammon's Horn (**Figure 2A**). Amongst the migrating neurons seen with the neuronal marker HuC/HuD, the *ACTG1* variant revealed whirls of densely positive neuronal heterotopia (**Figure 2B**). The heterotopia were absent in the control (not shown).

We then investigated the morphology of the radial glia in the intermediate zone using vimentin. In the control case, the linear radial glia were identified extending into the cortical regions (**Figure 2C**). The *ACTG1* variant had disjointed vimentin-positive extensions (**Figure 2D**) with nodular cell bodies.

Since the subcortical white matter had multiple heterotopia consistent with a band seen in the MRI, we investigated the morphology of the adjacent cortical region. We compared the neuronal and astroglial populations within the cortex seen in the control with those seen in the *ACTG1* variant. The control case demonstrated a six-layered isocortex with numerous neurons (**Figure 2E**) and astrocytes (**Figure 2F**) seen in each layer.

In the *ACTG1* variant, the cortical region was only sparsely positive for the neuronal marker HuC/HuD (**Figure 2H**). The vimentin-positive astroglia were only spread scantily throughout the cortex (**Figure 2I**) and was there was a deficit in cell numbers across all cortical layers.

We next assessed the ECM in the cortical region using the Colloidal Iron Stain. In the control the ECM was rich with hyaluronic acid components and was densely positive in the control (**Figure 2G**); this was absent in the *ACTG1* variant (**Figure 2J**) case.

The deficits of ECM in the cortex and the underlying white matter demonstrate that the neuronal environment may not be not conducive to structures that require hyaluronic acid components to facilitate their ability to navigate and therefore it may not be supportive of neuronal/glial migration and positioning within the cortex.

Deficits of Neuronal and Astroglial Proteins Are Seen in the CGE in the Case With ACC-H

The cellular morphologies seen in the cortical, subcortical and intermediate structures in the *ACTG1* variant (**Figure 2**) showed many histological abnormalities that could have been due to disturbances in radial glia or the ECM composition.

However, it is also possible that these changes were due to primary abnormalities in the germinal zones; the subventricular zone and the ganglionic eminence, the latter being highly active during mid-late fetal gestation. We identified cortical abnormalities in the *ACTG1* variant which may have been due to the disturbances seen in the radial glial or the lack of proliferation signals stemming from the subventricular zone in the early stages of development.

Therefore, in the next series of experiments, the distribution of proliferation markers, neuronal cells, and growth cone proteins were investigated in the CGE as this region is still highly active around 35 GA weeks. MIB-1 (Ki-67) antibodies are commonly used to identify proliferation densities in sections prepared with paraffin processing (Mathews et al., 2017).

MIB-1-positive cells were less densely stained and not as numerous in the *ACTG1* variant (**Figures 3B,B*i***) compared with the control (**Figures 3A,A*i***). The lack of MIB-1 protein translated to a decrease in neuronal cells within the CGE shown by the reduced number of HuC/HuD positive cells in the *ACTG1* variant (**Figures 3D,D*i***) compared with the control (**Figures 3C,C*i***).

Parallel microtubule bundles with MAP proteins (i.e., MAP1B or MAP2) are captured in the growing axons and dendrites via linking-proteins, such as Drebrin (Conde and Caceres, 2009). Drebrin is localized in two regions of the growth cone, in the T-zone and the filopodia. Drebrin protein is essential for forming links with EB3 to solidify synaptic connections and is required for neuritogenesis (Ivanov et al., 2009a,b). Although prominent drebrin leading edges were seen on the neurons in the CGE (**Figure 3E*i***) the number of drebrin positive cells was reduced in the *ACTG1* variant (**Figures 3F,F*i***) compared with the age-matched control (**Figures 3E,E*i***).

The ganglionic eminence relies on cells of the neuroepithelia to line the ventricles to support and protect the adjacent tissue in the lateral ventricles from edema. Radial glia and ependymal cells arise from the same lineage of neuroepithelial cells after the onset of neurogenesis. Radial glia guide migrating neurons while ependymal cells line the cavities of the CNS to help circulate the cerebrospinal fluid. We extended our investigation of the CGE to include the protein expression of nestin, vimentin, and GFAP as these are vital to the survival of the germinal matrix function.

The control demonstrated normal astroglial cell histology as along the ventricular wall, the cell bodies formed tight gap junctions, and in the CGE the astroglia were typical star-shaped (**Figures 4A–A*ii*,C,E**). With the *ACTG1* variant, nestin expression was remarkably decreased along the ventricle (**Figures 4B–B*ii***) compared to the control. In the control (**Figures 4C,D**), the cell bodies of the glia had radial processes with long protruding ends (**Figures 4C*i*,E*i***) which were identified with both vimentin and GFAP proteins. The vimentin and GFAP expression in the CGE were similar in morphology in the *ACTG1* variant (**Figures 4D,F**) and showed gaps between the cell bodies and the protruding end that normally forms the radial glia (**Figures 4D*i*,F*i***) were not as prominent.

The dense expression of vimentin and GFAP seen in the PVWM (adjacent to the CGE) in the *ACTG1* variant is indicative of astrogliosis (**Figures 4D*ii*,F*ii***), this pathology was not seen in the control (**Figures 4C*ii*,E*ii***). These finding suggests that

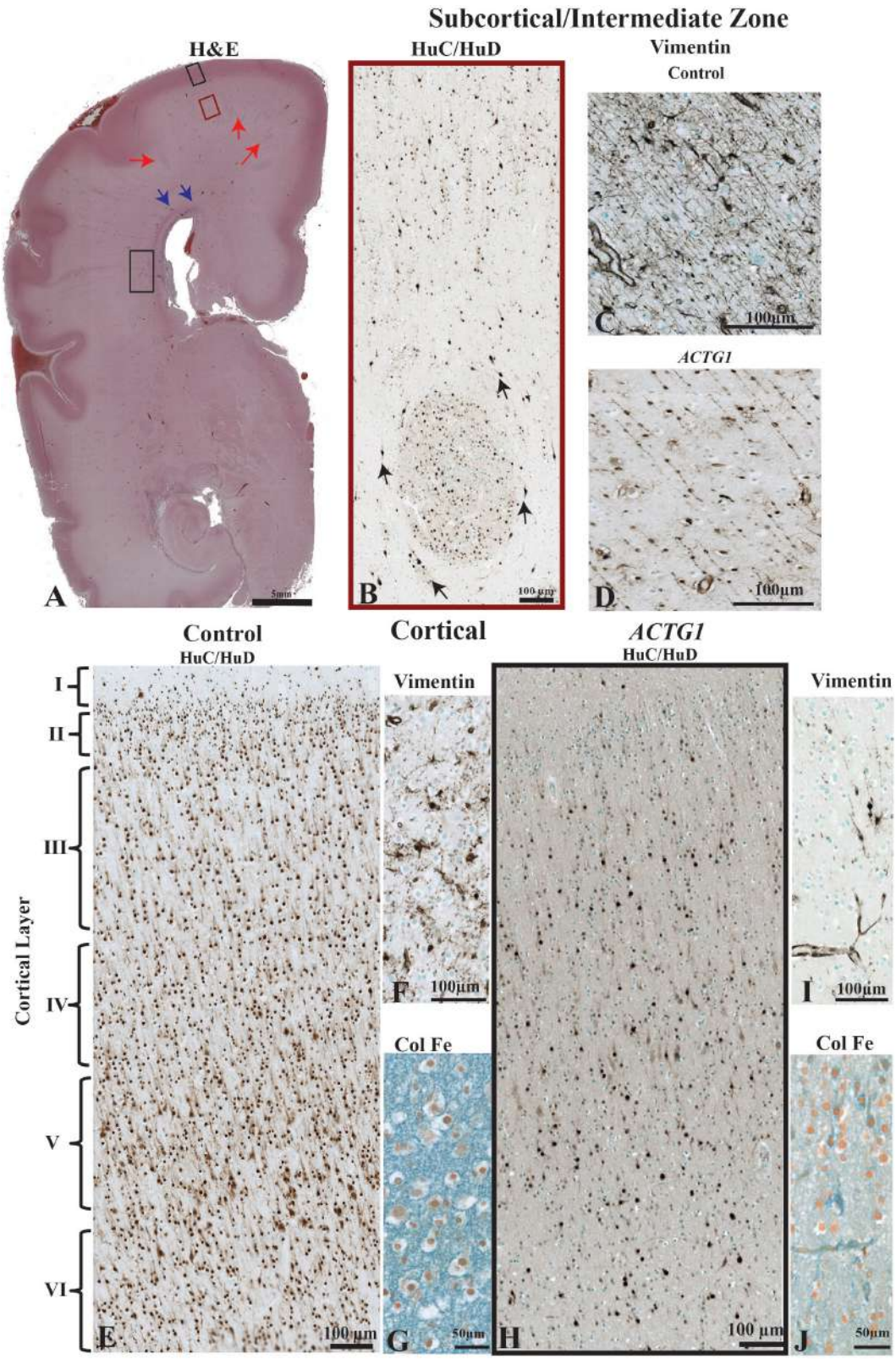


FIGURE 2 | The morphological differences between the age match control and the case with *ACTG1* variant, neuronal heterotopia, and agenesis of the corpus callosum. In image **(A)**, a coronally oriented scan from Haematoxylin and Eosin (H&E) staining of the frontal lobe of the brain of the *ACTG1* variant shows neuronal

(*Continued*)

FIGURE 2 | Continued
heterotopia in the subcortical, intermediate zone white matter (red arrows) and in the periventricular white matter regions (black rectangle). The blue arrows in image **(A)**, are pointing to a region where there are severe disruptions of the callosal fiber tracts. In image **(B)**, the immunoreactivity of anti-HuC/HuD from the subcortical region (from the red-boxed region on image **A**) shows an example of a whirling heterotopia with neurons located in and around a nodular structure. The black arrows are pointing to neurons adjacent to the heterotopia. Image **(C)**, show the immunoreactivity from using anti-vimentin, which identifies radial glia and astrocytes in the control. In the *ACTG1* variant **(D)**, the vimentin-positive radial glia are fragmented and sporadically situated. The black boxed seen in image **(A)** in the rostral frontal cortical region from the *ACTG1* variant is exemplified in image **(H)** using anti-mouse HuC/HuD. This image shows the poorly distribution of cortical neurons compared to the control in image **(E)**. Anti-vimentin staining of the cortex **(F,I)** show normal radial glia fiber end (**F**; control) which are nearly absent (**I**; *ACTG1* variant). Additional differences can be seen in the extracellular matrix in the cortex using the Colloidal Iron Stain. Image **(G)** shows a vast perineuronal network with hyaluronic acid complexes, which is not as dense in the *ACTG1* variant **(J)**.

in this case with *ACTG1* variant, deficits in the CGE may be related to a lack of migration cues and reduced expression of proliferation proteins.

The Expression of Oligodendroglia and Microglia Proteins Are Decreased in the ACTG1 Variant

As we saw a change in the astroglia morphology in the *ACTG1* variant case, we sought to determine if other glial populations were affected. Hence, we stained for oligodendroglia using anti-OLIG2 and microglia with anti-Iba-1. In the CGE of the control case, there was a moderate to frequent expression of the OLIG2 protein (**Figures 5A–A*ii***). Although oligodendrocytes are present in the *ACTG1* variant, OLIG2 immunolabeled cells labeled with mouse anti- OLIG2 immunoreactivity were scant (**Figures 5B–B*ii***). Surprisingly, with edema in the *ACTG1* variant seen around the ventricular endothelial and white matter regions, there was only a low level of Iba-1 expression (**Figures 5D–D*ii***). The control had ameboidal Iba1-positive microglia seen along the ventricular lining (**Figures 5C,C*i***) and in the CGE regions that were adjacent to the white matter (**Figures 5C,C*ii***). The absence of oligodendroglia and microglia may indicate that there may be significant deficits in this brain with heterotopia which may interfere with the migrating neurons and the proliferation of supporting glia; how this attributes to the formation of the heterotopia remains unclear.

White Matter Neuronal Heterotopia Are Positive for Synaptic Proteins

Since we identified a decrease of neurons in the CGE and clusters of heterotopias in the subcortical region of the *ACTG1* case, we next assessed the PVWM (black rectangle in **Figure 2A**).

The PVWM region adjacent to the CGE is an important part of the paracentral stratified transitional field and migratory stream and was investigated using H&E, anti-Vimentin and anti-HuC/HuD antibodies and. In the control case (**Figures 6A,B,E**) there were regular patterns of white matter fibers (see **Figure 6A**). Amongst the fibers were vimentin-positive astroglia and radial glia (**Figure 6B**) that either followed the white matter tracks or lay perpendicular to them. The HuC/HuD immunostaining in the control demonstrated that the white matter fibers house migratory streams of neurons well into the third trimester of gestation (**Figure 6E**).

In the *ACTG1* variant (**Figures 6C,D,F,G**) the H&E shows the morphology of the PVWM fibers that were less uniform, and there were round clusters of cells (**Figure 6C**). Immunostaining with the mouse anti-HuC/HuD antibody identified the cells in the clusters to be neurons (**Figures 6F,G**) consistent with neuronal heterotopia. Dense vimentin-positive astroglia were seen in the PVWM regions of the *ACTG1* variant (**Figure 6D**) and around the heterotopia (**Supplementary Data**), however, the astroglial processes were fragmented.

PVWM Neuronal Heterotopia Have Both GABAergic and Glutamatergic Neurons

We identified that the heterotopia contained neurons (i.e., HuC/HuD positive cells) therefore we sought to determine if the neurons were making synaptic connections, using the markers for growth cones (drebrin), synaptic processes (synaptophysin and SNAP-25) and interneuronal markers (calretinin and parvalbumin).

In the PVWM of the control case, the neuronal growth cones had long leading drebrin-positive ends (**Figure 7A**). Although the neurons inside the heterotopia expressed drebrin, the fine extensions seen in the control are lacking, and the expression is more dense and centered around the nucleus (**Figure 7B**). Synaptophysin, a marker for synaptic vesicle transmission, was identified around the perimeter of the heterotopia (**Figure 7C**). However, toward the center, the staining was more sporadic. Dense perinuclear and axonal SNAP-25 processes were identified within the heterotopia which may be indicative of glutamatergic neurons (**Figures 7D,D*i***). Of interest, linear SNAP-25 protein expression was also seen around the heterotopia which indicates that axons circumnavigate the heterotopia (**Figure 7D*ii***). In addition, GFAP and Iba-1 immunoreactivity also circumvented the heterotopia (**Supplementary Data**).

Finally, we used the interneuron markers calretinin and parvalbumin to identify any GABAergic neurons within the heterotopia. In the PVWM heterotopia, we identified calretinin-positive neurons (**Figure 7E**), but these were negative for parvalbumin proteins [**Figure 7F**; although parvalbumin fibers and neurons were seen in the putamen (see **Supplementary Data**)]. The significance of calretinin positive neurons opposed to parvalbumin neurons may be indicative of the origin of neurons seen in the heterotopia (i.e., the CGE) (Wonders and Anderson, 2006). Nevertheless, the data presented in this section show that within the heterotopia of this *ACTG1* variant, the neurons may have made synaptic connections and may be comprised of both glutamatergic and GABAergic neurons.

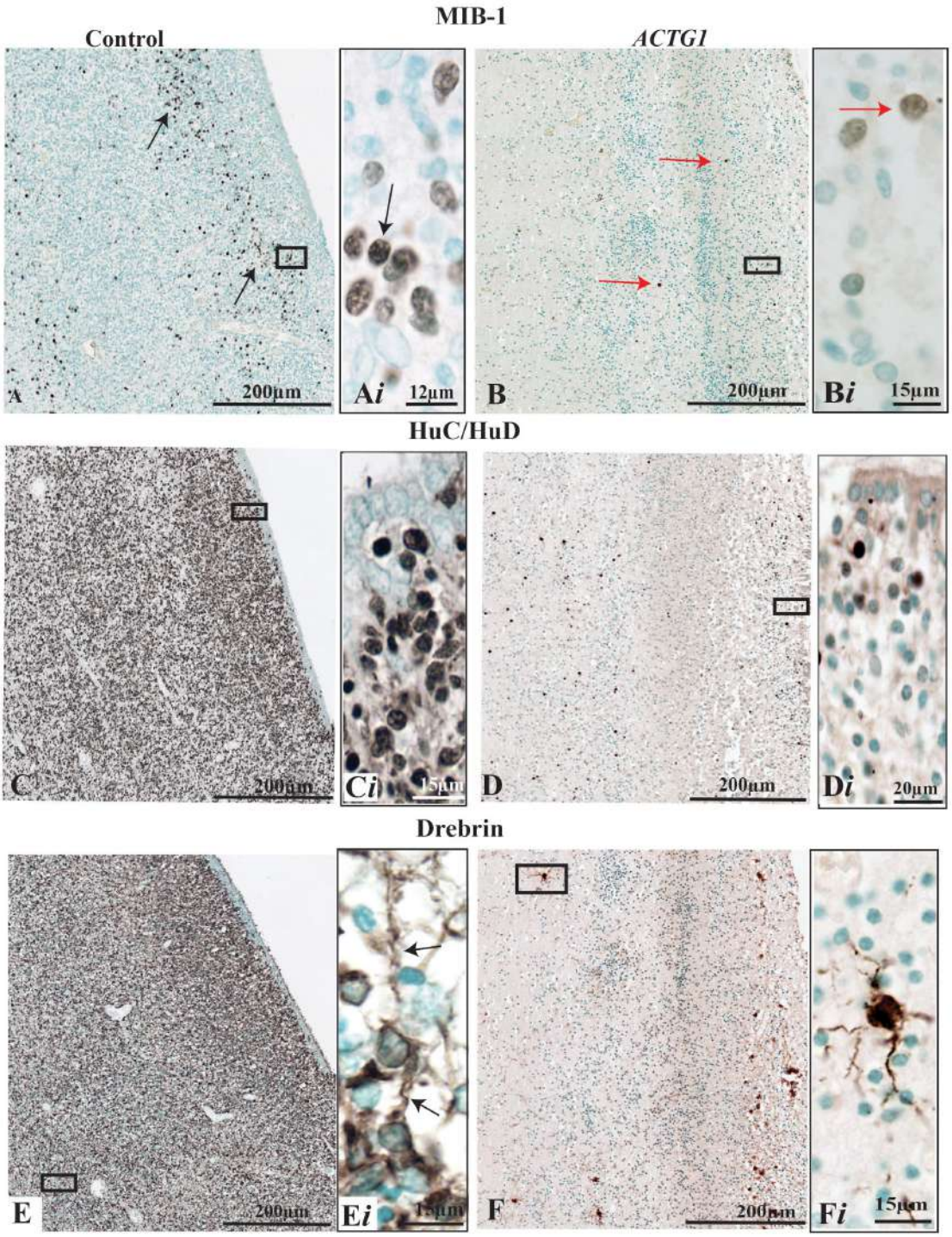


FIGURE 3 | Immunoreactivity of cell proliferation (MIB-1) neurons (HuC/HuD) and growth cone structures (Drebrin) from the caudal ganglionic eminence (CGE) of a control case (32 GA wks; **A,C,E**) and the case with heterotopia and agenesis of the corpus callosum (*ACTG1* variant; 35 GA wks; **B,D,F**). In image **(A)**, the black arrows are pointing to positive cells for mouse anti-MIB-1 (ki-67) and demonstrate a reasonable distribution of proliferating cells **(A*i*)**. Whereas, in image **(B)**, the red arrows are pointing to the sparse population of proliferative cells in the *ACTG1* variant that are not as strongly stained **(B*i*)**. HuC/HuD immunoreactivity shows the neuronal population in the CGE is seen in the control brain **(C,C*i*)** and in *ACTG1* variant **(D,D*i*)**. Drebrin positive, growth structures are seen in images **(E,F)**. The control brain is densely packed with growth cones **(E)** and the inset shows elongating extensions arising from the cell (black arrows; **E*i***). The *ACTG1* variant has neurons that are positive for drebrin **(F)**, but the cell shown in the inset **(F*i*)** exemplifies that the staining is dense and fragmented rather than perinuclear with long extensions.

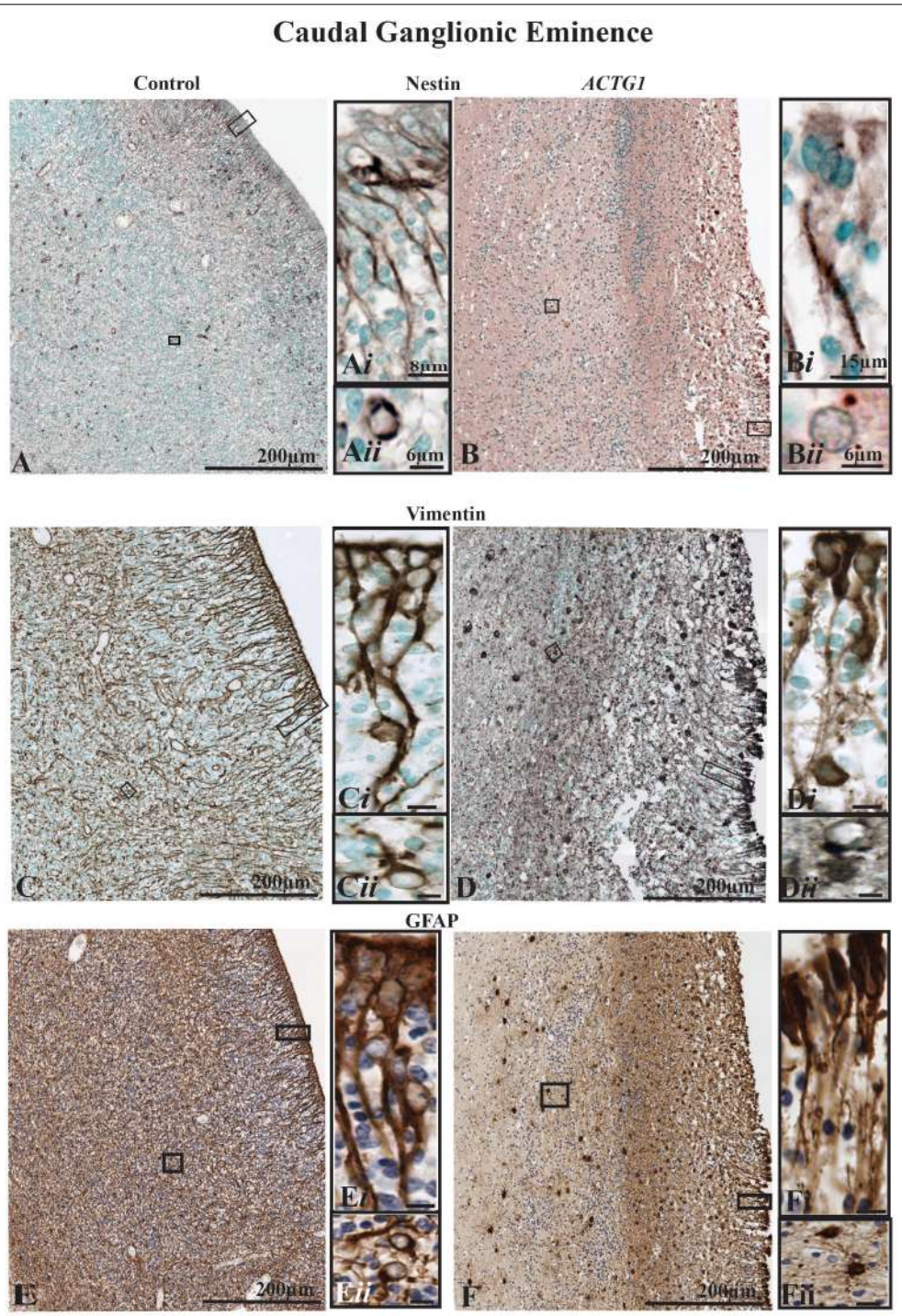


FIGURE 4 | The radial glia proliferation and filament marker, nestin (images **A,B**), and the radial glia and astroglia markers [vimentin (images **C,D**) and GFAP (images **E,F**)] in the caudal ganglionic eminence (CGE). Immunoreactivity of nestin in the control brain **(A)** demonstrates a normal distribution along the ependymal cells that develop from tanycytes, types of transitional cells with radially extending processes that extend from the lining of the ventricle **(A*i*)** into the lateral ganglionic eminence. Image **(A*ii*)** shows a newly form astrocyte, that was probably migrating out of the CGE with perinuclear nestin expression. The ependymal lining is fragmented in the case with heterotopia and agenesis of the corpus callosum (ACC-H; images **B,D,F**) and the nestin-positive radial glial are sparse and lack the long extending processes **(B*i*)** additionally astroglia seen in the CGE do not have strong perinuclear staining **(B*ii*)**. The mouse anti-vimentin and the mouse anti-GFAP immunostaining show a similar pattern to the Nestin in the cases. In the control case **(C,E)** there densely packed radial glia and the fibers are strongly positive for vimentin **(C,C*i*)** and GFAP **(E,E*i*)**. Immature astrocytes are seen in the CGE **(C*ii*,E*ii*)** with stout processes. Whereas, in the *ACTG1* variant **(D,F)** the radial glia fibers are thin **(D*i*,F*i*)** and fragmented as they emerge from the ventricular lining. In the CGE of the *ACTG1* variant, there are dense vimentin and GFAP positive cuboidal gliotic astrocytes **(D*ii*,F*ii*)**. Scale bar in images **(C*i*–F*ii*)** = 8 μm.

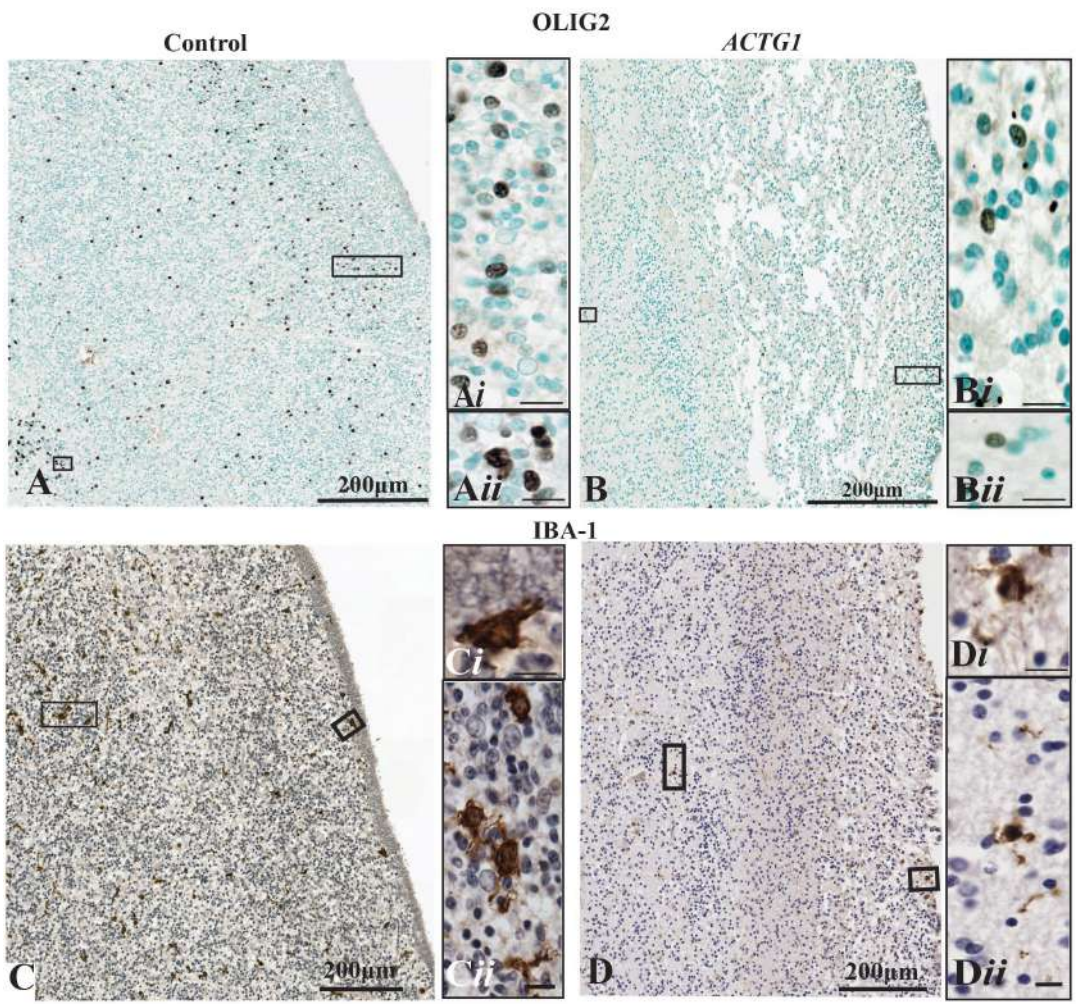


FIGURE 5 | Oligodendroglia and microglia populations shown with markers for mouse anti-OLIG2 and rabbit anti-Iba-1. In the control case (image **A**) the OLIG2 positive cells are dispersed throughout the caudal ganglionic eminence (CGE), and the insets show densely OLIG2 positive nuclei **(A*i*,A*ii*)** found throughout the CGE. Image **(B)** demonstrates that the OLIG2 positive nuclei in the CGE lack in the heterotopia and agenesis of the corpus callosum (ACC-H) case (insets **B*i*,B*ii***). The immunoreactivity of rabbit anti-Iba-1 shows ameboidal microglia **(C)** are seen in the beneath the ependymal cells **(C*i*)** of the CGE and in regions adjacent to the white matter **(C*ii*)** of the control case. The *ACTG1* variant **(D)** show Iba-1 immuno-stained microglia beneath the ependymal cells **(D*i*)**, but in the CGE adjacent to the white matter the microglia in the *ACTG1* variant are much smaller **(D*ii*)**. Scale bar in inset images = 9 μm.

DISCUSSION

In this case report, we have described deficits in the expression of essential proteins in a near-term fetus with a variant in the *ACTG1* gene and demonstrating microcephaly and ACC-H by comparing this case with an age-matched control.

We have shown that in this case of the *ACTG1* gene variant, there are disruptions in radial glia, which are comorbid with a loss of axonal fiber density in the corpus callosum. Additionally, we found disturbances in the subcortical and intermediate zone radial glia composition and a dramatic decrease in cortical neurons In the *ACTG1* variant, there is a marked lack of proliferative signals (MIB-1) and guidance structures (growth cones proteins and glia), which are normally found in the caudal ganglionic eminence. Moreover, these findings imply that neuronal heterotopia are more complex as we show that they are composed of both glutamatergic and GABAergic neurons. To the best of our knowledge, this is the first time that synaptic proteins, growth cone structures, and axonal morphology in and around neuronal heterotopia have been shown in the human neonatal brain.

The results presented in this study of an *ACTG1* variant, are compatible with other case studies that analyzed neuronal migration disturbances in type I lissencephaly (*LIS1*), doublecortin (*DCX*) and aristaless-related homeobox gene (*ARX*) variants (Ross et al., 2001; Paul et al., 2007; Marcorelles et al., 2010). *DCX* and *LIS1* genetic variants cause

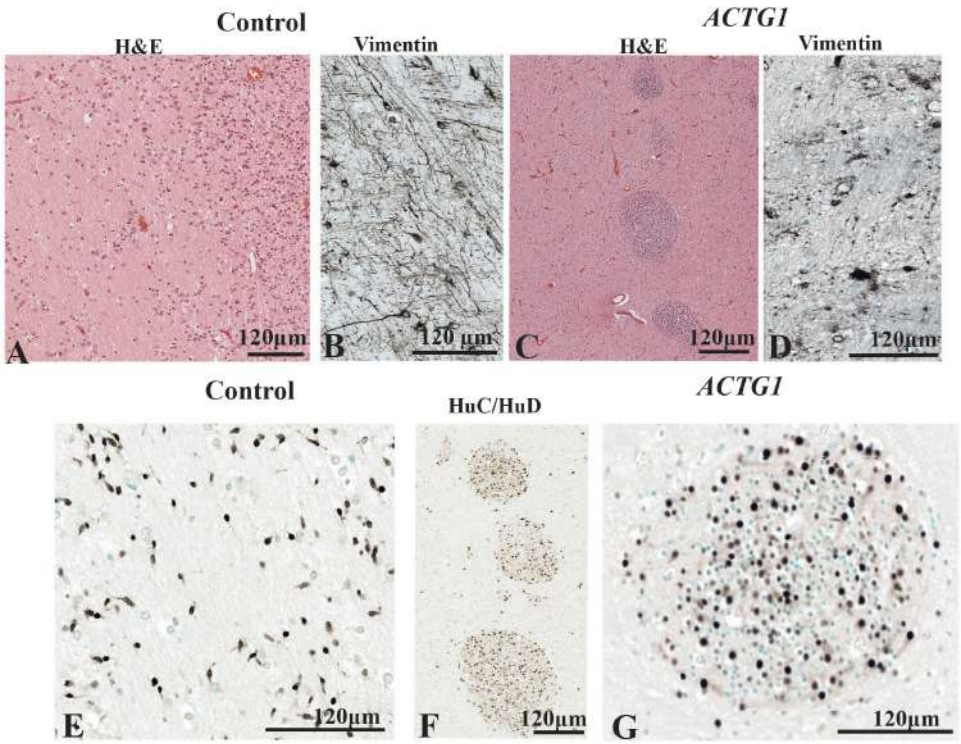


FIGURE 6 | The periventricular white matter (PVWM) adjacent to the caudal ganglionic eminence (CGE; see **Figure 2A**). In the control, the hematoxylin stain shows the normal morphology of the PVWM **(A)** whereas in the case *ACTG1* variant and heterotopia and agenesis of the corpus callosum (ACC-H) cellular clusters are found instead white matter fibers **(C)**. Inside the control **(B)** case there are linear radial glial along with perpendicular astroglia seen with anti-vimentin, however, in the *ACTG1* variant, the astroglia processes are disjointed **(D)**. The HuC/HuD immunostaining in the control case demonstrates migrating neurons within the PVWM **(E)** in the control case. Images **(F,G)** show that the cellular clusters are neuronal heterotopia seen the *ACTG1* variant using mouse anti-HuC/HuD.

a very similar phenotype of subcortical heterotopia. Although *LIS1* variants have been associated with isolated lissencephaly, where the parietooccipital brain region is more affected, *DCX* variants often give rise to malformations of the frontal cortex. Furthermore, variants in *DCX* result in lissencephaly in males and subcortical laminar heterotopia in females (Watrin et al., 2015). Variants in the *ARX* gene are characterized by abnormalities such as microcephaly and lissencephaly, plus ACC (Marcorelles et al., 2010). The specific mechanisms that relate ACC and neuronal heterotopia are still unknown, although other investigations show that *LIS1* and *DCX* deletions result in radial glial depletions and microtubule malfuctioning (Watrin et al., 2015). Recently, other candidates such as glial cell line-derived neurotrophic factor (GDNF) and the GDNF family receptor alpha-1 have shown that variants, even when heterozygous, can upset the proliferation of radial glia and disturb migration (Ledda et al., 2007) and synaptogenesis (Ibanez and Andressoo, 2016).

In this post-mortem, near-term fetal study, we showed microcephaly, ACC with comorbid heterotopia seen subcortically and in the PVWM. Furthermore, there was a decrease in cortical volume and radial glia structures compared with the aged-matched control. These findings imply that other supporting glia may be perturbed which would have serious consequences to the developing brain.

The GA at birth of the age-matched control was similar to the *ACTG1* variant, but we cannot exclude the possibility that the control would have developed neuronal pathologies if it had lived longer, however, this is highly unlikely as these are early developmental changes and one would have expected to see them. Nevertheless, our data suggest that compared to the age-matched control the mechanism underlying overt pathophysiological changes seen in *ACTG1* variants are related to radial glia disturbances that affect GABAergic and glutamatergic neuronal migration.

Neuronal Proliferation and Pathological Findings

Previous studies focusing on subcortical neuronal heterotopia have shown relationships between cortical lamination and the genetic abnormalities associated with the mechanisms of migration impairment in agyric/pachygyria syndromes (Marcorelles et al., 2010; Verloes et al., 2014; Yates et al., 2017). In this study with associated callosal agenesis and heterotopia, we have demonstrated an array of underlying pathologies, such as impairments in the proliferation of the progenitor cells, a noticeable reduction of neurons, and growth cones without leading edges in the CGE. We have identified that proteins involved in forming the radial glia and astroglia are

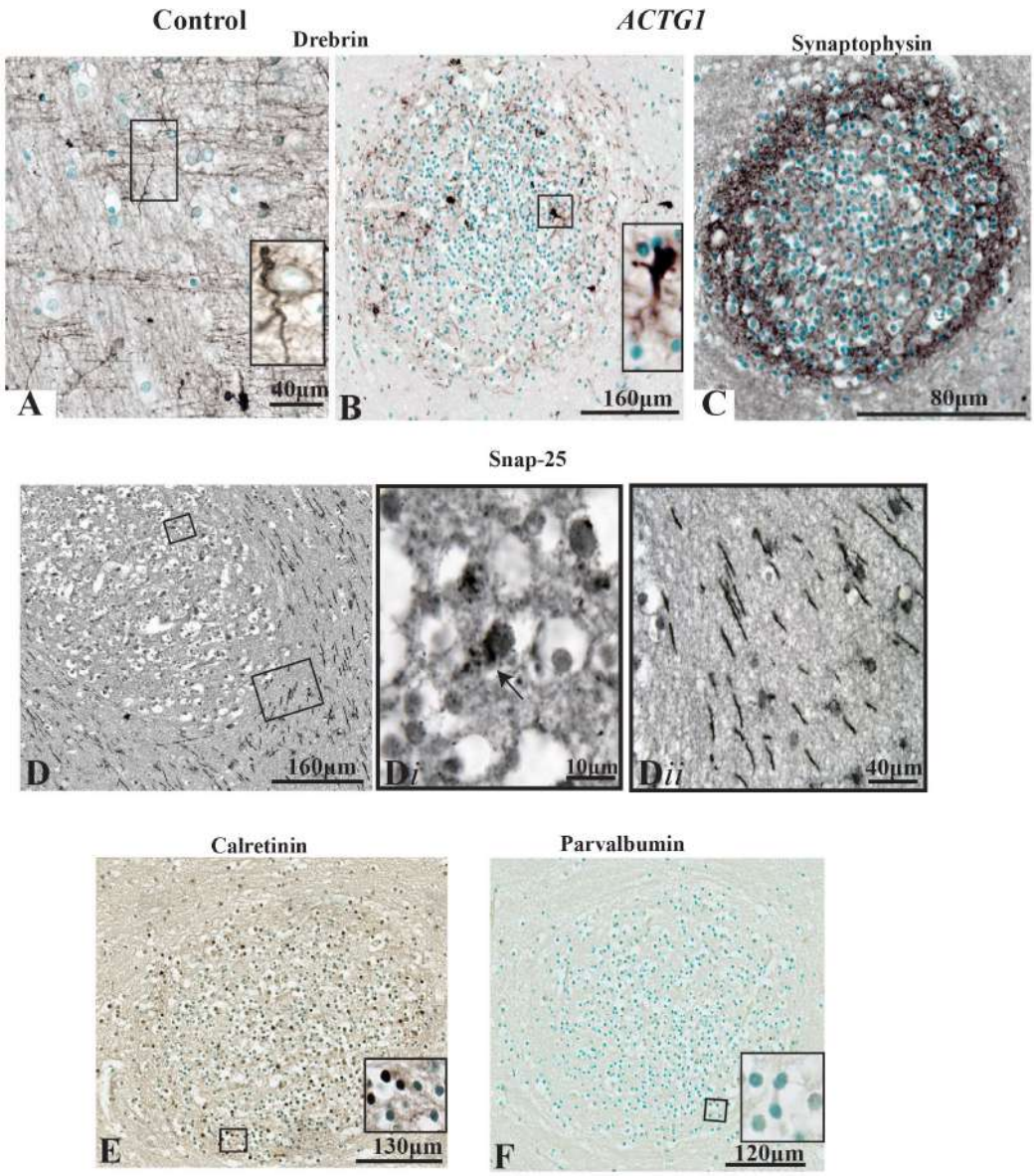


FIGURE 7 | Photomicrographs of the periventricular white matter (PVWM) region adjacent to the caudal ganglionic eminence (CGE; outlined in **Figure 2**) demonstrating the growth cone proteins (anti-drebrin), synaptic connections (anti-synaptophysin and anti-SNAP-25) and interneuronal proteins (anti-calretinin and anti-parvalbumin). In the control case **(A)** anti-drebin expression is seen on growth cones as long bipolar extensions. In contrast, the case with *ACTG1* variant, heterotopia are identified with neurons expressing dense drebrin protein with blunt and short extensions **(B)**. The immunoreactivity of synaptophysin densely surrounds the outer regions of the neuronal heterotopia **(C)**. Detection of glutamatergic cells inside the neuronal heterotopia is seen with anti-SNAP-25 staining **(D,D*i*)**. Of interest, the PVWM region surrounding the neuronal heterotopia have SNAP-25 positive axons **(D*ii*)**. Additionally, rabbit anti-calretinin neurons seen in the neuronal heterotopia **(E)** however, the neuronal heterotopia were negative for rabbit anti-parvalbumin **(F)**.

impaired. The lack of the neuroepithelial structures identified along the ventricular lining in the *ACTG1* variant substantiates that this can have a knock-on effect on the oligodendrocytes and the microglial formation. The mechanisms of *ACTG1* variants and other variants have recently been described in Ibanez and Andressoo (2016), where researchers have identified variants that were correlated to abnormalities seen in the brain (i.e., basal ganglia and hippocampus) and other organs such as the kidneys (i.e., renal agenesis). In the present study, we demonstrate that there was ACC and neuronal heterotopia that may have been due to malformation of the radial fibers. Additionally, the post-mortem report described a severely hypoplastic kidney.

The Composition of Neuronal Heterotopia

Previously, Marcorelles et al. (2010) demonstrated that PVWM heterotopia were positive for GABAergic interneurons. The

results presented in this study are compatible with (Marcorelles et al., 2010) as we found that in the PVWM the neuronal heterotopia contains calretinin-positive cells. Additionally, we have identified that inside the neuronal heterotopia clusters there are large SNAP-25 positive glutamatergic neurons. Furthermore, we have shown that the neuronal heterotopia are making synaptic vesicles, identified with synaptophysin protein. If the radial glia fails to project from the ventricular zone to the cortical plate, then guidance cues for the glutamatergic neurons would be compromised. This lack of guidance possibly causes these destined cortical neurons to negotiate another destination or to simply "get stuck" causing either obstacles or attraction forces for the migration of the GABAergic neurons. Misplaced neuronal populations can cause lifelong neurological disabilities such as epilepsy and cognitive impairments.

Identifying changes in proteins associated with neuronal heterotopia may improve our understanding of the molecular mechanisms associated with migrational disturbances.

ETHICS STATEMENT

Written and informed parental consent was acquired for post-mortem examination and post-mortem research according to National Health Service United Kingdom and Human Tissue Authority guidelines. Research study ethics was obtained from the National Research Ethics Service (West London), United Kingdom [ethics number, 07/H0707/139; Post-mortem Magnetic Resonance Imaging (MRI) Study of the Developing Brain].

AUTHOR CONTRIBUTIONS

RV, VS, CT, HH, and MR conceived and planned the experiments. VS, AM, RV, and AD contributed to the sample preparation. RV carried out the histochemical and immunohistochemical staining and prepared the photomicrographs. AD prepared the representative images from the MRI scans. MH-E requested the whole exome sequencing on the fetus's DNA-interpreted the results. SL, SY, and MJ contributed to the whole exome sequencing and analysis of the data. RV and MR took the lead in writing the manuscript. All authors provided the critical feedback and helped shape the research, analysis, and the manuscript.

FUNDING

This work was supported by the Medical Research Council strategic award (MRC; United Kingdom, MR/K006355/1), the Wellcome Trust (WT094823), Leducq Foundation, the Rosetrees Trust (A1563), the Swedish Medical Research Council (VR 2012-3500), the Wilhelm and Martina Lundgren Foundation, the Åhlén Foundation, the Frimurare Barnhus Foundation, the Byggmästare Olle Engqvist Foundation, The Brain Foundation (2013-0035) and Governmental Grants for University Hospitals in Sweden (ALFGBG-137601). The authors acknowledge financial support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust and the University of Miami Miller School of Medicine.

ACKNOWLEDGMENTS

We thank the families who consented to the "Post-mortem MRI study" and our colleague Dr. Surabhi Nanda from the Fetal Medicine Unit.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00623/full#supplementary-material

REFERENCES

Achiron, R., and Achiron, A. (2001). Development of the human fetal corpus callosum: a high-resolution, cross-sectional sonographic study. *Ultrasound Obstet. Gynecol.* 18, 343–347. doi: 10.1046/j.0960-7692.2001.00512.x

Antonucci, F., Corradini, I., Morini, R., Fossati, G., Menna, E., Pozzi, D., et al. (2013). Reduced SNAP-25 alters short-term plasticity at developing glutamatergic synapses. *EMBO Rep.* 14, 645–651. doi: 10.1038/embor.2013.75

Bramanti, V., Tomassoni, D., Avitabile, M., Amenta, F., and Avola, R. (2010). Biomarkers of glial cell proliferation and differentiation in culture. *Front. Biosci.* 2, 558–570. doi: 10.2741/s85

Brazel, C. Y., Romanko, M. J., Rothstein, R. P., and Levison, S. W. (2003). Roles of the mammalian subventricular zone in brain development. *Prog. Neurobiol.* 69, 49–69. doi: 10.1016/s0301-0082(03)00002-9

Bruckner, G., Brauer, K., Hartig, W., Wolff, J. R., Rickmann, M. J., Derouiche, A., et al. (1993). Perineuronal nets provide a polyanionic, glia-associated form of microenvironment around certain neurons in many parts of the rat brain. *Glia* 8, 183–200. doi: 10.1002/glia.440080306

Clowry, G. J. (2014). An enhanced role and expanded developmental origins for gamma-aminobutyric acidergic interneurons in the human cerebral cortex. *J. Anat.* 225, 2–10. doi: 10.1111/joa.12198

Conde, C., and Caceres, A. (2009). Microtubule assembly, organization and dynamics in axons and dendrites. *Nat. Rev. Neurosci.* 10, 319–332. doi: 10.1038/nrn2631

Condliffe, S. B., and Matteoli, M. (2011). Inactivation kinetics of voltage-gated calcium channels in glutamatergic neurons are influenced by SNAP-25. *Channels* 5, 304–307. doi: 10.4161/chan.5.4.16228

De Luca, C., and Papa, M. (2016). Looking inside the matrix: perineuronal nets in plasticity, maladaptiv plasticity and neurological disorders. *Neurochem. Res.* 41, 1507–1515. doi: 10.1007/s11064-016-1876-2

Edwards, T. J., Sherr, E. H., Barkovich, A. J., and Richards, L. J. (2014). Clinical, genetic and imaging findings identify new causes for corpus callosum development syndromes. *Brain* 137, 1579–1613. doi: 10.1093/brain/awt358

Erzurumlu, R. S., Guido, W., and Molnár, Z. (2006). *Development and Plasticity in Sensory Thalamus and Cortex*. New York, NY: Springer.

Geraldo, S., and Gordon-Weeks, P. R. (2009). Cytoskeletal dynamics in growth-cone steering. *J. Cell Sci.* 122(Pt 20), 3595–3604. doi: 10.1242/jcs.042309

Geraldo, S., Khanzada, U. K., Parsons, M., Chilton, J. K., and Gordon-Weeks, P. R. (2008). Targeting of the F-actin- binding protein drebrin by the microtubule plus-tip protein EB3 is required for neuritogenesis. *Nat. Cell Biol.* 10, 1181–1189. doi: 10.1038/ncb1778

Gill, G. W., Frost, J. K., and Miller, K. A. (1974). A new formula for a half-oxidized hematoxylin solution that neither overstains nor requires differentiation. *Acta Cytol.* 18, 300–311.

Hetts, S. W., Sherr, E. H., Chao, S., Gobuty, S., and Barkovich, A. J. (2006). Anomalies of the corpus callosum: an MR analysis of the phenotypic spectrum of associated malformations. *AJR* 187, 1343–1348. doi: 10.2214/ajr.05.0146

Ibanez, C. F., and Andressoo, J. O. (2016). Biology of GDNF and its receptors - relevance for disorders of the central nervous system. *Neurobiol. Dis.* 97(Pt B), 80–89. doi: 10.1016/j.nbd.2016.01.021

Ishii, K., Kubo, K.-I., Endo, T., Yoshida, K., Benner, S., Ito, Y., et al. (2015). Neuronal heterotopias affect the activities of distant brain areas and lead to behavioral deficits. *J. Neurosci.* 35, 12432–12445. doi: 10.1523/JNEUROSCI.3648-14.2015

Ivanov, A., Esclapez, M., and Ferhat, L. (2009a). Role of drebrin A in dendritic spine plasticity and synaptic function: Implications in neurological disorders. *Commun. Integr. Biol.* 2, 268–270. doi: 10.4161/cib.2.3.8166

Ivanov, A., Esclapez, M., Pellegrino, C., Shirao, T., and Ferhat, L. (2009b). Drebrin A regulates dendritic spine plasticity and synaptic function in mature cultured hippocampal neurons. *J. Cell Sci.* 122(Pt 4), 524–534. doi: 10.1242/jcs.033464

Kadhim, H. J., Lammens, M., Gosseye, S., Gadisseux, J. F., and Evrard, P. (1993). Brain defects in infants with Potter syndrome (oligohydramnios sequence). *Pediatr. Pathol.* 13, 519–536. doi: 10.3109/15513819309048240

Kato, M. (2015). Genotype-phenotype correlation in neuronal migration disorders and cortical dysplasias. *Front. Neurosci.* 9:181. doi: 10.3389/fnins.2015.00181

Kolasinski, J., Takahashi, E., Stevens, A. A., Benner, T., Fischl, B., Zollei, L., et al. (2013). Radial and tangential neuronal migration pathways in the human fetal brain: anatomically distinct patterns of diffusion MRI coherence. *NeuroImage* 79, 412–422. doi: 10.1016/j.neuroimage.2013.04.125

Kostoviæ, I., Sedmak, G., Vukšiæ, M., and Judaš, M. (2015). The relevance of human fetal subplate zone for developmental neuropathology of neuronal migration disorders and cortical dysplasia. *CNS Neurosci. Therapeu.* 21, 74–82. doi: 10.1111/cns.12333

Lagercrantz, H. (2010). *The Newborn Brain : Neuroscience and Clinical Applications*, 2nd Edn. New York, NY: Cambridge University Press.

Leach, E. H. (1946). Curtis' substitute for Van Gieson Stain. *Stain Technol.* 21, 107–109. doi: 10.3109/10520294609110359

Ledda, F., Paratcha, G., Sandoval-Guzman, T., and Ibanez, C. F. (2007). GDNF and GFR[alpha]1 promote formation of neuronal synapses by ligand-induced cell adhesion. *Nat. Neurosci.* 10, 293–300. doi: 10.1038/nn1855

Leviton, A., and Gressens, P. (2007). Neuronal damage accompanies perinatal white-matter damage. *Trends Neurosci.* 3, 473–476.

Marcorelles, P., Laquerrière, A., Adde-Michel, C., Marret, S., Saugier-Veber, P., Beldjord, C., et al. (2010). Evidence for tangential migration disturbances in human lissencephaly resulting from a defect in LIS1, DCX and ARX genes. *Acta Neuropathol.* 120, 503–515. doi: 10.1007/s00401-010-0692-z

Marin, O., and Rubenstein, J. L. R. (2001). A long, remarkable journey: tangential migration in the telencephalon. *Nat. Rev. Neurosci.* 2, 780–790. doi: 10.1038/35097509

Mathews, K. J., Allen, K. M., Boerrigter, D., Ball, H., Shannon Weickert, C., and Double, K. L. (2017). Evidence for reduced neurogenesis in the aging human hippocampus despite stable stem cell markers. *Aging Cell* 16, 1195–1199. doi: 10.1111/acel.12641

Moss, J., Gebara, E., Bushong, E. A., Sánchez-Pascual, I., O'Laoi, R., El, M., et al. (2016). Fine processes of Nestin-GFP–positive radial glia-like stem cells in the adult dentate gyrus ensheathe local synapses and vasculature. *Proc. Natl. Acad. Sci. U.S.A.* 113, E2536–E2545. doi: 10.1073/pnas.1514652113

Paul, L. K. (2011). Developmental malformation of the corpus callosum: a review of typical callosal development and examples of developmental disorders with callosal involvement. *J. Neurodev. Disord.* 3, 3–27. doi: 10.1007/s11689-010-9059-y

Paul, L. K., Brown, W. S., Adolphs, R., Tyszka, J. M., Richards, L. J., Mukherjee, P., et al. (2007). Agenesis of the corpus callosum: genetic, developmental and functional aspects of connectivity. *Nat. Rev. Neurosci.* 8, 287–299. doi: 10.1038/nrn2107

Pozzi, D., Corradini, I., and Matteoli, M. (2018). The control of neuronal calcium homeostasis by SNAP-25 and its Impact on neurotransmitter release. *Neuroscience* doi: 10.1016/j.neuroscience.2018.11.009 [Epub ahead of print].

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., et al. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and genomics and the association for molecular pathology. *Genet. Med.* 17, 405–424.

Ross, M. E., Swanson, K., and Dobyns, W. B. (2001). Lissencephaly with cerebellar hypoplasia (LCH): a heterogeneous group of cortical malformations. *Neuropediatrics* 32, 256–263. doi: 10.1055/s-2001-19120

Shu, T., and Richards, L. J. (2001). Cortical axon guidance by the glial wedge during the development of the corpus callosum. *J. Neurosci.* 21, 2749–2758. doi: 10.1523/jneurosci.21-08-02749.2001

Sonego, M., Oberoi, M., Stoddart, J., Gajendra, S., Hendricusdottir, R., Oozeer, F., et al. (2015). Drebrin regulates neuroblast migration in the postnatal mammalian brain. *PLoS One* 10:e0126478. doi: 10.1371/journal.pone.0126478

Sturrock, R. R. (1978). Development of the indusium griseum. III. an autoradiographic study of cell production. *J. Anat.* 126(Pt 1), 1–6.

Sultan, K. T., Shi, W., and Shi, S. H. (2014). Clonal origins of neocortical interneurons. *Curr. Opin. Neurobiol.* 26, 125–131. doi: 10.1016/j.conb.2014.01.010

Verloes, A., Di Donato, N., Masliah-Planchon, J., Jongmans, M., Abdul-Raman, O. A., Albrecht, B., et al. (2014). Baraitser–Winter cerebrofrontofacial syndrome: delineation of the spectrum in 42 cases. *Eur. J. Hum. Genet.* 23:292. doi: 10.1038/ejhg.2014.95

Volpe, J. (2008). *Neurology of the Newborn*, 5 Edn. Philadelphia, PA: Saunders Elsevier.

Vontell, R., Supramaniam, V., Thornton, C., Wyatt-Ashmead, J., Mallard, C., Gressens, P., et al. (2013). Toll- like receptor 3 expression in glia and neurons alters in response to white matter injury in preterm infants. *Dev. Neurosci.* 35, 130–139. doi: 10.1159/000346158

Vontell, R., Supramaniam, V., Wyatt-Ashmead, J., Gressens, P., Rutherford, M., Hagberg, H., et al. (2015). Cellular mechanisms of toll-like receptor-3 activation in the thalamus are associated with white matter injury in the developing brain. *J. Neuropathol. Exp. Neurol.* 74, 273–285. doi: 10.1097/NEN.0000000000000172

Watrin, F., Manent, J.-B., Cardoso, C., and Represa, A. (2015). Causes and consequences of gray matter heterotopia. *CNS Neurosci. Therapeu.* 21, 112–122. doi: 10.1111/cns.12322

Wonders, C. P., and Anderson, S. A. (2006). The origin and specification of cortical interneurons. *Nat. Rev. Neurosci.* 7:687. doi: 10.1038/nrn1954

Yang, H., Zhang, M., Shi, J., Zhou, Y., Wan, Z., Wang, Y., et al. (2017). Brain-specific SNAP-25 deletion leads to elevated extracellular glutamate level and schizophrenia-like behavior in mice. *Neural Plast.* 2017:11. doi: 10.1155/2017/4526417

Yates, T. M., Turner, C. L., Firth, H. V., Berg, J., and Pilz, D. T. (2017). Baraitser-Winter cerebrofrontofacial syndrome. *Clin. Genet.* 92, 3–9. doi: 10.1111/cge.12864

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Vontell, Supramaniam, Davidson, Thornton, Marnerides, Holder-Espinasse, Lillis, Yau, Jansson, Hagberg and Rutherford. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 05 June 2019
doi: 10.3389/fphys.2019.00634

Mild Neonatal Brain Hypoxia-Ischemia in Very Immature Rats Causes Long-Term Behavioral and Cerebellar Abnormalities at Adulthood

Eduardo Farias Sanches[1], Yohan van de Looij[1,2], Audrey Toulotte[1], Stéphane Vladimir Sizonenko[1†] and Hongxia Lei[3*†]*

[1] Division of Child Development and Growth, Department of Pediatrics, School of Medicine, University of Geneva, Geneva, Switzerland, [2] Laboratory for Functional and Metabolic Imaging, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, [3] Center for Biomedical Imaging, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

OPEN ACCESS

Edited by:
Carina Mallard,
University of Gothenburg, Sweden

Reviewed by:
Angela Leigh Cumberland,
RMIT University, Australia
Ana A. Baburamani,
King's College London,
United Kingdom

***Correspondence:**
Stéphane Vladimir Sizonenko
stephane.sizonenko@unige.ch
Hongxia Lei
Hongxia.lei@epfl.ch

† These authors have contributed equally to this work as senior authors

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: 22 January 2019
Accepted: 06 May 2019
Published: 05 June 2019

Citation:
Sanches EF, van de Looij Y, Toulotte A, Sizonenko SV and Lei H (2019) Mild Neonatal Brain Hypoxia-Ischemia in Very Immature Rats Causes Long-Term Behavioral and Cerebellar Abnormalities at Adulthood. Front. Physiol. 10:634. doi: 10.3389/fphys.2019.00634

Systemic hypoxia-ischemia (HI) often occurs during preterm birth in human. HI induces injuries to hinder brain cells mainly in the ipsilateral forebrain structures. Such HI injuries may cause lifelong disturbances in the distant regions, such as the contralateral side of the cerebellum. We aimed to evaluate behavior associated with the cerebellum, to acquire cerebellar abundant metabolic alterations using *in vivo* ^{1}H magnetic resonance spectroscopy (^{1}H MRS), and to determine GFAP, NeuN, and MBP protein expression in the left cerebellum, in adult rats after mild early postnatal HI on the right forebrain at day 3 (PND3). From PND45, HI animals exhibited increased locomotion in the open field while there is neither asymmetrical forelimb use nor coordination deficits in the motor tasks. Despite the fact that metabolic differences between two cerebellar hemispheres were noticeable, a global increase in glutamine of HI rats was observed and became significant in the left cerebellum compared to the sham-operated group. Furthermore, increases in glutamate, glycine, the sum of glutamate and glutamine and total choline, only occurred in the left cerebellum of HI rats. Remarkably, there were decreased expression of MBP and NeuN but no detectable reactive astrogliosis in the contralateral side of the cerebellum of HI rats. Taken together, the detected alterations observed in the left cerebellum of HI rats may reflect disequilibrium in the glutamate-glutamine cycle and a delay in the return of glutamine from astrocytes to neurons from hypoxic-ischemic origin. Our data provides *in vivo* evidence of long-term changes in the corresponding cerebellum following mild neonatal HI in very immature rats, supporting the notion that systemic HI could cause cell death in the cerebellum, a distant region from the expected injury site.

HIGHLIGHTS

- Neonatal hypoxia-ischemia (HI) in very immature rats induces hyperactivity toward adulthood.
- ^{1}H magnetic resonance spectroscopy detects long-term cerebellar metabolic changes in adult rats after neonatal HI at postnatal day 3.
- Substantial decreases of expression of neuronal and myelin markers in adult rats cerebellum after neonatal cortical mild HI.

Keywords: hypoxia-ischemia, prematurity, ^{1}H magnetic resonance spectroscopy, ^{1}H MRS, cerebellum, brain

INTRODUCTION

Complications derived from premature birth account for 29% of global neonatal deaths yearly and around 3% of total disability during the lifespan (Lawn et al., 2010; Howson et al., 2013). Premature newborns have a high incidence of neonatal brain injury (Gopagondanahalli et al., 2016) linked to subcortical white and gray matter lesions, impaired structural connectivity (Volpe et al., 2011; Salmaso et al., 2014) which cause lifelong neurodevelopment disturbances (Robinson, 2005; Allin et al., 2008; Delobel-Ayoub et al., 2009; Pyhälä, 2012; Breeman et al., 2015; Hübner et al., 2015; Thomason et al., 2017).

Neonatal hypoxia-ischemia (HI) contributes to pathologies such as cerebral palsy (CP), developmental delay, attention deficit and hyperactivity disorder (ADHD) learning deficits and others (Fatemi et al., 2009; Volpe, 2009a; Phillips et al., 2013). The most used experimental model of neonatal HI (Levine, 1960; Rice et al., 1981) consists of unilateral carotid ligation followed by a period of hypoxic exposure leading to deficits in motor coordination (Lubics et al., 2005), anxiety-related behavior and cognitive impairment in early and late development, due to lesions in hippocampus, striatum and cortex (Arteni et al., 2010; Sanches et al., 2015). Also, studies using HI at postnatal day 7 have shown that cell death occurs in brain regions that are not directly affected by ischemia, such as the cerebellum (Joyal et al., 1996; Kim et al., 2004; Northington et al., 2011) suggesting that neuronal connectivity may play a role in neurodegeneration following HI to the immature brain. The HI model performed at postnatal day 3 mimics the lesion observed in very preterm infants' brains (Sizonenko et al., 2003; Sanches et al., 2013; Ginet et al., 2016). HI in the very immature rat brain causes disruption in cell metabolism, development and in cortical cytoarchitecture (Sizonenko et al., 2008; van de Looij et al., 2011; Misumi et al., 2016), alters the myelination pattern and leads to behavioral impairments (Huang et al., 2009; Sanches et al., 2015; Misumi et al., 2016). HI injury characteristics can be detected in infants born preterm via magnetic resonance imaging. Alderliesten et al. (2013) found high correlation between neuropathological evidence of cerebellar injury and MRI analysis (Alderliesten et al., 2013). Since cerebellum has a major role in high order brain functions, lesions in its connections with cortical and sub-cortical centers could lead not only to motor and verbal impairments (Marr, 1969; Barradas et al., 2016) but also to cognitive, affective and social disturbances (Schmahmann et al., 2008; Limperopoulos et al., 2009; Kitai et al., 2015). Strikingly, pathologic evidence of cerebellar injury in neonates has gained valuable input with the advances in numerous magnetic resonance imaging (MRI) techniques (reviewed by Smyser et al., 2018) in which many HI injury characteristics (Schneider et al., 2009; Matsufuji et al., 2017) and other early-life cerebellar impairments associated with brain injury in premature infants can be detected (Limperopoulos, 2005a,b). Despite the improving imaging techniques, early diagnosis before the formation of MRI-detectable lesions remains challenging (Gopagondanahalli et al., 2016). In addition, ^{1}H MR spectroscopy (^{1}H MRS) offers abundant cerebral metabolites and are applicable to neonatal HI in preterm newborns (Cheong et al., 2006; Xu and Vigneron, 2010) but remains less explored in the cerebellum and even less so in the long-term perspective. Besides, ^{1}H MRS shows early alterations in brain structure and metabolism highly correlated to HI in clinical and preclinical settings (Roelants-Van Rijn et al., 2001; van de Looij et al., 2015; Xu et al., 2015) and could be used as a biomarker for late-term neurodevelopmental outcomes following HI.

The cerebellum is not classically considered a brain region vulnerable to hypoxic-ischemic insults mainly due to its relative distance from the injury site in initial phases of injury (only suffering from systemic hypoxia). However, recent data suggests the presence of cerebellar injury following experimental HI (Taylor et al., 2006; Biran et al., 2011). Since neonatal HI in very immature rats is highly variable and affects the cerebellum up to weeks later (Biran et al., 2011), and may not be detected by standard MRI in adulthood, we aimed to evaluate the long-term effects of mild HI (Sanches et al., 2018) on (1) cerebellar metabolism at adulthood using ^{1}H MRS; (2) locomotor function, and (3) expression of astrocytes, neurons and myelin proteins.

MATERIALS AND METHODS

Animals

Geneva State Animal Ethics Committee and the Swiss Federal Veterinary Service approved this study under GE/132/15 license. The experiments were performed at the EPFL (Centre d'Imagerie BioMédicale – CIBM) and CMU (UNIGE). Male and female Wistar rats were ordered from Charles River Laboratories (L'Arbresle, France). Animals were housed under standard

animal facility conditions (12-h-light, 12-h-dark cycle and room temperature at 22 ± 1°C).

Neonatal Hypoxia-Ischemia

At postnatal day 1 (PND1), the animals were counted, and the litters were culled to have between 8 and 12 animals (males and females were used in the study) to avoid differences regarding animal weights. At PND3, pups were submitted to a mild to a mild hypoxic-ischemic injury as previously described (Sizonenko et al., 2008; van de Looij et al., 2011; Sanches et al., 2018). Briefly, under isoflurane anesthesia (4% induction and 1.5–2.0% maintenance), the right carotid artery was isolated from the vagus nerve and surrounding tissue and permanently occluded with 6.0 silk thread. The surgical access was closed with Histoacryl™ and Steri-strip™. After a 30 min recovery period in a chamber at 37°C in room air, the flux of room air was replaced by 2 L/min of 6% O_2 at 37°C during 30 min to induce hypoxia. Sham-operated (SH) animals were anesthetized, had the incision without carotid occlusion or hypoxia.

Behavioral Analysis

At young adult/adolescence age (from PND45) animals performed locomotor tests. The same investigator performed all experimental sessions in a light and sound controlled room. The number of animals used for the behavioral analysis was SH = 13 and HI = 19.

Open Field (OF)

The test allows the observation of exploratory activity of animals in a novel environment. OF consisted of a circular wooden chamber (100 cm diameter × 30 cm high wall) with a floor divided into 21 fields. Using ANY-Maze software, the open field test was video recorded during 5 min. The latency to leave the central circle, number of crossings and rearings were considered as indicative of spontaneous motor activity (Sanches et al., 2017).

Cylinder Test (CYL)

This test is used to assess the asymmetrical use of forelimbs after hypoxia-ischemia (Grow et al., 2003). Animals were placed inside a Plexiglas cylinder (20 cm diameter × 40 cm high) and videotaped from the top. Spontaneous ipsilateral and contralateral forelimb wall contacts on the cylinder wall were recorded for 4 min. When the number of total contacts was less or equal to twelve, the animal was removed from the statistical analysis. The equation: (ipsilateral contacts/ipsilateral + contralateral) × 100 was used to calculate percentage of asymmetrical use of the forelimbs (Sanches et al., 2013).

Beam Balance (BB)

To access locomotor deficits, we modified the protocol described by Lotan et al. (2014). The rats were trained (three trials) to traverse a narrow wooden beam (width 2.5 cm, length 100 cm). The beam rested on two acrylic boxes at 50 cm above the floor. The animals were placed on one side, with a safe place (a black box) on the other side, allowing the animals to walk on the beam. In the test session, the number of hindpaw slips were counted (in three trials) 24 h after the training session.

In vivo ^{1}H Magnetic Resonance Spectroscopy (^{1}H MRS)

Following behavioral analysis, ^{1}H MRS was carried out in a horizontal, 14.1-T/26-cm magnet (Magnex Scientific, United Kingdom), equipped with a 12-cm inner-diameter gradient (400 mT/m in 200 μs, minimized eddy currents) and interfaced with a DirectDrive console (Varian Inc., Palo Alto, CA, United States). A home-built quadrature surface coil with two geometrically decoupled single-turn loops (16-mm inner diameter), resonating at 600 MHz radio frequency (RF), was used as RF transceiver. Briefly, as previously described (Lei et al., 2009), animals 15 females (7 SH and 8 HI) and 10 males (3 SH and 7 HI) were anesthetized with 5% isoflurane mixed in O_2 and air (1:1) and then maintained under 1.5–2.5% isoflurane during the entire of MR session (∼50–55 min). Once animal heads were stereotaxically fixed by two ear pieces and one bite bar, animals were secured into a home-built holder and transferred to the center of the magnet. During the entire experiment, the animals were monitored for breathing rates (∼60 breaths-per-min) and rectal temperature (∼37°C) through a MR-compatible monitor system (Model 1025, SA Instruments Inc., Stony Brook, NY, United States).

Multislice T_2-weighted images were acquired using the fast spin-echo technique [FSE (Hennig, 1988)], with effective echo time TE_{eff} = 50 ms, repetition time TR = 4000 ms and 4 averages (∼6 min). Thereafter, both first- and second-order shim terms over the VOI were altered accordingly using FASTMAP (Gruetter and Tkáč, 2000) and resulted in water linewidth <20 Hz for a 15 μL volume. Localized ^{1}H-MR spectra of both cerebellar hemispheres were obtained using the SPECIAL technique (Mlynárik et al., 2006), TE/TR = 2.8/4000 ms and 240 averages (16 min) in combination with outer volume suppression and VAPOR water suppression. The corresponding non-water suppressed spectra (eight averages) were acquired for further quantification (assuming 80% water in both hemispheres) of the cerebellum (McBride et al., 2018).

In this study, metabolites were processed and analyzed using the LCModel (Lei et al., 2009 and references therein). In particular, acetate (Ace), alanine (Ala), ascorbate (Asc), aspartate (Asp), creatine (Cr), myo-inositol (Ins), γ-aminobutyric acid (GABA), glutamine (Gln), glutamate (Glu), glycine (Gly), glycerophocholine (GPC), glutathione (GSH), lactate (Lac), *N*-acetyl-aspartate (NAA), *N*-acetyl-aspartyl-glutamate (NAAG), phosphocholine (PCho), phosphocreatine (PCr) phosphoethanolamine (PE), scyllo-inositol (scyllo), macromolecules (Mac), and taurine (Tau) were quantified. Summed concentrations, e.g., Glu+Gln, PCr+Cr, GPC+PCho, and NAA+NAAG, were also calculated. Based on our preliminary data, Scyllo and Ace were noticeably less than 0.2 μmol/g among all spectra of both groups and considered to be non-detectable. No cerebellar volumetric data was analyzed in the study.

Immunoblotting

Total RNA and proteins were extracted with PrepEase RNA/Protein Spin Kit (78871 1 KT; Affymetrix, Santa Clara, CA,

United States) according to the manufacturer's instructions. Protein pellets were resuspended in RIPA buffer (Cell Signaling, 9806S). For immunoblotting, protein extracts were sonicated and the protein concentration was determined using a Bradford assay. Proteins (25 μg) were separated by SDS-PAGE, transferred to nitrocellulose membrane and analyzed, as previously described (Sanches et al., 2018). Briefly, after overnight incubation with primary antibodies for neurons (NeuN, Sigma-Aldrich), astrocytes (GFAP, Sigma-Aldrich) and myelin (MBP, Abcam) were diluted (1:1000) in 0.1% casein (Sigma-Aldrich, C8654) membranes were incubated with the following secondary antibodies (1:10000): goat anti-mouse IgG conjugated with IRDye 680 (LI-COR, B70920-02), goat anti-rabbit IgG conjugated with IRDye 800 (LI-COR, 926-32210). Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR). ImageStudio™ Lite (LI-COR) was used to measure the optical densities of protein signals on all scans. The optical density of each sample was first estimated based on the optical density of a loading control (βIII-tubulin), and then normalized to the corresponding SH value (as 100%) (n = 6–8 animals/group). Original western blotting images are presented in the **Data Sheet S1** (clearly marked in **Supplementary Figure S1**) and the list of antibodies used in the study are presented in the **Supplementary Table S1**. RNA was not analyzed in the study.

Statistical Analysis

All statistical analyses were performed using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, United States). Data are presented as mean ± standard error of the mean (SEM). All comparisons between SH and HI groups were made using unpaired t-tests. For the comparisons between hemispheres, paired t–tests were performed. Two-way ANOVA (GraphPad Prism) was carried out for analyzing treatment factor (HI vs. SH) and one additional factor, e.g., behavior, metabolite and protein expression, respectively. The significance was accepted when $p < 0.05$.

RESULTS

Behavioral Analysis

Due to the role of the cerebellum in locomotion, motor function was evaluated from PND45 (**Table 1**). In the OF test, HI animals presented hyperactivity, i.e., increased number of crossings (t = −2.357, p = 0.026), compared to their controls. Other parameters evaluated in the OF test, such as latency to leave the center of the arena and the number of rearings, did not show significant differences between groups. Furthermore, there is no statistically significant differences in either the beam balance number of mistakes in the paw placements (t = −0.658, p = 0.515) or asymmetrical use of the forelimbs in the cylinder test (t = 0.025, p = 0.77). Taken together, two-way ANOVA on factors of treatment and behavioral outcomes revealed substantial differences in the treatment factor (p = 0.01).

TABLE 1 | Summary of behavioral analysis in the open field, beam balance errors, and asymmetrical use of forelimbs in the Cylinder test.

	SHAM (n = 13)	HI(†) (n = 19)
Open field (OF) – Latency to leave the center	1.4 ± 0.3	2.1 ± 0.5
Number of crossings	179 ± 11.3	207 ± 5.7*
Number of rearings	15.5 ± 2.7	18.5 ± 2.3
Cylinder (%) – asymmetrical use of the forelimbs	52.4 ± 1.7	51.4 ± 1.4
Beam balance – number of errors	1.3 ± 0.1	1.6 ± 0.3

Data are expressed as mean ± SEM. The results were analyzed by unpaired t-test between HI and SHAM. Significance was accepted when $p < 0.05$, as marked "". Two-way ANOVA of treatment and behavioral test factors resulted in significant difference in treatment (p = 0.01, "†").*

Cerebellar Metabolism

In order to search for clinically relevant information on long-term cerebellar metabolism alterations following neonatal HI at PND3 and evaluating potential *in vivo* biomarkers, non-invasive ^{1}H MRS was performed on both cerebella of neonatal HI and SH rats from PND50.

High quality anatomical images did not show brain abnormalities or T_2-hyperintensities in either cerebral cortex or cerebellum in HI rats compared to the SH group. The quality of the images allowed precise location of both volumes of interests, i.e., right and left hemispheres of cerebellum (**Figures 1A,B**). Spectral quality was evaluated based on the improvements of field homogeneities, efficiency of water suppression and sufficient signal-to-noise ratios (SNRs) by accumulation of scans, as explained in methods. For instance, in SH animals, the resulting metabolic linewidth of right hemisphere was 14.4 ± 0.9 Hz and that of left hemisphere was 12.6 ± 0.9 Hz after the improvement of field homogeneities. With averaging the sufficient number of scans (i.e., 240), SNRs of 14.9 ± 0.7 and 14.4 ± 0.7 were achieved in the spectra of the right and left hemispheres (**Figure 1C**), respectively. Such spectral data allowed up to 20 metabolites to be reliably quantified (**Figure 2** and **Table 2**).

Paired t-tests showed that most metabolites in the SH group were similar between right and left hemispheres of the cerebellum, except for Cr (5.2 ± 0.1 vs. 5.8 ± 0.2 μmol/g, p = 0.002), GSH (0.7 ± 0.1 vs. 0.4 ± 0.1 μmol/g, p = 0.027), Gly (0.9 ± 0.1 vs. 0.3 ± 0.1 μmol/g, p = 0.003), and Ins (5.8 ± 0.2 vs. 6.4 ± 0.1 μmol/g, p = 0.037).

In the HI group, metabolic differences between right and left hemispheres of cerebella were observed: e.g., Glu (9.2 ± 0.3 vs. 10.0 ± 0.2 μmol/g, p = 0.008), Ins (5.6 ± 0.1 vs. 6.4 ± 0.1 μmol/g, p = 0.001), Tau (4.2 ± 0.1 vs. 4.9 ± 0.1 μmol/g, p = 0.001), Glu+Gln (12.3 ± 0.3 vs. 13.1 ± 0.2 μmol/g, p = 0.034), GPC+PCho (0.9 ± 0.03 vs. 1.0 ± 0.05 μmol/g, p = 0.007), and Cr+PCr (10.3 ± 0.2 vs. 9.6 ± 0.2 μmol/g, p = 0.03). Interestingly, the observed differences of GSH and Gly between hemispheres in the SH group were abolished ($p \geq 0.8$) in the HI group (**Figure 2**).

Unpaired t-tests revealed noticeable metabolic changes in the left cerebellum two months after neonatal HI compared to the corresponding SH values: increases in the concentrations of Gln (HI vs. SH: 3.1 ± 0.1 vs. 2.6 ± 0.2 μmol/g, p = 0.02), Gly

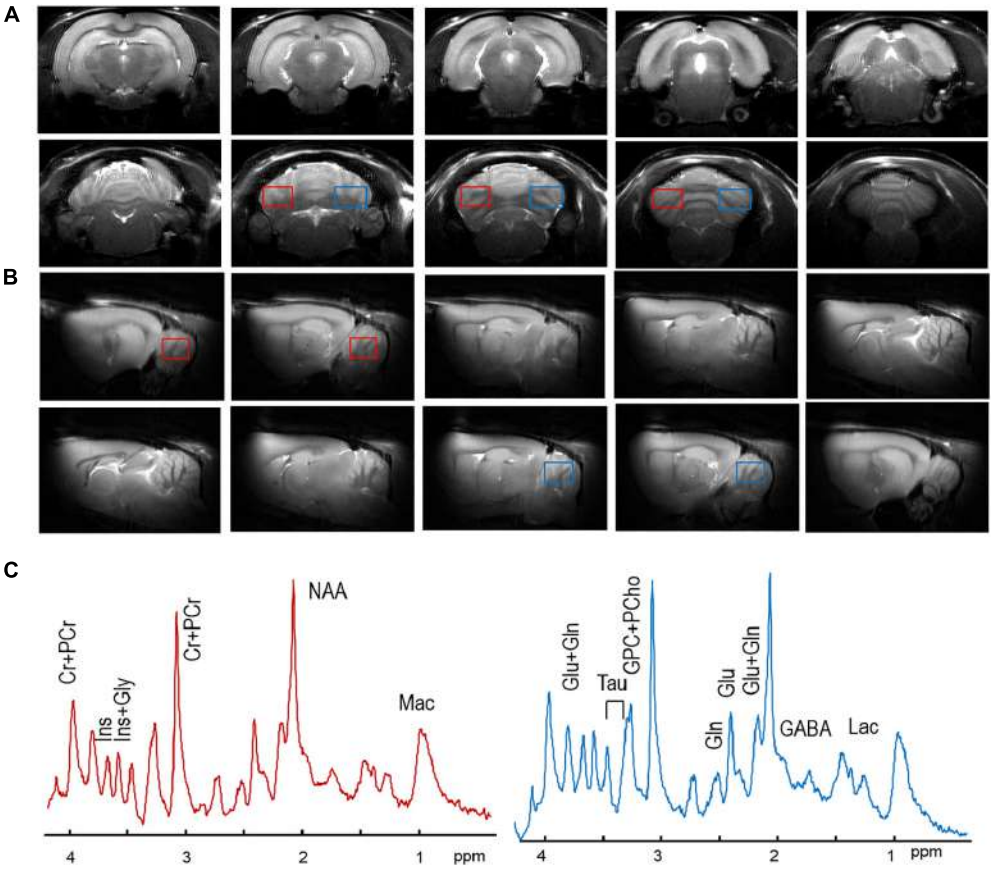


FIGURE 1 | Typical axial **(A)** and sagital **(B)** MR images, and one of each spectrum from the right cerebellum (red boxes) and the left cerebellum (blue boxes) in and **(B)** of one SH rat. In panel **(C)**, no visual difference was observed between the right cerebellar spectrum (in solid red line) and the left one (in solid blue line). No visual differences in MR spectra were observed. Major metabolic resonances were highlighted along with their abbreviations, as listed in the Section "Materials and Methods."

(0.9 ± 0.1 vs. 0.3 ± 0.1 μmol/g, $p = 0.003$), Glu+Gln (13.1 ± 0.2 vs. 11.9 ± 0.4 μmol/g, $p = 0.034$), and GPC+PCho (1.0 ± 0.5 vs. 0.8 ± 0.03 μmol/g, $p = 0.01$) along with decrease in PE (0.2 ± 0.1 vs. 0.7 ± 0.2 μmol/g, $p = 0.027$) (**Figure 2**). Additional alteration trends were noticeable in Glu (10.0 ± 0.2 vs. 9.3 ± 0.4 μmol/g, $p = 0.11$) and NAAG (1.0 ± 0.1 vs. 1.2 ± 0.1 μmol/g, $p = 0.053$) in the left cerebellum of HI animals compared to their respective SH group. However, all metabolites did not show significant differences between right cerebellum hemispheres in both HI and SH groups (**Figure 2** and **Table 2**). Further two-way ANOVA confirmed HI-induced differences in GPC+PCho ($p = 0.06$) and even more so in Gln ($p = 0.018$) and PE ($p = 0.025$), as shown in **Figure 2**.

Cell Markers Protein Expression

Immediately after ^{1}H MRS, protein expression analysis was performed in both cerebellum hemispheres to evaluate plausible anomalies in the expression of proteins related to neurons, myelin and astrocytes. A significant decrease in the expression of NeuN ($t = 2.59$, $p = 0.029$) was observed in the left cerebellum of HI animals (**Figures 3B,C**) compared to SH group. Differences between right and left cerebellar hemispheres were observed in the HI group for NeuN only ($t = 3.492$, $p = 0.006$) (**Figure 3B**). Similarly, hypomyelination was observed through the decrease in MBP expression in the contralateral left cerebellar hemisphere of HI animals compared to SH rats ($t = 2.381$, $p = 0.027$) (**Figure 3B**). Interestingly, despite the decrease in MBP expression in the right hemisphere, no significant differences were observed. Despite metabolic alterations observed in ^{1}H MRS, reactive astrogliosis was not significantly altered between groups; however, a trend to an increase in GFAP in the right hemisphere was observed in the HI group ($t = 2.322$, $p = 0.053$) (**Figure 3A**).

DISCUSSION

To our knowledge, this study is the first to report long-lasting effects of mild neonatal HI on cerebellar metabolism with extension to neuropathology of adult rats. Taking together behavior and motor performances, our *in vivo* ^{1}H MRS findings suggest that neonatal HI at PND3 has consequences on cerebellar metabolism and function of rats in adulthood. Furthermore,

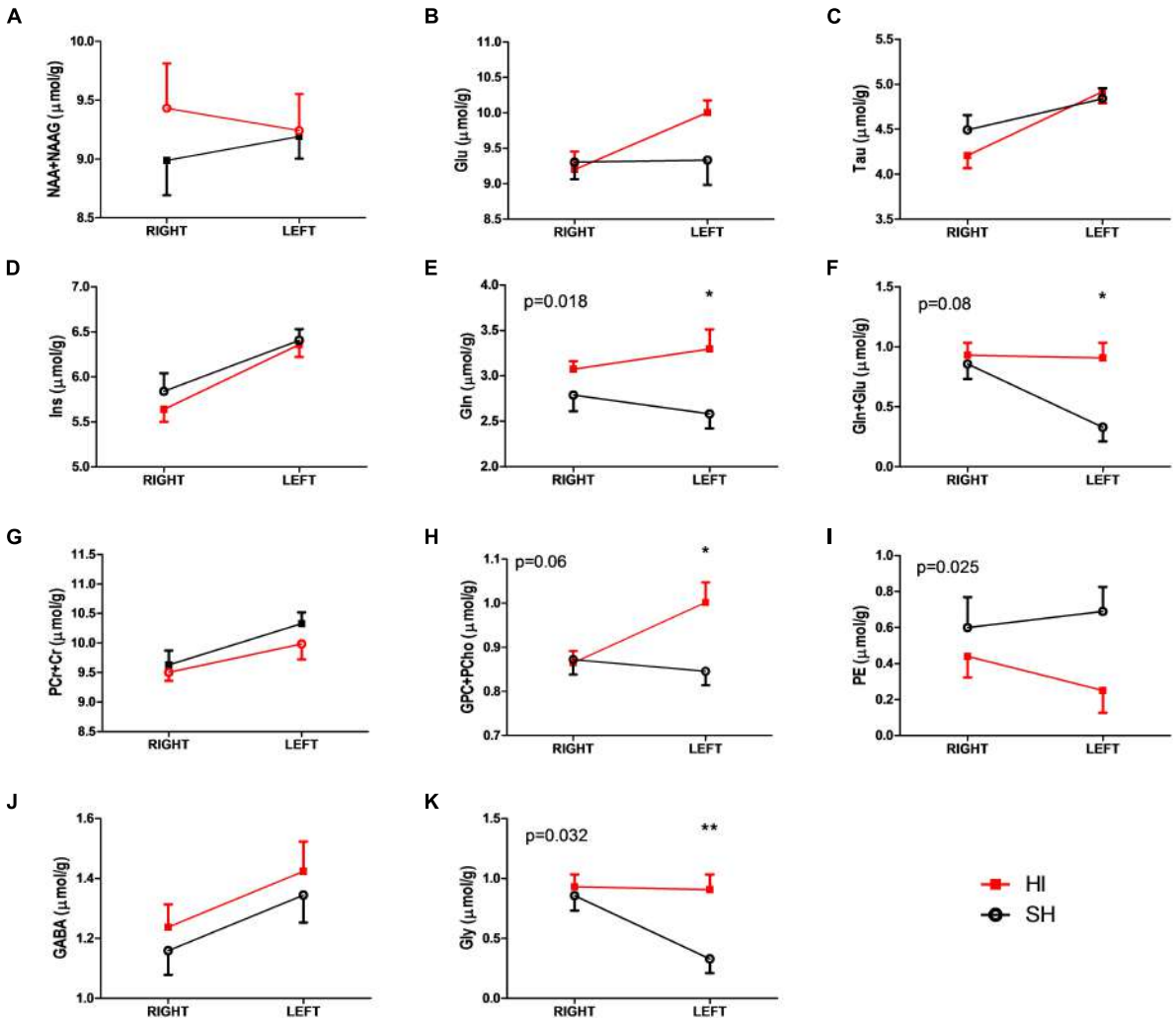


FIGURE 2 | Selected metabolic differences **(A–K)** between SH (open black circles) and HI (solid red squares) groups of adult rats following HI and SH at PND3. Two-way ANOVA analysis showed treatment differences with the corresponding *p*-values **(E,J,K)**. Total choline (GPC+PCho, **H**) and total glutamine and glutamate (Gln+Glu, **F**) showed different trends toward the significant level, i.e., $p < 0.05$. The further Bonferroni post-test revealed further differences, *p*-value <0.05 was marked with "*" and *p*-value <0.01 was marked with "**." Abbreviations were listed in the "Materials and Methods."

in the targeted cerebellum of HI rats, protein expression of both neuronal and myelin markers was reduced compared to their respective controls despite no evidence of damage. Collectively, our study provides relevant *in vivo* evidences that neonatal HI, one of the main causes of Periventricular Leukomalacia and Cerebral Palsy, affects brain pathology in adulthood and induces alterations distant from the injury site, such as in the cerebellum.

Neonatal HI Induces Long-Term Cerebellar Metabolic Alterations in ^{1}H MRS

With the aim of investigating potential effects of neonatal HI on abundant metabolites in the cerebellum, we studied both hemispheres in adult rats after neonatal HI and SH using non-invasive ^{1}H MRS.

Primary Energy Disturbances Right After Neonatal HI Are Restored in Adulthood

Following HI, there is a primary phase of energy failure up to 24 h following injury, with a decrease in energetic brain metabolites such as ATP and PCr (Blumberg et al., 1996; van de Looij et al., 2011; Thornton and Hagberg, 2014). Given the intensity and the initial cortical target injury following neonatal HI (i.e., 30 min hypoxia exposure) and the age at time of analysis of rats in this study, energy related substrates should return to normal levels (van de Looij et al., 2011). In particular, lactate in the targeted cerebellum of HI rats was not different from SH animals. In addition, PCr in the targeted cerebellar hemisphere of HI rats (4.2 µmol/g) was identical to the SH rats (4.2 µmol/g), while a slight increase in Cr in the left cerebellum of HI rats was observed. Thus, the normal levels of energy related substrates in adult rats after neonatal HI at PND3 were restored following the initial

TABLE 2 | Summary of additional cerebellar metabolic results obtained from localized ^{1}H-MRS.

		Right (SH vs. HI)	Left (SH vs. HI)	Right hemisphere	Right vs. left (paired *t*-test)	Left hemisphere
Mac	SH (n = 9)			1.38 ± 0.01		1.41 ± 0.04
	HI (n = 15)			1.33 ± 0.04		1.40 ± 0.05
Ala	SH			0.39 ± 0.09		0.39 ± 0.08
	HI			0.41 ± 0.08		0.39 ± 0.06
Asp	SH			1.12 ± 0.2		0.78 ± 0.20
	HI			1.25 ± 0.2	p = 0.09	0.85 ± 0.14
PCho	SH			0.63 ± 0.11		0.60 ± 0.12
	HI			0.66 ± 0.08		0.69 ± 0.07
Cr	SH	p = 0.08		5.24 ± 0.10	p = 0.002	5.77 ± 0.16
	HI			5.59 ± 0.16	p = 0.06	6.10 ± 0.17
PCr	SH			4.26 ± 0.18		4.21 ± 0.15
	HI			4.04 ± 0.12		4.22 ± 0.17
GABA	SH			1.16 ± 0.08	p = 0.055	1.34 ± 0.09
	HI			1.24 ± 0.08		1.42 ± 0.10
Lac	SH			0.87 ± 0.09		0.94 ± 0.10
	HI			0.67 ± 0.10		0.77 ± 0.08
NAA	SH			8.50 ± 0.34		8.05 ± 0.32
	HI			8.08 ± 0.29		8.20 ± 0.20
Asc	SH			0.90 ± 0.21	p = 0.057	1.55 ± 0.30
	HI			0.94 ± 0.17		1.03 ± 0.18
Glc	SH			2.41 ± 0.48		1.87 ± 0.22
	HI			1.92 ± 0.24		1.81 ± 0.30
GPC	SH			0.24 ± 0.11		0.24 ± 0.11
	HI			0.20 ± 0.08		0.32 ± 0.08

All data were shown as mean ± SEM. Abbreviations were listed in methods. Paired t-test was performed in the same animals. Unpaired t-test was performed between groups, i.e., SH vs. HI.

phase of energy failure immediately after neonatal HI, e.g., drop in PCr and increase in Lac (van de Looij et al., 2011).

Hemispherical Differences in Cerebellar Metabolites Altered by Neonatal HI

Although selected metabolites were noticeably different between the two hemispheres of the cerebellum (**Figure 2** and **Table 2**), substantial metabolic changes in the targeted cerebellum of HI rats remained observable when compared to their respective SH (**Figure 2** and **Table 2**). It is also interesting to note that some hemisphere differences occurring in the SH animals disappeared in HI rats, e.g., GSH and Gly. Although we could not exclude the fact that Gly is highly overlapped with one Ins resonance in the typical ^{1}H MR spectrum, the very similar quality of spectral data (SNR >10, metabolic linewidth ∼14 Hz) and another non-overlapping resonance of Ins provides reliable quantification of both Gly and Ins at 14.1T (Gambarota et al., 2008; Mlynárik et al., 2008). Thus, SH discrepancies could be largely due to some inheriting technical differences, e.g., coil sensitivity or chemical shift error (Mlynárik et al., 2006).

Astrocytic and Neuronal Specific Metabolic Pool Alterations

Myo-inositol (Ins), a glial specific metabolite, exhibited hemispherical differences but was not significantly different between groups in our study (**Figure 2**). In addition, the concentration of putative neuronal markers, NAA and its downstream product (NAAG), did not level off from their control values (**Table 2**). Altogether, this suggests an absence of neuronal death or suffering as well as glial reaction 2 months after HI. Furthermore, while Glu was not reduced in the left hemisphere of HI cerebellum, Gln in both cerebellar hemispheres of the HI rats were elevated compared to their control levels, even more so in the left hemisphere ($p < 0.05$). Since Gln is mainly located in astrocytes, this elevation in Gln may be associated with astrocytic uptake of excessive extracellular glutamate, which has been shown to occur shortly after transient ischemia (Lei et al., 2009).

Membrane Phospholipid Changes in the Cerebellum

It is noteworthy that PE decreases in the cerebellum of adult rats after neonatal HI (**Figure 2**). PE is a precursor to one of the major constituents of the phospholipid bilayer of cellular membranes, phosphatidylethanolamine, which restricts primarily in the inner leaflet. Since PE decreases with brain development in rodents paralleling the progression of myelination and cell proliferation (Gyulai et al., 1984; Blüml et al., 1999; Tkáč et al., 2003), the lower cerebellar PE here may not be fully explained by delayed brain development, given the lower expression of neurons and myelin markers observed in our study (**Figure 3**).

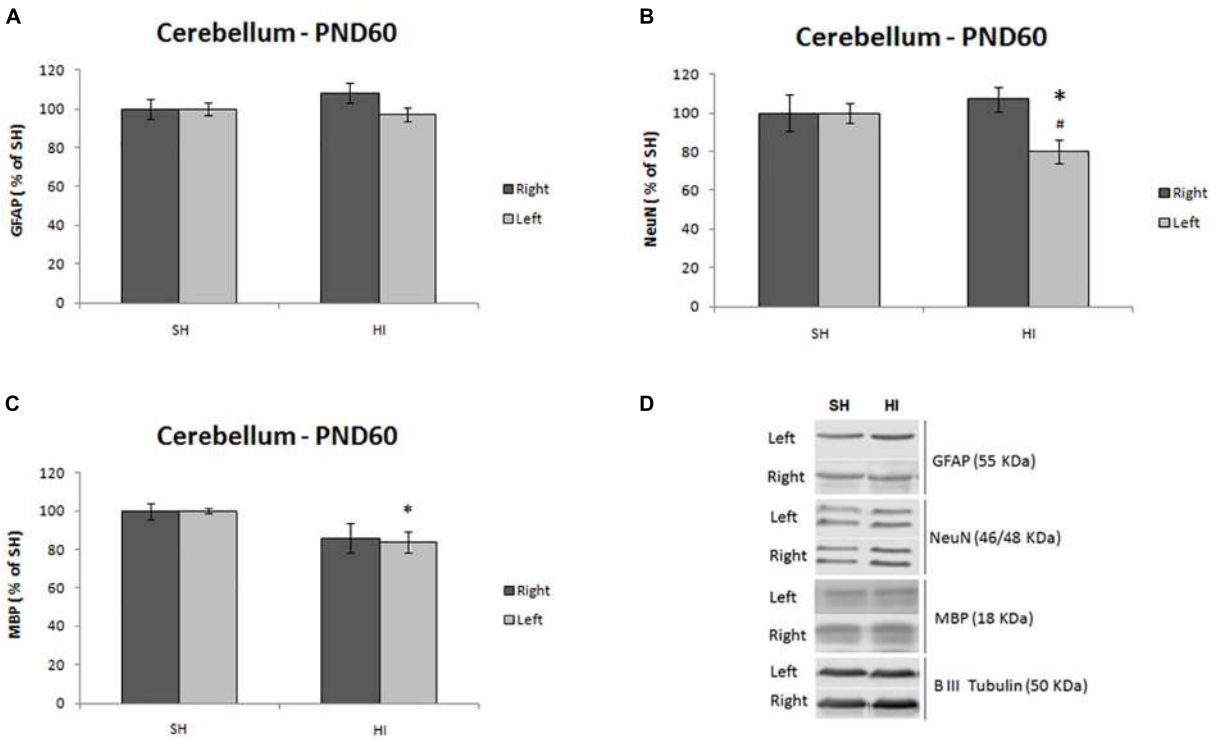


FIGURE 3 | Summary of protein expression of astrocytes (GFAP, **A**), neurons (NeuN, **B**), and myelin (MBP, **C**) in both hemispheres of the cerebellum in adult rats (PND60) after HI or SH at PND3. The representative blots for the NeuN, GFAP, and MBP, for SH and HI groups, were shown in panel **(D)**. Western blotting results are plotted normalized to SH expression level (100%) (mean ± SEM). "*" Stands for difference between HI and SH (n = 6–8 animals per group), and "#" represents difference between right and left hemispheres. Significance was determined using unpaired t-test when $p < 0.05$.

In addition, the sum of two other choline containing compounds of membrane lipid synthesis, i.e., GPC+PCho, is noticeably elevated in the left hemisphere of HI rats (**Figure 2** and **Table 2**). Since PE is the substrate of membrane synthesis and GPC is one membrane breakdown product, our ^{1}H MRS data suggests that membrane synthesis is likely incapable of compensating membrane breakdown in the cerebellum of HI rats. Further reduction of PCho/GPC (∼2.2) in the left cerebellum of HI rats compared to that of SH rats (∼2.5) reinforces such a notion, indicating a possible decrease in cell turnover. Together, *in vivo* ^{1}H MRS of PE is in agreement with the expression of myelin basic protein (MBP) marker.

Glutamatergic Neurotransmission Is Altered in the Cerebellum of Adult Rats After Neonatal HI

Cerebral glutamine is synthesized from extracellular Glu and/or ammonia by glutamine synthase (GS) in the astrocytes. Given that the ammonia present in the blood stream has been detoxified by the liver, it has been postulated that the elevated Gln levels upregulate uptake of extracellular glutamate, especially upon the restoration of reperfusion after acute stroke (Lei et al., 2009). Glu, on the other hand, is mainly located in the neurons and its concentration increase may be associated with numerous factors, namely extracellular glutamate accumulation from cerebellar and/or other efferent neurons, reduction of glutamate transporter and increase in glutamatergic neuron population.

In the absence of significant GFAP changes (**Figure 3A**), elevated concentrations of Gln in the cerebellum of adult rats after neonatal HI doesn't seem to be associated with astrogliosis. Instead, such Gln increase may be related to the uptake of extracellular glutamate into astrocytes. In addition, the reduction of protein expression of NeuN did not fully support the notion that accumulation of Glu may be due to the increase in glutamatergic neuron population. Although we could not exclude either some loss of glutamate transporters at this stage (Torp et al., 1995; Raymond et al., 2011) or the reduction of NeuN in specific cells, e.g., Purkinje cells (Biran et al., 2011), and given the role of the cerebellum in the motor control system (Volpe, 2009b), the accumulation of Glu might partially occur due to the afferent and efferent trans-synaptic connections between cerebellum and other brain regions, e.g., motor cortex and brainstem, respectively.

Given the vulnerability of the cerebellum (Volpe, 2009b), cerebellar damage may be independent of the extend of forebrain injury (Biran et al., 2011). Thus, we hypothesize that the cerebellum after mild neonatal HI (Sizonenko et al., 2008; Sanches et al., 2018) continues to be affected for prolonged periods into adulthood. As no protein overexpression of either astrocytes or neurons was observed, and that the enlarged metabolic pool sizes of glutamatergic neurotransmission with

no reduction in either GABAergic or glycinergic pools occurred in the cerebellum even in adulthood following the very mild neonatal HI injury (**Figure 2**), our data suggests that glutamatergic neurotransmission in the left cerebellum of rats (after right forebrain HI) is altered mostly due to trans-synaptic connections.

Although neurogenesis is nearly complete after birth, Purkinje cells start elaborating their characteristic of dendritic arbors up to PND5 (Sotelo, 2004). In the present study, the protein expression of NeuN was noticeably decreased. This observation is in line with the aforementioned Purkinje cell loss weeks after HI at PND2 and further supported by increased number of apoptotic cells in the internal granular layer day(s) after neonatal HI at PND7 and PND14 (Peng et al., 2005; Taylor et al., 2006).

Altogether, these results indicate that HI injury to the right forebrain, irrespective of the underlying mechanism, induces cell loss in the left cerebellar hemisphere, a brain region not experiencing hypoxic-ischemic insult (Vannucci et al., 1988) and distant from the primary injury.

Metabolic Alterations May Be Associated With Hyperactivity

Premature birth impedes cerebellar development even in the absence of detectable brain injury (Limperopoulos, 2005b). Children suffering from perinatal HI exhibit neurological disorders, learning disabilities, hyperactivity, visual impairments and other limitations that compromise their life quality (Aarnoudse-Moens et al., 2009; Volpe, 2009a). In particular, preterm babies suffering unilateral cerebral injuries show cerebellar damage in the contralateral hemisphere (Limperopoulos, 2005a). Similarly, in rats following neonatal HI at PND7, mild to marked locomotor abnormalities were reported, including shorter intervals for falling from rotarod, impairments in beam walking (McQuillen and Ferriero, 2004) and delayed motor abilities (Lubics et al., 2005). Despite being less investigated than HI at PND7 (Ikeda et al., 2001; McQuillen and Ferriero, 2004; Lubics et al., 2005; Lu et al., 2014), studies using HI at PND3 have shown delays in neurological reflex maturation that lead to motor deficits weeks after the insult near adulthood (Misumi et al., 2016; Durán-Carabali et al., 2017; Sanches et al., 2017). Regardless the influence of immaturity on HI brain damages (Rice et al., 1981; Towfighi et al., 1997), motor dysfunctions remain largely dependent on the severity of injury (Ikeda et al., 2001; McQuillen and Ferriero, 2004; Lubics et al., 2005; Lu et al., 2014; Misumi et al., 2016; Durán-Carabali et al., 2017). However, to date, no *in vivo* ^{1}H MRS study has investigated adulthood cerebellum consequences of HI in the very immature rat.

Thus, non-invasive methodologies that enable identification of ischemic core and penumbra, while seeking plausible biomarkers and therapeutic targets, and longitudinal follow-up treatment in the very same subjects would intrinsically improve diagnosis and prognosis of neonatal HI. Here, we incorporated some well-established behavior and motor function tests (Aarnoudse-Moens et al., 2009) in adult rats after mild neonatal HI, i.e., 30 min hypoxia at PND3 (Sanches et al., 2018) in addition to abundant metabolic information that provides non-invasive biomarkers (Lei et al., 2009; Berthet et al., 2011, 2014). In the present study, after mild neonatal HI, rats showed behavior hyperactivity accompanied by very mild motor dysfunction at adult age (**Table 1**). Indeed, deficits in motor coordination and locomotion tasks associated with cortical damage have been reported shortly after neonatal HI (Ten et al., 2003), e.g., a disorganization of oligodendrocyte development in the sensorimotor cortex (Misumi et al., 2016).

These alterations in cerebellar metabolites (**Figure 2**) accompanied by cellular abnormalities (**Figure 3**), hyperactivity and mild motor dysfunction observed in HI rats at adulthood could be associated with (1) a disturbance in neuron-glial interaction due to myelin loss that was evidenced by altered membrane phospholipids (**Figure 2**) and confirmed by reduced myelination (**Figure 3C**); and (2) an imbalance in glutamatergic neurotransmission with increases in both glutamine and glutamate (**Figure 2**). In parallel, neither substantial cellular changes in the neonatal HI injured cortex (Sanches et al., 2018) nor ^{1}H-MRS-detectable metabolic changes (**Supplementary Figure S2**) were found at this age, supporting the notion that myelin loss may change conduction velocity in trans-synaptic connections, and any remaining alterations resulting from the forebrain HI-insult may contribute toward other interneuron networks. Furthermore, our results indicate that the neuronal loss and hypomyelination due to the combination between hypoxia and ischemia was mainly observed in the left hemisphere of HI group, which confirms the unilateral injury profile, since both hemispheres are affected by systemic hypoxia.

CONCLUSION

Although the precise neuropathologic characteristics, involved in the long-term damage observed in remote areas from injury site in the developing brain following mild neonatal HI, remain to be further explored, our results provide insights into the long-term cerebellar abnormalities in metabolism, cellular damage and functional alterations after mild HI in the very immature rat. Therefore, the capability of ^{1}H MRS in providing useful diagnostic biomarkers after stroke and other diseases in combination with behavior and motor performance tests may improve diagnosis and prognosis in neonatal HI.

ETHICS STATEMENT

Geneva State Animal Ethics Committee and the Swiss Federal Veterinary Service approved this study under GE/132/15 license.

AUTHOR CONTRIBUTIONS

ES, SS, and HL designed the study. ES performed the HI, behavioral analysis, and WB quantification. HL performed the ^{1}H MRS experiments and analyzed the data. ES and AT performed the WB. ES and HL wrote the manuscript. ES, YvdL, SZ, and HL revised the manuscript.

FUNDING

ES received a Swiss Excellence Scholarship for Foreign Scholars to perform the study in our laboratory. This study was supported by the Swiss National Fund N° 33CM30-124101/140334, the Fondation pour Recherches Médicales, Geneva, and the Center for Biomedical Imaging (CIBM) of the UNIL, UNIGE, HUG, CHUV and EPFL, and the Leenaards and Jeantet Foundations.

ACKNOWLEDGMENTS

The authors thank Analina da Silva, Jacqueline Romero, and all other technicians and staff of the CIBM institute for their cooperation in performing the experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00634/full#supplementary-material

FIGURE S1 | Original western blotting runs. Note that only the 7 ladders localized in the left side of the gels were used in the study (n = 6–8 animals/group). Antibodies used in the study were NeuN **(a)**, GFAP **(b)**, βIII Tubulin **(c)**, and MBP **(d)**.

FIGURE S2 | Metabolite concentrations in the brain cortex (R: right and L: left) after neonatal HI compared to the sham-operated animals (SH). Metabolite abbreviations were presented in the Methods section. No substantial differences were observed.

TABLE S1 | Antibodies used in the study.

DATA SHEET S1 | The raw data of typical western blotting runs.

REFERENCES

Aarnoudse-Moens, C. S., Weisglas-Kuperus, N., van Goudoever, J. B., and Oosterlaan, J. (2009). Meta-analysis of neurobehavioral outcomes in very. preterm and/or very low birth weight children. *Pediatrics* 124, 717–728. doi: 10.1542/peds.2008-2816

Alderliesten, T., Nikkels, P. G. J., Benders, M. J. N. L., De Vries, L. S., and Groenendaal, F. (2013). Antemortem cranial MRI compared with postmortem histopathologic examination of the brain in term infants with neonatal encephalopathy following perinatal asphyxia. *Arch. Dis. Child. Fetal Neonatal Ed.* 98, F304–F309. doi: 10.1136/archdischild-2012-301768

Allin, M., Walshe, M., Fern, A., Nosarti, C., Cuddy, M., Rifkin, L., et al. (2008). Cognitive maturation in preterm and term born adolescents. *J. Neurol. Neurosurg. Psychiatry* 79, 381–386. doi: 10.1136/jnnp.2006.110858

Arteni, N. S., Pereira, L. O., Rodrigues, A. L., Lavinsky, D., Achaval, M. E., and Netto, C. A. (2010). Lateralized and sex-dependent behavioral and morphological effects of unilateral neonatal cerebral hypoxia-ischemia in the rat. *Behav. Brain Res.* 210, 92–98. doi: 10.1016/j.bbr.2010.02.015

Barradas, P. C., Savignon, T., Manhães, A. C., Tenório, F., da Costa, A. P., Cunha-Rodrigues, M. C., et al. (2016). Prenatal systemic hypoxia-ischemia and oligodendroglia loss in cerebellum. *Adv. Exp. Med. Biol.* 949, 333–345. doi: 10.1007/978-3-319-40764-7_16

Berthet, C., Lei, H., Gruetter, R., and Hirt, L. (2011). Early predictive biomarkers for lesion after transient cerebral ischemia. *Stroke* 42, 799–805. doi: 10.1161/STROKEAHA.110.603647

Berthet, C., Xin, L., Buscemi, L., Benakis, C., Gruetter, R., Hirt, L., et al. (2014). Non-invasive diagnostic biomarkers for estimating the onset time of permanent cerebral ischemia. *J. Cereb. Blood Flow Metab.* 34, 1848–1855. doi: 10.1038/jcbfm.2014.155

Biran, V., Heine, V. M., Verney, C., Sheldon, R. A., Spadafora, R., Vexler, Z. S., et al. (2011). Cerebellar abnormalities following hypoxia alone compared to hypoxic-ischemic forebrain injury in the developing rat brain. *Neurobiol. Dis.* 41, 138–146. doi: 10.1016/j.nbd.2010.09.001

Blumberg, R. M., Cady, E. B., Wigglesworth, J. S., McKenzie, J. E., and Edwards, A. D. (1996). Relation between delayed impairment of cerebral energy metabolism and infarction following transient focal hypoxia ischemia in the developing brain. *Exp. Brain Res.* 113, 130–137. doi: 10.1007/bf02454148

Blüml, S., Seymour, K. J., and Ross, B. D. (1999). Developmental changes in choline- and ethanolamine-containing compounds measured with proton-decoupled 31P MRS in in vivo human brain. *Magn. Reson. Med.* 42, 643–654. doi: 10.1002/(sici)1522-2594(199910)42:4<643::aid-mrm5>3.0.co;2-n

Breeman, L. D., Jaekel, J., Baumann, N., Bartmann, P., and Wolke, D. (2015). Preterm cognitive function into adulthood. *Pediatrics* 136, 415–423. doi: 10.1542/peds.2015-0608

Cheong, J. L. Y., Cady, E. B., Penrice, J., Wyatt, J. S., Cox, I. J., and Robertson, N. J. (2006). Proton MR spectroscopy in neonates with perinatal cerebral hypoxic-ischemic injury: metabolite peak-area ratios, relaxation times, and absolute concentrations. *Am. J. Neuroradiol.* 27, 1546–1554.

Delobel-Ayoub, M., Arnaud, C., White-Koning, M., Casper, C., Pierrat, V., Garel, M., et al. (2009). Behavioral problems and cognitive performance at 5 years of age after very preterm birth: the EPIPAGE Study. *Pediatrics* 123, 1485–1492. doi: 10.1542/peds.2008-1216

Durán-Carabali, L. E., Sanches, E. F., Marques, M. R., Aristimunha, D., Pagnussat, A., and Netto, C. A. (2017). Longer hypoxia–ischemia periods to neonatal rats causes motor impairments and muscular changes. *Neuroscience* 340, 291–298. doi: 10.1016/j.neuroscience.2016.10.068

Fatemi, A., Wilson, M. A., and Michael, V. J. (2009). Hypoxic ischemic encephalopathy in the term infant. *Clin. Perinatol.* 36:835. doi: 10.1016/j.clp.2009.07.011

Gambarota, G., Xin, L., Perazzolo, C., Kohler, I., Mlynárik, V., and Gruetter, R. (2008). In vivo 1H NMR measurement of glycine in rat brain at 9.4 t at short echo time. *Magn. Reson. Med.* 60, 727–731. doi: 10.1002/mrm.21695

Ginet, V., van de Looij, Y., Petrenko, V., Toulotte, A., Kiss, J., Hüppi, P. S., et al. (2016). Lactoferrin during lactation reduces lipopolysaccharide-induced brain injury. *BioFactors* 42, 323–336. doi: 10.1002/biof.1278

Gopagondanahalli, K. R., Li, J., Fahey, M. C., Hunt, R. W., Jenkin, G., Miller, S. L., et al. (2016). Preterm hypoxic-ischemic encephalopathy. *Front. Pediatr.* 4:114. doi: 10.3389/fped.2016.00114

Grow, J. L., Liu, Y. Q., and Barks, J. D. E. (2003). Can lateralizing sensorimotor deficits be identified after neonatal cerebral hypoxia-ischemia in rats? *Dev. Neurosci.* 25, 394–402. doi: 10.1159/000075665

Gruetter, R., and Tkáč, I. (2000). Field mapping without reference scan using asymmetric echo-planar techniques. *Magn. Reson. Med.* 43, 319–323. doi: 10.1002/(sici)1522-2594(200002)43:2<319::aid-mrm22>3.0.co;2-1

Gyulai, L., Bolinger, L., Leigh, J. S., Barlow, C., and Chance, B. (1984). Phosphorylethanolamine - the major constituent of the phosphomonoester peak observed by 31P-NMR on developing dog brain. *FEBS Lett.* 178, 137–142. doi: 10.1016/0014-5793(84)81257-81250

Hennig, J. (1988). Multiecho imaging sequences with low refocusing flip angles. *J. Magn. Reson.* 78, 397–407. doi: 10.1016/0022-2364(88)90128-x

Howson, C. P., Kinney, M. V., McDougall, L., and Lawn, J. E. (2013). Born toon soon: preterm birth matters. *Reprod. Health* 10(Suppl. 1), 1–9. doi: 10.1186/1742-4755-10-S1-S1

Huang, Z., Liu, J., Cheung, P. Y., and Chen, C. (2009). Long-term cognitive impairment and myelination deficiency in a rat model of perinatal hypoxic-ischemic brain injury. *Brain Res.* 1301, 100–109. doi: 10.1016/j.brainres.2009.09.006

Hübner, S., Reich, B., and Heckmann, M. (2015). Role of sex steroids and their receptors in human preterm infants: impacts on future treatment strategies

for cerebral development. *Biochem. Pharmacol.* 98, 556–563. doi: 10.1016/j.bcp.2015.08.093

Ikeda, T., Mishima, K., Yoshikawa, T., Iwasaki, K., Fujiwara, M., Xia, Y. X., et al. (2001). Selective and long-term learning impairment following neonatal hypoxic-ischemic brain insult in rats. *Behav. Brain Res.* 118, 17–25. doi: 10.1016/s0166-4328(00)00287-4

Joyal, C. C., Meyer, C., Jacquart, G., Mahler, P., Caston, J., and Lalonde, R. (1996). Effects of midline and lateral cerebellar lesions on motor coordination and spatial orientation. *Brain Res.* 739, 1–11. doi: 10.1016/s0006-8993(96)00333-2

Kim, Y. P., Kim, H., Shin, M. S., Chang, H. K., Jang, M. H., Shin, M. C., et al. (2004). Age-dependence of the effect of treadmill exercise on cell proliferation in the dentate gyrus of rats. *Neurosci. Lett.* 355, 152–154. doi: 10.1016/j.neulet.2003.11.005

Kitai, Y., Hirai, S., Ohmura, K., Ogura, K., and Arai, H. (2015). Cerebellar injury in preterm children with cerebral palsy after intraventricular hemorrhage: prevalence and relationship to functional outcomes. *Brain Dev.* 37, 758–763. doi: 10.1016/j.braindev.2014.12.009

Lawn, J. E., Kerber, K., Enweronu-Laryea, C., and Cousens, S. (2010). 3. 6 Million neonatal deaths-what is progressing, and what is not? *Semin. Perinatol.* 34, 371–386. doi: 10.1053/j.semperi.2010.09.011

Lei, H., Berthet, C., Hirt, L., and Gruetter, R. (2009). Evolution of the neurochemical profile after transient focal cerebral ischemia in the mouse brain. *J. Cereb. Blood Flow Metab.* 29, 811–819. doi: 10.1038/jcbfm.2009.8

Levine, S. (1960). Anoxic-ischemic encephalopathy in rats. *Am. J. Pathol.* 36, 1–17.

Limperopoulos, C. (2005b). Late gestation cerebellar growth is rapid and impeded by premature birth. *Pediatrics* 115, 688–695. doi: 10.1542/peds.2004-1169

Limperopoulos, C. (2005a). Impaired trophic interactions between the cerebellum and the cerebrum among preterm infants. *Pediatrics* 41, 1–8.

Limperopoulos, C., Robertson, R. L., Sullivan, N. R., Bassan, H., and du Plessis, A. J. (2009). Cerebellar injury in term infants: clinical characteristics, magnetic resonance imaging findings, and outcome. *Pediatr. Neurol.* 41, 1–8. doi: 10.1016/j.pediatrneurol.2009.02.007

Lotan, D., Benhar, I., Alvarez, K., Mascaro-Blanco, A., Brimberg, L., Frenkel, D., et al. (2014). Behavioral and neural effects of intra-striatal infusion of anti-streptococcal antibodies in rats. *Brain Behav. Immun.* 38, 249–262. doi: 10.1016/j.bbi.2014.02.009

Lu, J., Jiang, L., Zhu, H., Zhang, L., and Wang, T. (2014). Hypoxia-inducible factor-1α and erythropoietin expression in the hippocampus of neonatal rats following hypoxia-ischemia. *J. Nanosci. Nanotechnol.* 14, 5614–5619. doi: 10.1166/jnn.2014.8728

Lubics, A., Reglodi, D., Tamás, A., Kiss, P., Szalai, M., Szalontay, L., et al. (2005). Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic-ischemic injury. *Behav. Brain Res.* 157, 157–165. doi: 10.1016/j.bbr.2004.06.019

Marr, D. (1969). A theory of cerebellar cortex. *J. Physiol.* 202, 437–470. doi: 10.1113/jphysiol.1969.sp008820

Matsufuji, M., Sano, N., Tsuru, H., and Takashima, S. (2017). Neuroimaging and neuropathological characteristics of cerebellar injury in extremely low birth weight infants. *Brain Dev.* 39, 735–742. doi: 10.1016/j.braindev.2017.04.011

McBride, D. W., Nowrangi, D., Kaur, H., Wu, G., Huang, L., Lekic, T., et al. (2018). A composite neurobehavioral test to evaluate acute functional deficits after cerebellar haemorrhage in rats. *J. Cereb. Blood Flow Metab.* 38, 433–446. doi: 10.1177/0271678X17696509

McQuillen, P. S., and Ferriero, D. M. (2004). Selective vulnerability in the developing central nervous system. *Pediatr. Neurol.* 30, 227–235. doi: 10.1016/j.pediatrneurol.2003.10.001

Misumi, S., Ueda, Y., Nishigaki, R., Suzuki, M., Ishida, A., Jung, C. G., et al. (2016). Dysfunction in motor coordination in neonatal white matter injury model without apparent neuron loss. *Cell Transplant.* 25, 1381–1393. doi: 10.3727/096368915X689893

Mlynárik, V., Cudalbu, C., Xin, L., and Gruetter, R. (2008). 1H NMR spectroscopy of rat brain in vivo at 14.1 tesla: improvements in quantification of the neurochemical profile. *J. Magn. Reson.* 194, 163–168. doi: 10.1016/j.jmr.2008.06.019

Mlynárik, V., Gambarota, G., Frenkel, H., and Gruetter, R. (2006). Localized short-echo-time proton MR spectroscopy with full signal-intensity acquisition. *Magn. Reson. Med.* 56, 965–970. doi: 10.1002/mrm.21043

Northington, F. J., Chavez-Valdez, R., and Martin, L. J. (2011). Neuronal cell death in neonatal hypoxia-ischemia. *Ann. Neurol.* 69, 743–758. doi: 10.1002/ana.22419

Peng, J. H. F., Feng, Y., LeBlanc, M. H., Rhodes, P. G., and Parker, J. C. (2005). Apoptosis and necrosis in developing cerebellum and brainstem induced after focal cerebral hypoxic-ischemic injury. *Dev. Brain Res.* 156, 87–92. doi: 10.1016/j.devbrainres.2005.02.002

Phillips, O. R., Clark, K. A., Luders, E., Azhir, R., Joshi, S. H., Woods, S. H., et al. (2013). Superficial white matter: effects of age, sex, and hemisphere. *Brain Connect.* 3, 146–159. doi: 10.1089/brain.2012.0111

Pyhälä, R. (2012). *Psychological and Psychophysiological Functioning of Young Adults Born Preterm: The Helsinki Study of Very Low Birth Weight Adults*. Doctoral dissertation University of Helsinki, Finland.

Raymond, M., Li, P., Mangin, J.-M., Huntsman, M., and Gallo, V. (2011). Chronic perinatal hypoxia reduces glutamate-aspartate transporter function in astrocytes through the janus kinase/signal transducer and activator of transcription pathway. *J. Neurosci.* 31, 17864–17871. doi: 10.1523/JNEUROSCI.3179-11.2011

Rice, J. E., Vannucci, R. C., and Brierley, J. B. (1981). The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann. Neurol.* 9, 131–141. doi: 10.1002/ana.410090206

Robinson, S. (2005). Systemic prenatal insults disrupt telencephalon development: implications for potential interventions. *Epilepsy Behav.* 7, 345–363. doi: 10.1016/j.yebeh.2005.06.005

Roelants-Van Rijn, A. M., Van Der Grond, J., De Vries, L. S., and Groenendaal, F. (2001). Value of1H-MRS using different echo times in neonates with cerebral hypoxia-ischemia. *Pediatr. Res.* 49, 356–362. doi: 10.1203/00006450-200103000-00009

Salmaso, N., Jablonska, B., Scafidi, J., Vaccarino, F. M., and Gallo, V. (2014). Neurobiology of premature brain injury. *Nat. Neurosci.* 17, 341–346. doi: 10.1038/nn.3604

Sanches, E. F., Arteni, N., Nicola, F., Aristimunha, D., and Netto, C. A. (2015). Sexual dimorphism and brain lateralization impact behavioral and histological outcomes following hypoxia-ischemia in P3 and P7 rats. *Neuroscience* 290, 581–593. doi: 10.1016/j.neuroscience.2014.12.074

Sanches, E. F., Arteni, N. S., Scherer, E. B., Kolling, J., Nicola, F., Willborn, S., et al. (2013). Are the consequences of neonatal hypoxia-ischemia dependent on animals' sex and brain lateralization? *Brain Res.* 1507, 105–114. doi: 10.1016/j.brainres.2013.02.040

Sanches, E. F., Durán-Carabali, L. E., Tosta, A., Nicola, F., Schmitz, F., Rodrigues, A., et al. (2017). Pregnancy swimming causes short- and long-term neuroprotection against hypoxia-ischemia in very immature rats. *Pediatr. Res.* 82, 544–553. doi: 10.1038/pr.2017.110

Sanches, E. F., van de Looij, Y., Toulotte, A., da Silva, A. R., Romero, J., and Sizonenko, S. V. (2018). Brain metabolism alterations induced by pregnancy swimming decreases neurological impairments following neonatal hypoxia-ischemia in very immature rats. *Front. Neurol.* 9:480. doi: 10.3389/fneur.2018.00480

Schmahmann, J. D., Smith, E. E., Eichler, F. S., and Filley, C. M. (2008). Cerebral white matter: neuroanatomy, clinical neurology, and neurobehavioral correlates. *Ann. N. Y. Acad. Sci.* 1141, 266–309. doi: 10.1196/annals.1444.017

Schneider, M. M., Berman, J. I., Baumer, F. M., Glass, H. C., Jeng, S., Jeremy, R. J., et al. (2009). Normative apparent diffusion coefficient values in the developing fetal brain. *Am. J. Neuroradiol.* 30, 1799–1803. doi: 10.3174/ajnr.a1661

Sizonenko, S. V., Camm, E. J., Dayer, A., and Kiss, J. Z. (2008). Glial responses to neonatal hypoxic-ischemic injury in the rat cerebral cortex. *Int. J. Dev. Neurosci.* 26, 37–45. doi: 10.1016/j.ijdevneu.2007.08.014

Sizonenko, S. V., Sirimanne, E., Mayall, Y., Gluckman, P. D., Inder, T., and Williams, C. (2003). Selective cortical alteration after hypoxic-ischemic injury in the very immature rat brain. *Pediatr. Res.* 54, 263–269. doi: 10.1203/01.pdr.0000072517.01207.87

Smyser, C. D., Wheelock, M. D., Limbrick, D. D., and Neil, J. J. (2018). Neonatal brain injury and aberrant connectivity. *NeuroImage* 185, 609–623. doi: 10.1016/j.neuroimage.2018.07.057

Sotelo, C. (2004). Cellular and genetic regulation of the development of the cerebellar system. *Prog. Neurobiol.* 72, 295–339. doi: 10.1016/j.pneurobio.2004.03.004

Taylor, D. L., Joashi, U. C., Sarraf, C., Edwards, A. D., and Mehmet, H. (2006). Consequential apoptosis in the cerebellum following injury to the developing rat forebrain. *Brain Pathol.* 16, 195–201. doi: 10.1111/j.1750-3639.2006.00017.x

Ten, V. S., Bradley-Moore, M., Gingrich, J. A., Stark, R. I., and Pinsky, D. J. (2003). Brain injury and neurofunctional deficit in neonatal mice with hypoxic-ischemic encephalopathy. *Behav. Brain Res.* 145, 209–219. doi: 10.1016/S0166-4328(03)00146-3

Thomason, M. E., Scheinost, D., Manning, J. H., Grove, L. E., Hect, J., Marshall, N., et al. (2017). Weak functional connectivity in the human fetal brain prior to preterm birth. *Sci. Rep.* 7:39286. doi: 10.1038/srep39286

Thornton, C., and Hagberg, H. (2014). Role of mitochondria in apoptotic and necroptotic cell death in the developing brain. *Clin. Chim. Acta* 451, 35–38. doi: 10.1016/j.cca.2015.01.026

Tkáč, I., Rao, R., Georgieff, M. K., and Gruetter, R. (2003). Developmental and regional changes in the neurochemical profile of the rat brain determined by in vivo 1H NMR spectroscopy. *Magn. Reson. Med.* 50, 24–32. doi: 10.1002/mrm.10497

Torp, R., Lekieffre, D., Levy, L. M., Haug, F. M., Danbolt, N. C., Meldrum, B. S., et al. (1995). Reduced postischemic expression of a glial glutamate transporter, GLT1, in the rat hippocampus. *Exp. Brain Res.* 103, 51–58. doi: 10.1007/BF00241964

Towfighi, J., Mauger, D., Vannucci, R. C., and Vannucci, S. J. (1997). Influence of age on the cerebral lesions in an immature rat model of cerebral hypoxia-ischemia: a light microscopic study. *Brain Res. Dev. Brain Res.* 100, 149–160. doi: 10.1016/s0165-3806(97)00036-9

van de Looij, Y., Chatagner, A., Hüppi, P. S., Gruetter, R., and Sizonenko, S. V. (2011). Longitudinal MR assessment of hypoxic ischemic injury in the immature rat brain. *Magn. Reson. Med.* 65, 305–312. doi: 10.1002/mrm.22617

van de Looij, Y., Dean, J. M., Gunn, A. J., Hüppi, P. S., and Sizonenko, S. V. (2015). Advanced magnetic resonance spectroscopy and imaging techniques applied to brain development and animal models of perinatal injury. *Int. J. Dev. Neurosci.* 45, 29–38. doi: 10.1016/j.ijdevneu.2015.03.009

Vannucci, R. C., Lyons, D. T., and Vasta, F. (1988). Regional cerebral blood flow during hypoxia-ischemia in immature rats. *Stroke* 19, 245–250. doi: 10.1161/01.STR.19.2.245

Volpe, J. J. (2009a). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 8, 110–124. doi: 10.1016/S1474-4422(08)70294-1

Volpe, J. J. (2009b). Cerebellum of the premature infant: rapidly developing, vulnerable, clinically important. *J. Child. Neurol.* 24, 1085–1104. doi: 10.1177/0883073809338067

Volpe, J. J., Kinney, H. C., Jensen, F. E., and Rosenberg, P. A. (2011). Reprint of "The developing oligodendrocyte: key cellular target in brain injury in the premature infant.". *Int. J. Dev. Neurosci.* 29, 565–582. doi: 10.1016/j.ijdevneu.2011.07.008

Xu, D., and Vigneron, D. (2010). Magnetic resonance spectroscopy imaging of the newborn brain—a technical review. *Semin. Perinatol.* 34, 20–27. doi: 10.1053/j.semperi.2009.10.003

Xu, S., Waddell, J., Zhu, W., Shi, D., Marshall, A. D., McKenna, M. C., et al. (2015). In vivo longitudinal proton magnetic resonance spectroscopy on neonatal hypoxic-ischemic rat brain injury: neuroprotective effects of acetyl-L-carnitine. *Magn. Reson. Med.* 74, 1530–1542. doi: 10.1002/mrm.25537

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Sanches, van de Looij, Toulotte, Sizonenko and Lei. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 11 June 2019
doi: 10.3389/fphys.2019.00718

Childhood Neurodevelopmental Outcome in Low Birth Weight Infants With Post-ligation Cardiac Syndrome After Ductus Arteriosus Closure

Maria Carmen Bravo[1], Marta Ybarra[1], Rosario Madero[2] and Adelina Pellicer[1]*

[1] Department of Neonatology, La Paz University Hospital, Madrid, Spain, [2] Division of Statistics, La Paz University Hospital, Madrid, Spain

OPEN ACCESS

Edited by:
Mary Tolcos,
RMIT University, Australia

Reviewed by:
Brigitte Vollmer,
University of Southampton, United Kingdom
Patrick McNamara,
The Hospital for Sick Children, Canada

****Correspondence:***
Maria Carmen Bravo
mcarmen.bravo@salud.madrid.org

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *15 October 2018*
Accepted: *23 May 2019*
Published: *11 June 2019*

Citation:
Bravo MC, Ybarra M, Madero R and Pellicer A (2019) Childhood Neurodevelopmental Outcome in Low Birth Weight Infants With Post-ligation Cardiac Syndrome After Ductus Arteriosus Closure. Front. Physiol. 10:718. doi: 10.3389/fphys.2019.00718

Background: Post-ligation cardiac syndrome (PLCS) is a common complication of patent ductus arteriosus (PDA) surgical closure in low birth weight infants. It has been associated with mortality, but there is a lack of information about the neurodevelopmental outcome of the survivors. We aimed to explore the prevalence of PLCS and to assess whether this clinical condition is a risk factor for adverse outcome, (moderate or severe neurodevelopmental disabilities).

Methods: We retrospectively reviewed the medical charts of all the infants < 30 weeks of gestation who underwent ductus arteriosus ligation at our unit between 2005 and 2009.

Results: During the study period, 39 preterm infants [mean gestational age 26.4 (2) weeks] underwent surgical closure of the PDA at a mean postnatal age of 25.3 (2.3) days. Twenty six percent of the study population developed PLCS. Five infants died and the follow-up was accomplished in 24 infants (70% of the survivors) at a mean age of 5.3 (1.5) years (range 2–9 years). Neurodevelopmental impairment was observed in 6 in the PLCS group (75%) and in 6 infants in the no PLCS group (37%), $p = 0.08$]. Multiple regression analyses showed that the best fitting model for predicting adverse outcome included PLCS and low birth weight, $p = 0.018$.

Conclusion: Preterm infants undergoing surgical closure of PDA who fulfill the criteria of PLCS according to this study seem to have a tendency toward higher risk of long-term neurodevelopmental impairment. Prospective clinical trials reporting long-term follow-up data should be designed to confirm the hypotheses generated in this pilot study.

Keywords: ductus arteriosus, preterm, cardiorespiratory instability, ductus ligation, neurodevelopment

Abbreviations: CP, cerebral palsy; IS, inotrope score; IVH, intraventricular hemorrhage; MPC, mental processing composite of the kaufman assessment battery for children; PDA, patent ductus arteriosus; PVHI, periventricular haemorrhagic infarction; PLCS, post-ligation cardiac syndrome; WMD, white matter damage.

INTRODUCTION

Post-ligation cardiac syndrome (PLCS), characterized by hypotension requiring cardiovascular support and ventilation or oxygenation failure, complicates the postoperative course of the surgically closed patent ductus arteriosus (PDA). The condition is particularly common in the youngest and most immature infants (McNamara et al., 2010; Sehgal et al., 2010), or in those who required cardiovascular support before surgery (Teixeira et al., 2008; Sehgal et al., 2010). Compromised ventricular performance as a result of increased systemic vascular resistance in combination with a decrease in left ventricular preload, are thought to underlie the pathophysiology of PLCS (McNamara et al., 2010; El-Khuffash et al., 2013, 2014). Increased mortality associated with PLCS has been shown (Harting et al., 2008). An increased risk of neurosensory impairment delay has been reported in infants who underwent PDA surgical closure (Kabra et al., 2007; Weisz et al., 2014). However, the primary determinants of adverse outcomes in those who undergo surgical PDA closure are confounded by the indication for this particular surgery that may be associated with adverse outcome.

We hypothesize that PLCS itself is a risk factor for impaired neurodevelopmental outcome. Therefore, we conducted a retrospective review in a cohort of infants who underwent surgical closure of the PDA. Our primary aim was to assess the prevalence of PLCS and to evaluate the impact of this condition on the long-term neurodevelopmental outcome.

MATERIALS AND METHODS

Clinical charts were reviewed of all infants born younger than 30 weeks of gestation who were free of congenital heart disease, and who underwent PDA ligation at the La Paz University Hospital in Madrid, Spain, between January 2005 and November 2009. The study was approved by the hospital's Ethics Committee. All the infants underwent a muscle-sparing posterolateral thoracotomy and clip occlusion. Surgery was conducted at the NICU in all cases. Anesthetic management was standardized using total intravenous anesthesia with fentanyle and neuromuscular blockade with cisatracurium. Changes in ventilation parameters or cardiovascular support were done at the discretion of the attending clinician. Cardiovascular management after surgery was guided by a constellation of routine clinical or biochemical data, mainly blood pressure, following a standardized protocol: dopamine (first line), epinephrine (second line), and dobutamine (third line) plus/minus volume expansion; and hydrocortisone in case of refractory shock. The following perinatal variables potentially influencing neonatal outcomes were considered: gestational age, birth weight, sex, antenatal steroids, cord pH, Apgar score, need for advanced resuscitation at birth, and cesarean section. Pre-surgery clinical and echocardiographic features of pulmonary over-circulation and systemic hypoperfusion that supported the indication of PDA ligation, and the postnatal age at surgery were also recorded. The severity of ductal disease before surgery was retrospectively classified by one of the study researchers (MCB) using the Teixeira's grading system (Teixeira et al., 2008). Accordingly, category I included infants with profuse pulmonary hemorrhage or cardiocirculatory failure requiring $>$ 2 inotropes; category II, included babies with deteriorating respiratory status, preterm babies $<$ 26 weeks of gestation with large PDA and clinical contraindication for medical treatment, cardiocirculatory failure requiring $>$ 1 inotropes, or necrotizing enterocolitis and a large PDA; and finally, category III was reserved for inability to extubate or wean respiratory support or cardiocirculatory failure associated with failure to thrive (Teixeira et al., 2008). The pre- and immediate postoperative (24-h period after PDA ligation) respiratory and circulatory status were systematically evaluated including the following data: type of ventilator support, supplemental oxygen, blood gasses and acid-base status, blood pressure, heart rate, and inotrope score (IS) [(dopamine dose $\times$ 1) + (dobutamine dose $\times$ 1) + (epinephrine dose $\times$ 10) in μg/kg/min] (Wernovsky et al., 1995). PLCS was defined as the need for cardiovascular support escalation, and either oxygenation or ventilation failure that were unexplained by other surgery-related complications. Cardiovascular support escalation was considered in the case of more than 20% increase in IS or need for fluid bolus administration (Jain et al., 2012). Oxygenation failure was considered when a non-transient (longer than 1 h) $\geq$ 20% increase in the inspired oxygen fraction or mean airway pressure was observed. Finally, ventilation failure was defined as need to switching to high frequency oscillatory ventilation or a consistent (longer than 1 h) $\geq$ 20% amplitude increase if already on high frequency oscillatory ventilation. Other surgery-related complications such as pneumothorax, vocal cord palsy, surgical wound infection, or immediate postoperative mortality were recorded. The main neonatal clinical outcomes were documented, that included the most severe cranial ultrasound diagnoses made by an expert neonatologist on brain ultrasonography who was blind to which group the patients belonged [(intraventricular hemorrhage (IVH), periventricular haemorrhagic infarction (PVHI), or white matter damage (WMD) at term equivalence)]. WMD was defined as porencephalic cysts, cystic periventricular leukomalacia, cerebral atrophy or persistent periventricular echogenicity (PVE) at term age.

Follow-Up

The surviving infants were included in the routine follow-up program and underwent regular physical and motor exams by an experienced team of neonatologist, psychologist, pediatric neurologist and pediatric psychiatrists if needed that were blinded to whether the infants had PLCS or not. Neurodevelopment was evaluated by the Bayley scales of infants development, second edition (BSID-II) (Bayley, 1993) at 2 years' corrected age. Test results were adjusted for prematurity. Cognitive assessment was performed in children aged 4–6 years using the Wechsler preschool and primary scale of intelligence (WPSI), Third Edition (Wechsler, 2010) or Kaufman Brief Intelligence Test, Second Edition (KBIT-2) (Kaufman and Kaufman, 2011). The history of vision, hearing

function and behavioral problems was collected from the medical records. Cerebral palsy (CP) was scored according to the Gross Motor Function Classification System (Cans, 2000). Neurodevelopmental disabilities were classified as: (1) absent or mild (no CP or GMFCS I; Bayley II mental and psychomotor scores > 84, global intelligence quotient, IQ, >70; normal hearing and normal vision); (2) moderate or severe (GMFCS level $\geq$ II; or Bayley II scores $\leq$ 84; or global IQ $\leq$ 70; or sensorineural hearing loss defined as a hearing threshold $\geq$ 20 dB by auditory brainstem responses; or visual deficit; or behavioral/emotional problems including affective problems, anxiety, somatic complaints, attention deficit/hyperactivity, oppositional defiant or rule-breaking behavior or pervasive developmental disorder (Alarcon et al., 2016).

The data were analyzed using the statistical software SPSS for windows, version 25. The quantitative data are expressed as means (standard deviation) and qualitative data as counts (percentages). The perinatal data, perioperative variables, and outcome variables of the study population (PLCS vs. no PLCS groups) were compared using the Mann-Whitney *U* test and Fisher's exact test. There were also studied, using a multiple regression analyses, the perinatal variables that could be associated with moderate or severe neurodevelopmental disabilities in order to create the best model for predicting adverse outcome. All the statistical analyses were considered bilateral, and values of $p < 0.05$ were considered significant.

RESULTS

Forty-two infants underwent surgical closure of the PDA during the investigation period. The clinical charts of 3 infants were poor or incomplete; thus, only 39 preterm infants with a mean gestational age of 26.4 (2) weeks formed the studied cohort. The surgery lasted a median of 45 min with a range of 25–140 min. The perinatal variables between the 10 infants who developed PLCS (26%, PLCS group) and the 29 infants who did not develop the condition (74%, no PLCS group) did not differ (**Table 1**). Eighty-seven per cent (n = 34) of the study population received medical treatment for PDA closure before surgery (17 indomethacin, 14 ibuprofen, and 3 both medications). Thus, for 5 infants (13% of the cohort) surgery was primarily indicated due to a contraindication for medical treatment (renal insufficiency, severe IVH, necrotising enterocolitis, or bowel perforation). PDA category I before surgery was more common in the PLCS group, whereas those in the no PLCS group were more frequently classified as PDA category III (**Table 1**). Before surgery, infants in the PLCS group had higher mean airway pressure than those in the no PLCS group, although not statistically significant [PLCS group 9.4 (2.3) cm H_2O; no PLCS group 7.7 (2) cm H_2O; p = 0.06]. No other haemodynamic or respiratory variables differed between the study groups before surgery. The evolution of the IS after surgery is shown in **Table 1**. All the infants in the PLCS group and 11 (38%) infants in the no PLCS group developed oxygenation failure (p = 0.002); one infant in the PLCS group also had ventilation failure. Other surgery-related complications were pneumothorax (n = 2, 5%) and vocal cord palsy (n = 5, 13%), without differences between study groups. No immediate postoperative mortality or surgical wound infection were found in this series.

TABLE 1 | Perinatal, perioperative, and outcome variables.

	PLCS group (n = 10)	No PLCS group (n = 29)	p
Perinatal and pre-ligation variables			
Gestational age, wks (SD)	25.6 (1.8)	26.6 (1.9)	0.1
Birth weight, g (SD)	822 (124)	884 (253)	0.3
SGA, n (%)	0 (0)	3 (10)	0.5
Male, n (%)	7 (70)	20 (69)	1
Multiple birth, n (%)	4 (40)	10 (34)	1
Antenatal steroids, n (%)	6 (60)	17 (59)	0.9
Cesarean section, n (%)	6 (60)	20 (69)	0.7
Advanced resuscitation, n (%)	8 (80)	21 (72)	1
Apgar 5 min (SD)	7.2 (1.2)	6.9 (1.2)	0.5
Cord pH (SD)	7.27 (1.4)	7.31 (0.04)	0.5
HMD, n (%)	10 (100)	25 (86)	0.5
IVH grade III or PVHI, n (%)	1 (10)	4 (14)	1
Perioperative variables			
PDA medical treatment, n (%)	9 (90)	24 (83)	1
Postnatal age at surgery, days (SD)	22 (12)	26 (25)	0.4
Ductus diameter, mm (SD)	2.5 (0.7)	2.6 (1)	0.8
Ductus category I, n (%)	4 (40)	3 (10)	0.06
Ductus category II, n (%)	5 (50)	10 (34)	0.4
Ductus category III, n (%)	1 (10)	15 (52)	0.03
IS before surgery (SD)	10.2 (12)	3.6 (5)	0.1
IS 1 h after surgery (SD)	11.8 (14)	3.9 (5.7)	0.1
IS 8 h after surgery (SD)	13 (10.7)	3.2 (5.2)	0.02
IS 12 h after surgery (SD)	12.3 (12.3)	2.8 (5)	0.052
IS 24 h after surgery (SD)	16 (13.4)	1.5 (3)	0.008
Outcome variables at term equivalence			
Mortality, n (%)	1 (10)	4 (14)	1
ROP requiring laser therapy, n (%)	5 (50)	10 (34)	0.2
Necrotizing enterocolitis, n (%)	3 (30)	7 (24)	0.7
BPD, n (%)	6 (85)	14 (64)	0.4
WMD, n (%)	7 (70)	14 (52)	0.5

Mann-Whitney U test and Fisher's exact test. Quantitative data are presented as means (SD) and qualitative data as count (percentages). SGA, small for gestational age; antenatal steroids, complete course; advanced resuscitation, endotracheal intubation in the delivery room; HMD, hyaline membrane disease; IS, inotropic score (Wernovsky et al., 1995) [(dopamine dose × 1) + (dobutamine dose × 1) + (epinephrine dose × 10)] ROP, retinopathy of prematurity; BPD, bronchopulmonary dysplasia defined as supplemental oxygen at 36 weeks of gestation; IVH, intraventricular hemorrhage; PVHI, periventricular haemorrhagic infarction; WMD, white matter damage.

Five infants (13%) died, without differences between groups. Among survivors, 70% (n = 24) were followed until a mean age of 5.3 (1.5) years (range 2–9 years). Patients lost to follow-up were primarily due to transfer to the referral hospital after PDA ligation [n = 1 (10%) in the PLCS group; and n = 9 (31%) in the no PLCS group; p = 0.3]. No differences in the perinatal or pre-ligation variables were observed between the infants that were followed and those who were not (gestational age, 26.4 (1.8) and 26.3 (2.2) weeks, p = 0.9; birth weight, 870 (210) and 864 (284)

g, p = 0.9; PDA category I, n = 5 (17.2%) and n = 2 (20%), p = 0.8). The main neonatal clinical outcomes at term equivalence are shown in **Table 1**.

At follow-up, moderate or severe neurodevelopmental impairment was more prevalent in those infants who developed PLCS after ductus surgery [n = 6 in the PLCS group (75%) and in 6 infants in the no PLCS group (37%), p = 0.08. Low birth weight was also associated with neurodevelopmental disability (p = 0.013). We found that the cut-off value with 67% of sensitivity and specificity for neurodevelopmental impairment was 850 gr at birth, p = 0.1. Thus, the best fitting model for predicting moderate or severe neurodevelopmental disabilities included PLCS and birth weight, p = 0.018. Neither the gestational age nor the ductus category before surgery improved the predictive model (only one of the infants with neurodevelopmental disabilities was classified as ductus category I).

The follow-up of the infants with adverse outcome (death or any grade of moderate or severe neurodevelopmental impairment) is described in **Table 2**.

TABLE 2 | Infants with adverse outcome defined as death or any grade of moderate or severe neurodevelopmental impairment.

Patient	GA (weeks)	Ductus category	PLCS	Most severe CUS diagnose	Death	Long-term outcome: findings	Follow up (years)
7	25.3	I	No	IVH grade II and WMD (PVE)	Yes		–
11	24	II	Yes	WMD (PVE)	No	MDI 75 at 2 years' corrected age PDI 88 at 2 years' corrected age SNHL CP (GMFCS level III): spastic diplegia	5
13	24.7	II	No	WMD (cerebral atrophy and PVE)	No	MDI 93 at 2 years' corrected age PDI 83 at 2 years' corrected age Motor and language delay at 5 years old	5
12	24	II	Yes	WMD and IVH grade II	No	Attention deficit/hyperactivity Language delay at 5 years old	5
14	27.6	II	Yes	IVH grade II	No	MDI 90 at 2 years' corrected age PDI 80 at 2 years' corrected age CP (GMFCS level I): spastic diplegia Attention deficit/hyperactivity	4
17	24	I	Yes	WMD (cerebral atrophy and PVE)	No	SNHL and visual deficit Autism spectrum disorder Motor, cognitive and language delay at 8 years old	8
19	26.7	I	No	IVH grade III and PHH	Yes		–
23	24.1	III	No	IVH grade II and WMD (PVE)	No	MDI 91 at 2 years' corrected age PDI 83 at 2 years' corrected age Motor delay at 5 years old	5
24	25.4	III	No	IVH grade II and WMD	No	MDI 71 at 2 years' corrected age PDI 58 at 2 years' corrected age Motor and language delay at 6 years old	6
25	26	III	No	WMD (PVE)	No	MDI 95 at 2 years' corrected age PDI 80 at 2 years' corrected age	2
28	27.1	III	No	IVH grade III and PVHI	Yes		–
31	30.3	II	No	WMD (PVE)	Yes		–
32	28.6	III	No	Normal	No	Attention deficit/hyperactivity CI 73 Motor and language delay at 6.5 years old	6.5
34	24.2	I	Yes	IVH grade II	Yes		–
36	26.1	II	Yes	IVH grade II	No	MDI 83 at 2 years' corrected age PDI 89 at 2 years' corrected age Rule-breaking behavior	5
37	26	III	Yes	WMD (PVE)	No	Motor and language delay Attention deficit/hyperactivity Rule-breaking behavior	7
40	24.7	III	No	IVH grade II and WMD (PVE)	No	MDI 72 at 2 years' corrected age PDI 79 at 2 years' corrected age Language delay at 5 years old	5

GA, gestational age; PLCS, postligation cardiac syndrome; CUS, cranial ultrasound; WMD, white matter damage; PVE, persistent periventricular echogenicity at term age; IVH, intraventricular hemorrhage; PVHI, periventricular haemorrhagic infarction; posthaemorrhagic hydrocephalus; MDI, Bayley scale II-mental developmental index; PMI, Bayley scale II-psychomotor developmental index; SNHL, sensorineural hearing loss; CP, cerebral palsy; GMFCS, gross motor function classification system; WPSI, Wechsler preschool and primary scale of intelligence.

DISCUSSION

This is the first study reporting on the impact that PLCS itself has on the long-term outcome of preterm infants undergoing PDA surgical closure. PLCS has been associated with mortality (Harting et al., 2008) and we have shown that the survivors who developed this condition after surgery seem to be at higher risk of neurodevelopmental impairment, although we could not find a statistical association probably due to the small sample size of this study. These findings have two implications. First, surgical ligation of the PDA could be a risk factor for neurodevelopmental impairment as the association between this kind of treatment and poor neurologic outcome has been reported (Kabra et al., 2007; Weisz et al., 2014). However, most infants undergoing surgery are also the sickest; thus, in the absence of randomized clinical trials addressing the causal role of the surgical procedure on the adverse outcome, it is essential to further characterize which infants undergoing PDA ligation are exposed to an increased risk for neurodevelopmental impairment. This step is crucial, given that surgery is considered a rescue treatment in the case of medical treatment failure or contraindication for cyclooxygenase inhibitor prescription. In this report we have observed that infants with profuse pulmonary hemorrhage or cardiocirculatory failure requiring > 2 inotropes before surgery are at higher risk of developing PLCS. Although PLCS is commonly observed in the sickest infants, we hypothesize whether PLCS, especially when is observed in the infants with a birth weight below 850 gr, could be an independent risk factor for neurodevelopmental impairment (neither the gestational age nor the ductus category before surgery were associated with poor neurodevelopmental outcome). Second, the primary determinants for the development of PLCS are the combination of impaired myocardial performance together with increased systemic vascular resistance and a sudden reduction in preload (Teixeira et al., 2008; McNamara et al., 2010; Jain et al., 2012; Ting et al., 2016). Regrettably, this retrospective study did not systematically evaluate cardiac performance by echocardiography; however, the escalation in cardiovascular treatment that was observed during the immediate postoperative period supports this notion. In fact, our patients clearly shown differential IS after surgery that was statistically significant across the whole postoperative observation period. To date, milrinone has been proposed as a preventive intervention in this population (Jain et al., 2012; Ting et al., 2016). Randomized clinical trials to address the efficacy and safety of this inodilator in this indication would be of utmost interest. It is, therefore, essential to fully characterize which infants are at greater risk of PLCS in the face of implementing preventive or treatment strategies.

Our study and previous reports (Harting et al., 2008) suggest that PLCS occurs in one in 4 or 5 preterm infants who undergo PDA surgical closure. PLCS has been associated with immaturity (Harting et al., 2008; McNamara et al., 2010; Sehgal et al., 2010) and greater need for cardiovascular support before surgery (Harting et al., 2008; Teixeira et al., 2008; Sehgal et al., 2010). We did not observe differences in either the perinatal or the perioperative variables among those who developed PLCS and those who did not, probably due to the small sample size. However, the infants in this series who suffered PLCS had a PDA that was categorized as being more severe than that of the infants without PLCS.

After analyzing the potential confounders for predicting neurodevelopmental impairment, we observed that, as the low birth weight was the only perinatal risk factor associated with adverse outcome in this study, the best predicted model for moderate or severe neurodevelopmental disability included PLCS and birth weight < 850 gr.

There are several limitations to our study. First of all, the small sample size could explain the lack of association between the gestational age and the neurodevelopmental impairment. Secondly, by the time this cohort underwent the surgery, the cardiovascular management was guided by a constellation of routine clinical or biochemical data, mainly blood pressure. Now, in our unit, the cardiovascular support after ductus surgery follows a pathophysiological approach as published recently (El-Khuffash et al., 2014), being also guided by functional echocardiography. Furthermore, this is a retrospective study with a small sample size, and a proportion of patients were lost to follow-up. However, the followed cohort appropriately represents the entire cohort, given the patients who were lost to follow-up are balanced between the two groups (PLCS group and no PLCS group). In addition, it is difficult to comply with a full follow-up program when the standard of care consists on transfer back to the referral hospital after PDA ligation in uncomplicated surgery. For this reason, the follow-up up to the mean age of 5.3 years in this cohort is a strength of the study.

In summary, preterm infants undergoing surgical PDA closure that fulfill the criteria of PLCS according to this study seem to have a tendency toward higher risk of moderate or severe long-term neurodevelopmental impairment, especially those with a birth weight below 850 gr. However, this is a pilot study, so prospective clinical trials reporting long-term follow-up data should be designed to confirm this data. Randomized clinical trials on preventive or treatment strategies for PLCS are also warranted.

ETHICS STATEMENT

This study was approved by The Ethics Committee for Human Studies at La Paz University Hospital as it was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. This Ethics Committee also concluded that it was not necessary to obtain an individual informed consent for all participants as this is a retrospective reviewed of the clinical charts of the infants born between 2005 and November 2009.

AUTHOR CONTRIBUTIONS

MB conceived and designed the study, drafted the initial manuscript, collected the information from the medical charts, performed the initial analyses, and approved the final manuscript as submitted. MY collected the information from the medical charts and approved the final manuscript as submitted.

RM performed the statistical analysis, reviewed the manuscript and approved the final manuscript as submitted. AP conceived and designed the study, drafted the initial manuscript and approved the final manuscript as submitted.

ACKNOWLEDGMENTS

We acknowledge the scientific advice of the SAMID Network (RD12/0026/004).

REFERENCES

Alarcon, A., Martinez-Biarge, M., Cabañas, F., Quero, J., and García-Alix, A. (2016). A prognostic neonatal neuroimaging scale for symptomatic congenital cytomegalovirus infection. *Neonatology* 110, 277–285.

Bayley, N. (1993). *Bayley Scales of Infant Development*. 2nd Edn. San Antonio: Psychological Corporation.

Cans, C. (2000). Surveillance of cerebral palsy in Europe: a collaboration of cerebral palsy surveys and registers. *Dev. Med. Child Neurol.* 42, 816–824.

El-Khuffash, A. F., Jain, A., and McNamara, P. J. (2013). Ligation of the patent ductus arteriosus in preterm infants: understanding the physiology. *J. Pediatr.* 162, 1100–1106.

El-Khuffash, A. F., Jain, A., Weisz, D., Mertens, L., and McNamara, P. J. (2014). Assessment and treatment of post patent ductus arteriosus ligation syndrome. *J. Pediatr.* 165, 46–52. doi: 10.1016/j.jpeds.2014.03.048

Harting, M. T., Blakely, M. L., Cox, C. S. Jr., Lantin-Hermoso, R., Andrassy, R. J., and Lally, K. P. (2008). Acute hemodynamic decompensation following patent ductus arteriosus ligation in premature infants. *J. Invest. Surg.* 21, 133–138. doi: 10.1080/08941930802046469

Jain, A., Sahni, M., El-Khuffash, A., Khadawardi, E., Sehgal, A., and McNamara, P. J. (2012). Use of targeted neonatal echocardiography to prevent postoperative cardiorespiratory instability after patent ductus arteriosus ligation. *J. Pediatr.* 160, 584–589. doi: 10.1016/j.jpeds.2011.09.027

Kabra, N. S., Schmidt, B., Roberts, R. S., Doyle, L. W., Papile, L., and Fanaroff, A. (2007). Neurosensory impairment after surgical closure of patent ductus arteriosus in extremely low birth weight infants: results from the trial of indomethacin prophylaxis in preterms. *J. Pediatr.* 150, 229–234.

Kaufman, A. S., and Kaufman, N. L. (2011). *Kaufman Brief Intelligence Test*, 2nd Edn (KBIT-2). Madrid: Pearson Education.

McNamara, P. J., Stewart, L., Shivananda, S. P., Stephens, D., and Sehgal, A. (2010). Patent ductus arteriosus ligation is associated with impaired left ventricular systolic performance in premature infants weighing less than 1000 g. *J. Thorac. Cardiovasc. Surg.* 140, 150–157. doi: 10.1016/j.jtcvs.2010.01.011

Sehgal, A., Francis, J. V., James, A., and McNamara, P. J. (2010). Patent ductus arteriosus ligation and post-operative hemodynamic instability: case report and framework for enhanced neonatal care. *Indian. J. Pediatr.* 77, 905–907. doi: 10.1007/s12098-010-0137-7

Teixeira, L. S., Shivananda, S. P., Stephens, D., Van Arsdell, G., and McNamara, P. J. (2008). Postoperative cardiorespiratory instability following ligation of the preterm ductus arteriosus is related to early need for intervention. *J. Perinatol.* 28, 803–810. doi: 10.1038/jp.2008.101

Ting, J. Y., Resende, M., More, K., Nicholls, D., Weisz, D. E., El-Khuffash, A., et al. (2016). Predictors of respiratory instability in neonates undergoing patient ductus arteriosus ligation after the introduction of targeted milrinone treatment. *J. Thorac. Cardiovasc. Surg.* 152, 498–504. doi: 10.1016/j.jtcvs.2016.03.085

Wechsler, D. (2010). *Wechsler Preschool and Primary Scale of Intelligence*. Madrid: Ediciones.

Weisz, D. E., More, K., McNamara, P. J., and Shah, P. S. (2014). PDA ligation and health outcomes: a meta-analysis. *Pediatrics* 133, e1024–e1046. doi: 10.1542/peds.2013-3431

Wernovsky, G., Wypij, D., Jonas, R. A., Mayer, J. E. Jr., Hanley, F. L., Hickey, P. R., et al. (1995). Postoperative course and hemodynamic profile after the arterial switch operation in neonates and infants. a comparison of low-flow cardiopulmonary bypass and circulatory arrest. *Circulation* 92, 2226–2235.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Bravo, Ybarra, Madero and Pellicer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

SYSTEMATIC REVIEW
published: 25 June 2019
doi: 10.3389/fphys.2019.00800

Cerebellar Hemorrhage in Preterm Infants: A Meta-Analysis on Risk Factors and Neurodevelopmental Outcome

Eduardo Villamor-Martinez[1†], *Monica Fumagalli*[2,3†], *Yaser Ibrahim Alomar*[1], *Sofia Passera*[2], *Giacomo Cavallaro*[2], *Fabio Mosca*[2,3] *and Eduardo Villamor*[1*]

[1] *Department of Pediatrics, School for Oncology and Developmental Biology (GROW), Maastricht University Medical Center, Maastricht, Netherlands,* [2] *Neonatal Intensive Care Unit, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy,* [3] *Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy*

OPEN ACCESS

Edited by:
Carina Mallard,
University of Gothenburg, Sweden

Reviewed by:
Andrew Whitelaw,
University of Bristol, United Kingdom
Nelly Padilla,
Karolinska Institute (KI), Sweden
Sylke Steggerda,
Leiden University Medical Center, Netherlands

***Correspondence:**
Eduardo Villamor
e.villamor@mumc.nl

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *25 January 2019*
Accepted: *06 June 2019*
Published: *25 June 2019*

Citation:
Villamor-Martinez E, Fumagalli M, Alomar YI, Passera S, Cavallaro G, Mosca F and Villamor E (2019) Cerebellar Hemorrhage in Preterm Infants: A Meta-Analysis on Risk Factors and Neurodevelopmental Outcome. Front. Physiol. 10:800. doi: 10.3389/fphys.2019.00800

Cerebellar hemorrhage (CBH) represents the most commonly acquired lesion of the posterior fossa in the neonatal period. We aimed to perform a systematic review and meta-analysis of studies exploring the perinatal risk factors and neurological outcome of CBH in preterm infants. A comprehensive literature search was conducted using PubMed/MEDLINE and EMBASE. Studies were included if they examined preterm infants and reported primary data on maternal, obstetric, or perinatal characteristics, and/or outcomes of infants with and without CBH. A random-effects model was used to calculate mean differences (MD), odds ratios (OR), and 95% confidence intervals (CI). We found 231 potentially relevant studies, of which 15 met the inclusion criteria (4,236 infants, 347 CBH cases). Meta-analysis could not demonstrate a significant association between CBH and multiple gestation, chorioamnionitis, pre-eclampsia, placental abruption, use of antenatal corticosteroids, mode of delivery, or infant sex. Infants with CBH had a significantly lower gestational age (6 studies, MD −1.55 weeks, 95% CI −1.93 to −1.16) and birth weight (6 studies, MD −173 g, 95% CI −225 to −120), and significantly higher rates of intubation at birth, hypotension, patent ductus arteriosus, intraventricular hemorrhage, sepsis, necrotizing enterocolitis, and bronchopulmonary dysplasia. CBH was significantly associated with delayed mental (6 studies, OR 2.95, 95% CI 1.21 to 7.20) and psychomotor (6 studies, OR 3.62, 95% CI 1.34 to 9.76) development, and higher rates of cerebral palsy (4 studies, OR 3.09, 95% CI 1.55 to 6.19). In conclusion, the present meta-analysis shows that the youngest and sickest preterm infants are at higher risk of developing CBH. Our results highlight the multifactorial nature of CBH and reinforce the idea that cerebellar injury in very preterm newborns has important neurodevelopmental consequences among survivors.

Keywords: cerebellar hemorrhage, prematurity, meta-analysis, systematic review, risk factors, neurodevelopmental outcome, cerebral palsy

INTRODUCTION

In the last decade, imaging of the posterior fossa in preterm infants has gained increased attention. This has been due to the growing awareness of the occurrence of cerebellar injury (Volpe, 2009; Limperopoulos et al., 2018) and the recent evidence highlighting the impact of perinatal acquired cerebellar lesions on a wide spectrum of cerebral functions including cognition, language, and memory (Brossard-Racine et al., 2015; Hortensius et al., 2018). Cerebellar hemorrhage (CBH) represents the most commonly acquired lesion of the posterior fossa in the neonatal period with the youngest infants being at the highest risk (Volpe, 2009; Brossard-Racine et al., 2015; Fumagalli et al., 2015; Hortensius et al., 2018; Limperopoulos et al., 2018).

During the third trimester of pregnancy, the cerebellum undergoes a rapid and precisely programmed growth resulting in a 3.5-fold increase in volume and a 30-fold increase in surface area (Limperopoulos et al., 2005b; Volpe, 2009; Du Plessis et al., 2018). This growth is mainly related to the development of the external granule cell layer (Dobbing, 1974; Volpe, 2009). Between 24 and 30 weeks' gestation a friable capillary network with poor supportive stroma is observed in the germinal matrix of the fourth ventricle and in the subpial region of the external granular layer. These structures are very vulnerable to perfusion–reperfusion injury and have been accounted as a possible source of CBH. More recently, the potential role of internal granule cell layer has also been highlighted, considering its dense vascularization and cellularity (Pierson and Al Sufiani, 2016).

The above-described critical phase of development renders the cerebellum very vulnerable, especially when preterm birth induces early exposure to the extrauterine environment (Limperopoulos et al., 2005a; Volpe, 2009). Indeed, the transitory presence of immature vasculature in a context of impaired cerebral autoregulation and hemodynamic disturbances, such as fluctuations in venous pressure, renders very, and extremely preterm infants highly susceptible to develop CBHs. Moreover, the well-documented association of CBH with supratentorial germinal matrix–intraventricular hemorrhage (GM-IVH), suggests possible common risk factors and pathogenetic mechanisms (Fumagalli et al., 2015).

There is a broad spectrum in the severity of CBHs reported, ranging from mild punctate lesions, focal unilateral lesions, to the less common and more extensive bihemispheric and vermian hemorrhages (Parodi et al., 2015; Limperopoulos et al., 2018). Recently, a grading scheme was proposed: grade 1 consisted of unilateral small ($\leq$3 mm) punctate lesions; grade 2 consisted of bilateral small punctate lesions; grade 3 consisted of extensive ($>$3 mm) unilateral lesions; and grade 4 consisted of extensive bilateral lesions (Neubauer et al., 2017). The addition of the mastoid fontanel window in cranial ultrasound (cUS) improved the detection of CBH compared to detection by the anterior and posterior fontanel cUS views alone (Limperopoulos et al., 2018). However, the majority of punctate hemorrhages may remain undetected, even when the mastoid fontanel approach is used (Steggerda et al., 2009; Steggerda and Van Wezel-Meijler, 2016). These small lesions are only detected by magnetic resonance imaging (MRI) scan, in particular on Susceptibility Weighted Imaging (SWI) sequence (Steggerda et al., 2009; Parodi et al., 2015; Steggerda and Van Wezel-Meijler, 2016; Limperopoulos et al., 2018). The reported incidence of focal CBHs detected by cUS ranged from 3.7 % in infants below 30 weeks of gestation (Sehgal et al., 2009) to 9% in infants below 32 weeks of gestation, when using the mastoid fontanel windows (Steggerda et al., 2009), but increased to 19% when MRI was used (Steggerda et al., 2009). Long-term neurodevelopmental prognosis of CBH is related to the location and extension of the lesion and, although still debated, growing evidence shows a wide range of neurodevelopmental impairments, including high-order cerebral functions (Volpe, 2009; Brossard-Racine et al., 2015; Hortensius et al., 2018; Limperopoulos et al., 2018).

Etiopathogenetic mechanisms may differ between large and small CBHs but it is generally accepted that the incidence of CBH is strikingly dependent on the degree of prematurity (Volpe, 2009; Limperopoulos et al., 2018). Nevertheless, very and extremely preterm infants have specific perinatal risk factors and some of these factors, such as lack of antenatal steroids, intubation at birth, patent ductus arteriosus (PDA), need for inotropic drugs acidosis during the first days of life, or sepsis, have been reported to confer additional risk for the development of CBH (Limperopoulos et al., 2005a, 2018; Sehgal et al., 2009; Volpe, 2009; Mccarthy et al., 2011; Chau et al., 2012; Zayek et al., 2012; Neubauer et al., 2017). However, the evidence is still unclear mainly due to the heterogeneity of the studied populations, the small sample sizes, and the different risk factors analyzed. A more accurate identification of risk factors for CBH would help in defining infants at high risk who may deserve in-depth neuroimaging investigation of posterior fossa and who may benefit from early intervention strategies. Therefore, we aimed to perform a systematic review and meta-analysis of studies exploring the perinatal risk factors and neurological outcomes of CBH in preterm infants.

METHODS

The study was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (Moher et al., 2009). A protocol was developed prospectively that detailed the specific objectives, criteria for study selection, the approach to assessing study quality, clinical outcomes, and statistical methodology. The study is reported according to the PRISMA checklist (see **Supplementary Material**).

Sources and Search Strategy

We searched PubMED (MEDLINE) and EMBASE for relevant studies. We searched from the inception of the databases until May 2017. The strategy for EMBASE was "exp cerebellum hemorrhage/ AND exp prematurity/," in addition to related free text terms ("cerebellar hemorrhage," "cerebellum hemorrhage," "cerebellar hemorrhage," "pre-mature infant," "pre-term baby," "pre-term child," "pre-term infant," "pre-term neonate," "pre-term newborn," "premature," "prematuritas," "preterm baby," "preterm child," "preterm infant," "preterm neonate," "preterm newborn"). We used a similar strategy for PubMED, with the MeSH terms for "cerebellar hemorrhage" and "neonatal

prematurity" in addition to related free text terms. We reviewed reference lists of relevant articles and reviews for additional studies. We did not exclude studies based on language, and studies were translated where necessary.

Study Selection

We included studies that compared infants with CBH to infants without CBH and reported on risk factors for and/or outcomes of CBH. We included studies which only included preterm (<37 weeks) and low birth weight (<2,500 g) infants. Studies without data on a control group were excluded. Two reviewers (EV-M, YA) screened studies for inclusion independently on title and abstract. In case of disagreement, the study was included and reevaluated in the second round of inclusion. The second round of inclusion was based on full-text screening, and discrepancies between reviewers were resolved through discussion or in consultation with a third reviewer (EV).

Data Extraction

Using a predefined worksheet, one researcher (YA) extracted data from included studies. We extracted the following data from each study: citation information, location of study, study period, primary objective, criteria for inclusion/exclusion of infants, definitions used for CBH, baseline characteristics, risk factors and outcomes (including raw numbers, summary statistics and adjusted analyses when available). Two researchers (SP, EV-M.) checked extracted data for accuracy and completeness. We resolved discrepancies through discussion and by consulting the primary report.

Quality Assessment

We used the Newcastle-Ottawa Scale (NOS) for cohort or case control studies to assess the methodological quality of included studies (Wells et al., 2001). This scale uses a rating system (range: 0–9 points) that scores three aspects of a study: selection of the study groups (0–4 points), comparability of the study groups (0–2 points) and ascertainment of exposure/outcome (0–3 points) (Wells et al., 2001). Two researchers (EV-M. and EV) independently used the NOS to evaluate the quality of each study, and discrepancies were discussed and resolved by consensus.

Statistical Analysis

We combined and analyzed studies using COMPREHENSIVE META-ANALYSIS V 3.0 software (CMA, RRID:SCR_012779, Biostat Inc., Englewood, NJ, USA). We calculated the odds ratio (OR) and 95% confidence intervals (CI) for dichotomous outcomes from the data extracted from the studies. We calculated the mean difference (MD) and 95% CI for continuous outcomes. We used the method of Wan and colleagues (Wan et al., 2014) to estimate the mean and standard deviation when continuous variables were reported as median and range/interquartile range in studies. We used a random-effects model to calculate summary statistics, due to anticipated heterogeneity. This method accounts for both intra-study and inter-study variability. We considered a probability value below 0.05 as statistically significant (0.10 for heterogeneity). We carried out publication bias analyses for outcomes reported in at least 15 studies (visual inspection of the funnel plot and Egger's regression test).

RESULTS

Description of Studies

Of 231 potentially relevant studies, 15 met the inclusion criteria (Limperopoulos et al., 2005a, 2007; Dyet et al., 2006; O'Shea et al., 2008; Fumagalli et al., 2009; Biran et al., 2011; Tam et al., 2011; Chau et al., 2012; Zayek et al., 2012; Duerden et al., 2013; Haines et al., 2013; Steggerda et al., 2013; Kidokoro et al., 2014; Gano et al., 2016; Neubauer et al., 2017). The PRISMA flow diagram of the search is shown in **Figure 1**. The included studies evaluated 4,236 infants, including 347 cases of CBH. The included studies and their characteristics are summarized in **Table 1**. Of the 15 studies, one (Steggerda et al., 2013) analyzed cerebellar punctate lesions, 7 analyzed focal lesions, 5 analyzed all type of lesions, and 2 did not clarify which types of CBH lesions they studied (**Table 1**). Two studies included infants with GA<28 weeks (O'Shea et al., 2008; Zayek et al., 2012), 3 studies included infants with GA<30 weeks (Dyet et al., 2006; Biran et al., 2011; Kidokoro et al., 2014), 5 studies included infants with GA<32 weeks (Limperopoulos et al., 2007; Chau et al., 2012; Duerden et al., 2013; Steggerda et al., 2013; Neubauer et al., 2017), 2 studies included infants with GA<33weeks (Fumagalli et al., 2009; Gano et al., 2016), one study included infants with GA<34weeks (Tam et al., 2011), and 2 studies included infants with GA<37weeks (Limperopoulos et al., 2005a; Haines et al., 2013).

Quality Assessment and Publication Bias

The quality assessment for each study according to the NOS is shown in **Supplementary Table 1**. Studies were downgraded in quality for not adjusting/matching for confounders ($k = 7$) and for not clearly defining CBH ($k = 3$). We did not carry out an analysis of publication bias for any of the included outcomes due to the low number of studies per outcome ($k < 15$).

Meta-Analyses on Demographic Characteristics and Maternal and Obstetric Risk Factors

Infants with CBH had significantly lower GA (MD in weeks −1.55, 95% CI −1.93 to −1.16, **Figure 2**) and significantly lower BW (MD in g −173, 95% CI −225 to −120, **Figure 3**). In contrast, meta-analysis could not find a significant association between CBH and infant sex (**Figure 4** and **Supplementary Figure 1**), multiple gestation (**Figure 4** and **Supplementary Figure 2**), preeclampsia (**Figure 4** and **Supplementary Figure 3**), use of antenatal steroids (**Figure 4** and **Supplementary Figure 4**), placental abruption (**Figure 4** and **Supplementary Figure 5**), chorioamnionitis (**Figure 4** and **Supplementary Figure 6**), or cesarean section (**Figure 4** and **Supplementary Figure 7**). In addition, maternal age was not significantly different between the CBH and the control group (3 studies, MD −0.44, 95% CI −2.51 to 1.63, **Supplementary Figure 8**).

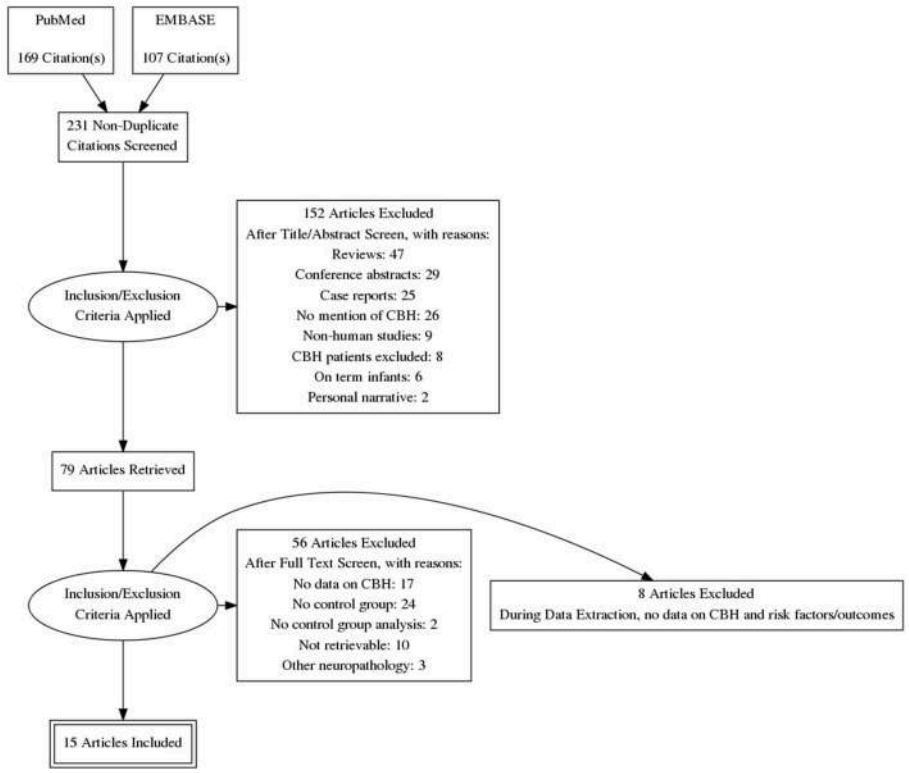


FIGURE 1 | PRISMA flow diagram of the systematic search.

Clinical Conditions and Short-Term Outcomes

We found in meta-analysis that CBH had a positive association with intubation at birth (**Figure 4** and **Supplementary Figure 9**), hypotension (**Figure 4** and **Supplementary Figure 10**), patent ductus arteriosus (**Figure 4** and **Supplementary Figure 11**), necrotizing enterocolitis (NEC, **Figure 4** and **Supplementary Figure 12**), sepsis (**Figure 4** and **Supplementary Figure 13**), bronchopulmonary dysplasia (BPD, **Figure 4** and **Supplementary Figure 14**), grade 1–2 IVH (**Figure 4** and **Supplementary Figure 15**), and grade 3-4 IVH (**Figure 4** and **Supplementary Figure 16**). CBH was also significantly associated with a lower pH in the first week of life, though only one study reported on this outcome (Limperopoulos et al., 2005a). Meta-analysis on Apgar score could not be performed due to the heterogeneity of reported data but two studies (Limperopoulos et al., 2005a; Neubauer et al., 2017) reported significant lower Apgar scores in infants with CBH.

Neurodevelopmental Outcome

Six studies reported data on neurodevelopmental outcome of CBH-exposed and control infants. The assessment methods, timing and summary of findings are summarized in **Supplementary Table 2**. We found through meta-analysis that CBH was significantly associated with delayed mental (6 studies, OR 2.95, 95% CI 1.21 to 7.20, **Figure 5**) and psychomotor (6 studies, OR 3.62, 95% CI 1.34 to 9.76, **Figure 6**) development, and higher rates of cerebral palsy (4 studies, OR 3.09, 95% CI 1.55 to 6.19, **Figure 7**).

DISCUSSION

To the best of our knowledge, this is the first meta-analysis providing a comprehensive estimation of antenatal, perinatal, and early postnatal risk factors associated with the development of CBH in preterm infants. The current meta-analysis strengthens and quantifies the previous concept of the multifactorial etiopathogenesis of CBH highlighting the central role of preterm birth and its related complications, while the relevance of maternal and obstetric factors remains unclear. Infants with CBH were born earlier, they had a lower BW, and they were more frequently exposed to systemic hypotension, PDA, low (1–2) and high (3–4) grade IVH, NEC, sepsis, and BPD. In addition, our meta-analysis confirms the findings of previous systematic reviews (Brossard-Racine et al., 2015; Hortensius et al., 2018) that showed that the presence of CBH is associated with a higher risk of impaired neurodevelopment in preterm infants. However, the presence of undiagnosed minor brain abnormalities or impaired brain growth associated with preterm birth may partially account for the long-term neurodevelopmental disorders observed in preterm infants with CBH.

TABLE 1 | Synoptic table of study characteristics.

Study	Study type	Included infants (centers)	Max GA	Max BW	Method of CBH diagnosis	CBH lesions studied
Biran et al., 2011	Retrospective case-control	30 (1)	29 6/7	N/A	MRI	Focal lesions
Chau et al., 2012	Prospective cohort	117 (1)	32	N/A	MRI	Unclear
Duerden et al., 2013	Prospective cohort	133 (1)	32	N/A	MRI	Unclear
Dyet et al., 2006	Prospective cohort	119 (1)	29 6/7	N/A	MRI	Focal lesions
Fumagalli et al., 2009	Prospective cohort	75 (1)	33	1000	MRI	Punctate and focal lesions
Gano et al., 2016	Prospective cohort	73 (1)	32 6/7	N/A	MRI	Punctate and focal lesions
Haines et al., 2013	Retrospective cohort	45 (1)	36 6/7	N/A	Autopsy	Focal lesions
Kidokoro et al., 2014	Prospective cohort	325 (3)	29 6/7	N/A	MRI	Punctate and focal lesions
Limperopoulos et al., 2005a	Retrospective case-control	105 (1)	36 6/7	N/A	cUS, mastoid view	Focal lesions
Limperopoulos et al., 2007	Retrospective case-control	86 (2)	31 6/7	N/A	cUS, mastoid view and MRI in early childhood	Focal lesions
Neubauer et al., 2017	Retrospective cohort	300 (1)	31 6/7	N/A	MRI	Punctate and focal lesions
O'Shea et al., 2008	Prospective cohort	1445 (14)	27 6/7	N/A	cUS, anterior fontanel	Focal lesions
Steggerda et al., 2013	Prospective cohort	132 (1)	31 6/7	N/A	MRI scan and cUS	Punctate lesions
Tam et al., 2011	Prospective cohort	131 (1)	33 6/7	N/A	MRI, and cranial ultrasound	Punctate and focal lesions
Zayek et al., 2012	Retrospective cohort	1120 (1)	27 6/7	N/A	cUS, anterior and posterior fontanels	Focal lesions

GA, gestational age; BW, birth weight; CBH, cerebellar hemorrhage; MRI, magnetic resonant imaging; cUS, cranial ultrasound.

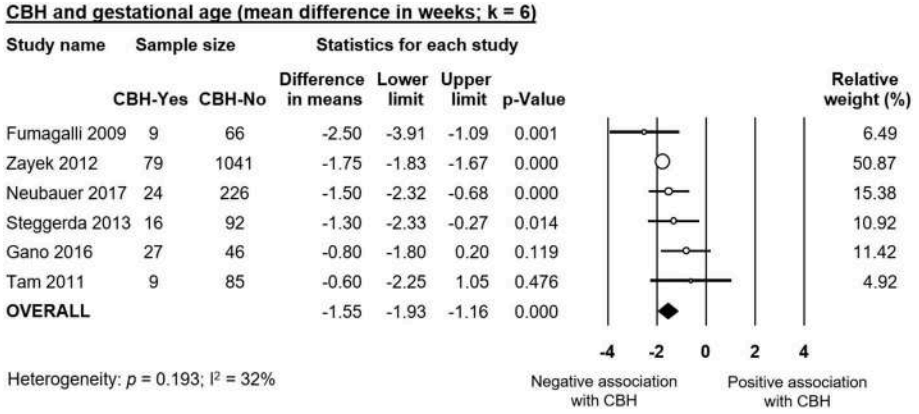


FIGURE 2 | Meta-analysis of mean difference in gestational age, comparing cerebellar hemorrhage (CBH)-group and control group.

The higher incidence of both large (Limperopoulos et al., 2005a; Gano et al., 2016) and small CBHs (Steggerda et al., 2013) in infants with lower GA and BW may be related to the anatomic characteristics of the developing cerebellum. In particular, the immature vessels of the preterm cerebellum are more sensitive to hemodynamic disturbances (Pierson and Al

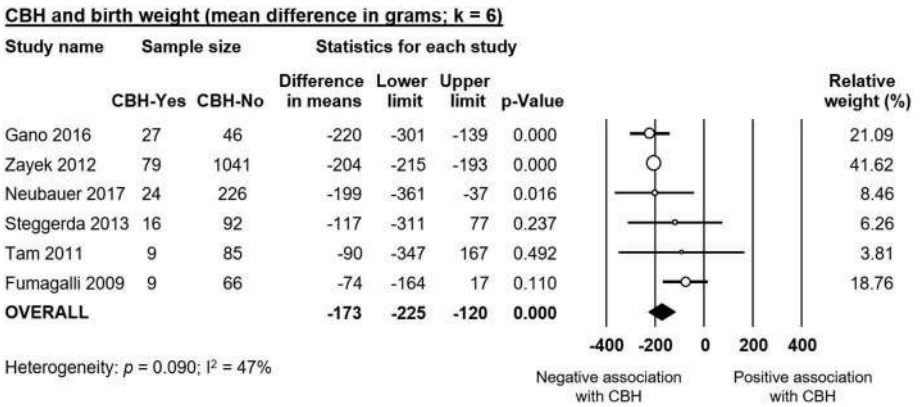


FIGURE 3 | Meta-analysis of mean difference in birth weight, comparing cerebellar hemorrhage (CBH)-group and control group.

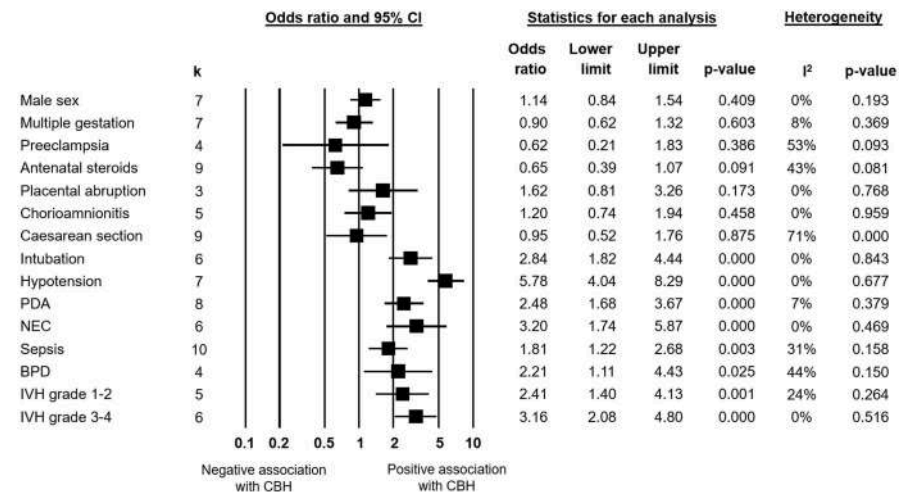


FIGURE 4 | Meta-analysis of the association between cerebellar hemorrhage (CBH) and maternal, obstetric and perinatal characteristics, and short-term neonatal outcomes. PDA, patent ductus arteriosus; NEC, necrotizing enterocolitis; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage.

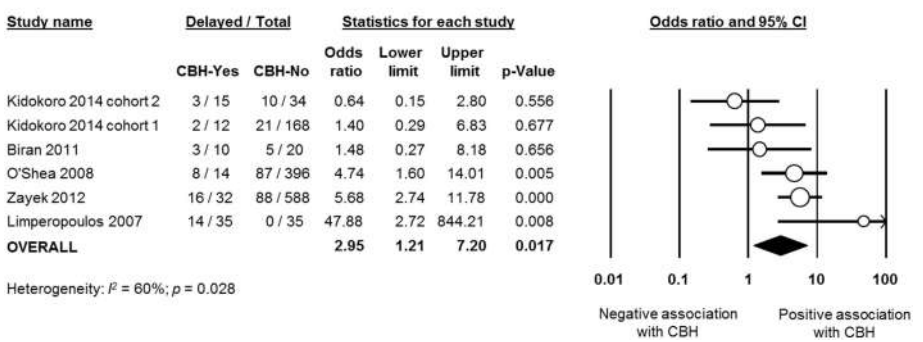


FIGURE 5 | Meta-analysis of the association between cerebellar hemorrhage (CBH) and risk of delayed mental development. CI, confidence interval.

Sufiani, 2016). The central role of hemodynamic disturbances (hypotension and PDA) in the context of an extreme immature anatomic and functional brain structure is supported by the timing of occurrence of CBH, with usually in the first days of life, when preterm infants experienced a critical period in terms of hemodynamic and respiratory adaptation. Moreover, agents that

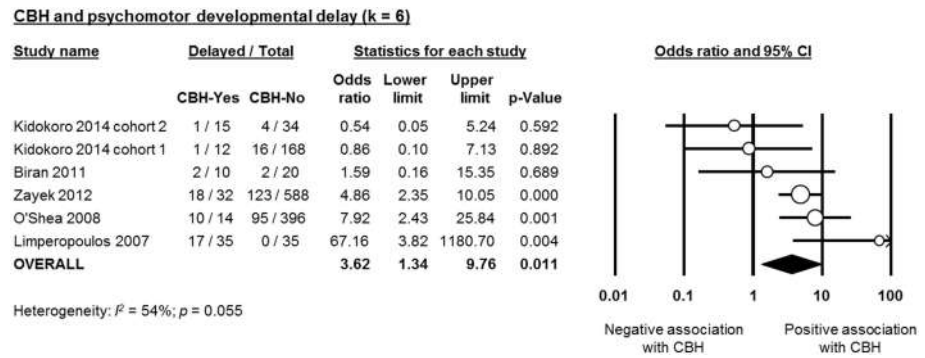


FIGURE 6 | Meta-analysis of the association between cerebellar hemorrhage (CBH) and risk of delayed psychomotor development. CI, confidence interval.

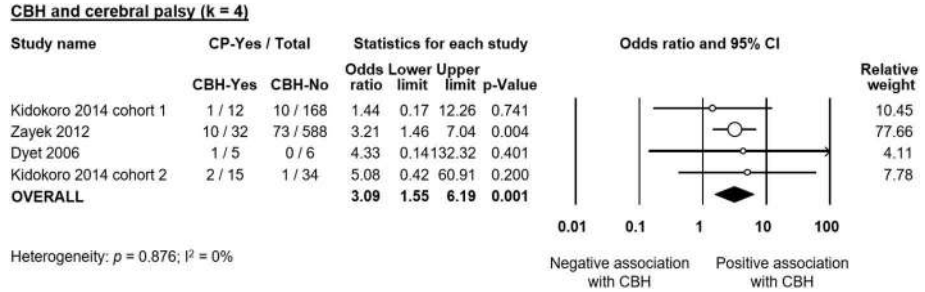


FIGURE 7 | Meta-analysis of the association between cerebellar hemorrhage (CBH) and risk of cerebral palsy (CP). CI, confidence interval.

reduce fluctuations in blood pressure, as magnesium sulfate, have been demonstrated to exert a neuroprotective effect (Gano et al., 2016), while interventions, like high dose caffeine, (Mcpherson et al., 2015), or surgery (Stolwijk et al., 2017), which are likely to impact on systemic hemodynamics are associated with a higher risk of developing CBH.

Among preterm infants, males appear to have a higher incidence and increased severity of brain lesions, such as IVH and periventricular leukomalacia (PVL) (Mohamed and Aly, 2010; O'Driscoll et al., 2018). It has been suggested that intrauterine differences in hormonal environment (Nuñez and Mccarthy, 2003) as well as the higher cerebral blood flow present in preterm male infants (Baenziger et al., 1994) are responsible for the higher susceptibility of preterm male neonates to brain injury (O'Driscoll et al., 2018). In contrast, in the present study, neither the individual studies (**Supplementary Figure 1**) nor the meta-analysis results suggested sex differences in the incidence of CBH.

Maternal and Obstetric Factors

Considering the multifactorial etiology of CBH, we analyzed the contribution of maternal and obstetric factors (**Figure 4**). Meta-analysis could not find that any of these factors, including maternal age, chorioamnionitis, preeclampsia, multiple pregnancy, mode of delivery, or use of antenatal corticosteroids is significantly associated with the development of CBH.

Chorioamnionitis is a well-known risk factor for preterm birth and prematurity-associated morbidities due to the activation of the inflammatory pathways (Hartling et al., 2012; Mitra et al., 2014; Behbodi et al., 2016; Villamor-Martinez et al., 2018a,b). The role of intrauterine inflammation is well-known as an underlying pathogenetic mechanism of white matter disease of prematurity (Strunk et al., 2014). Very recently, we showed in a meta-analysis that both clinical and histological chorioamnionitis were associated with an increased risk for developing IVH in very preterm infants (Villamor-Martinez et al., 2018b). Interestingly, and in contrast to other complications of prematurity, such as PDA, ROP, or BPD (Hartling et al., 2012; Mitra et al., 2014; Behbodi et al., 2016; Villamor-Martinez et al., 2018a), the effect of chorioamnionitis on IVH appeared to be independent of chorioamnionitis as a causative factor of prematurity. Considering that IVH and CBH share common pathogenetic mechanisms, we expected to see an effect of chorioamnionitis on the risk of developing CBH but this was not the case. A small number of studies reporting on chorioamnionitis, and the heterogeneity of the inclusion criteria for chorioamnionitis, varying from clinical (Kidokoro et al., 2014; Gano et al., 2016) to histological (Limperopoulos et al., 2005a) or not specified (Sehgal et al., 2009; Haines et al., 2013 may account for this discrepancy.

Only four studies included in meta-analysis reported data on pre-eclampsia (Limperopoulos et al., 2005a; Fumagalli et al., 2009; Haines et al., 2013; Gano et al., 2016) and all of them observed a trend, albeit a non-significant one, toward reduction

of CBH in infants born from mothers with pre-eclampsia. Recently, pre-eclampsia has been suggested as a protective factor for supratentorial bleeding in very (Morsing et al., 2018) and extremely (Gemmell et al., 2016) preterm infants. However, evidence is still controversial considering the potential role of many confounding factors such as the higher likelihood of planned delivery, cesarean section, and of receiving antenatal steroids, alongside greater prenatal surveillance of mothers with pre-eclampsia. Of note, although cesarean section is suggested to protect against IVH (Inder et al., 2018), our meta-analysis could not find a similar protective effect against CBH.

One important limitation in interpreting our negative results on the association between multiple gestation and CBH is the lack of data on chorionicity. Monochorionic pregnancy are likely to be complicated by placental hemodynamic alterations which may result in fetal hemodynamic disturbances (as twin-to-twin-transfusion) playing a role in the pathogenesis of CBH.

Maternal administration of corticosteroids in case of anticipated preterm delivery reduces neonatal mortality and morbidity, including IVH (Handley et al., 2018), and has become standard of care in current obstetric practice (Miracle et al., 2008). Many mechanisms have been suggested for the protective effects of antenatal corticosteroids, among which the induction of lung maturation and the reduction of severity of postnatal respiratory disease (Xu et al., 2008; Vinukonda et al., 2010; Wapner, 2013). However, and although one of the included studies suggested a protective effect of antenatal corticosteroids on CBH development (Neubauer et al., 2017), meta-analysis could not demonstrate a significant effect (**Figure 4**). Nevertheless, due to the low robustness of the results, the inclusion of future studies may produce relevant changes in the effect size of the association between antenatal corticosteroids and CBH.

Clinical Conditions and Short-Term Outcomes

Our analysis confirms that the need of resuscitation with intubation at birth represents a risk factor for CBH. Meta-analysis on Apgar score could not be performed due to the heterogeneity of reported data but two studies (Limperopoulos et al., 2005a; Neubauer et al., 2017) reported significant lower Apgar scores in infants with CBH. Major early postnatal complications were significantly associated with CBH confirming that the most immature and sickest infants, who suffer the most severe morbidities, are the ones more prone to develop CBH (Sehgal et al., 2009; Neubauer et al., 2017). Sepsis, systemic hypotension, PDA and acidosis in the first 7 days increase the risk of CBH. All these factors, directly or indirectly, induce fluctuations in cerebral blood flow which in turn increase the risk of bleeding. These factors play a role in the complex interplay of pathogenetic mechanisms of CBH but unfortunately, we were not able to assess their independent role and to disentangle the potential effect of prematurity itself.

The association between CBH and supratentorial IVH is well-documented (Fumagalli et al., 2015) suggesting common pathogenetic mechanisms. Indeed, the germinal matrix, which is supposed to be the origin of both IVH and CBH, is present both in the cerebellum and supratentorially (Volpe, 2009; Limperopoulos et al., 2018). Moreover, large CBH can also occur secondary to extension of intraventricular or sub- arachnoid blood into the cerebellum as a consequence of a dissection of blood through the fourth ventricle or subarachnoid spaces following massive IVH (Limperopoulos et al., 2008). Similarly, Parodi et al. speculated that cerebellar microhemorrhages, which are mostly located in the external portion of cerebellar parenchyma, might represent haemosiderin depositions originating from a supratentorial bleeding and conveyed by the cerebrospinal fluid to the subarachnoid space of the posterior fossa (Parodi et al., 2015).

Neurodevelopmental Outcome

As mentioned in the introduction, the systematic review of Hortensius et al. showed a high incidence of severe neurodevelopmental impairment in preterm infants with isolated CBH in the cognitive, motor, language, and behavioral domains (Hortensius et al., 2018). Our meta-analysis confirmed that the presence of CBH in preterm infants increased the risk of impaired mental and psychomotor development as well as the rate of CP. However, our results are limited by the low number of studies and the marked heterogeneity that did not allow us to investigate the effect of the location (unilateral, bilateral, or with vermis involvement) and the size (punctate or large) of CBH on neurodevelopmental outcome. Vermis involvement and large CBHs have been related with higher rates of neurodevelopmental impairment (Hortensius et al., 2018).

In addition, some of the short-term complications that are significantly more frequent in infants with CBH, such as sepsis, NEC, or BPD may have a marked, CBH-independent, influence on neurodevelopmental outcome through both additive and interactive roles (Hortensius et al., 2018). Moreover, the studies used different follow-up periods and different scales, or different editions of the Bayley scale, for the assessment of neurodevelopmental outcome (**Supplementary Table 2**) and the correlation between the cut-off points of the different scales to define neurodevelopmental delay is controversial (Moore et al., 2012; Johnson et al., 2014).

Limitations

Our meta-analysis has some important limitations that hamper the practical application of the results. Firstly, and as mentioned above, we were only able to include a limited number of studies ($k = 15$), and studies reported on different risk factors and outcomes. Consequently, we probably lacked the power to find some associations. Secondly, studies showed heterogeneity in inclusion criteria, as well as in definition and assessment of CBH, risk factors, and outcomes, and 6 studies out of 15 did not control for confounding factors. Some of the included studies did not have the investigation of CBH as primary aim. In addition, it should be noted that only 2 studies exclusively included extremely preterm infants (GA $<$ 28 weeks) (O'Shea et al., 2008; Zayek et al., 2012) and 2 studies included all preterm infants (GA $<$ 37 weeks) (Limperopoulos et al., 2005a; Haines et al., 2013). The limited number of studies did not

make it possible to stratify studies in subgroups to analyze the potential sources of heterogeneity, and we could not adjust for confounders through meta-regression. Finally, we did not define baseline characteristics and outcomes to include and analyze a priori but, due to the limited number of studies, decided on a case-by-case basis whether there was enough homogeneity to carry out meta-analysis, which has some potential to introduce bias.

CONCLUDING REMARKS

The present meta-analysis highlights the multifactorial nature of CBH and reinforces the idea that cerebellar injury in very preterm newborns has important neurodevelopmental consequences among survivors. However, further research is warranted to understand the complex relationship between hemorrhagic cerebellar injury and its influence on neurodevelopmental outcome (Brossard-Racine et al., 2015; Hortensius et al., 2018). The cerebellum does not participate as an isolated entity in the integration of neural information, since the cerebral cortex and cerebellum of the mature brain are connected by a myriad, closed loop circuit, with afferent and efferent limbs, forming distinct functional, and structural units (Volpe, 2009; Limperopoulos et al., 2014). In very and extremely preterm infants, cerebellar injury occurs prior to maturation of cerebello-cerebral connectivity and, therefore, the remote effects of primary cerebellar injury may continue to influence cerebral development and plasticity over months to years (Volpe, 2009; Limperopoulos et al., 2014). Future studies should include large cohorts, with clear description of the topography and the size of CBHs, and other cerebellar and supratentorial injuries, and with risk factors and outcomes described on an individual patient level (Hortensius et al., 2018). In addition, research is needed in order to understand the relationship between prematurity-related cerebellar injury and other long-term outcomes such as autism spectrum disorders (Brossard-Racine et al., 2015).

AUTHOR CONTRIBUTIONS

EV-M selected studies for inclusion, carried out data collection, carried out statistical analyses, assessed methodological quality, contributed to interpretation of results, drafted part of the initial manuscript, and reviewed and revised the manuscript. MF contributed to the design of the study, the statistical analysis and interpretation of results, supervised data collection, drafted part of the initial manuscript, and reviewed and revised the manuscript. YA selected studies for inclusion, carried out data collection, contributed to statistical analyses and interpretation of results, and reviewed and revised the manuscript. SP checked extracted data for accuracy and completeness, contributed to interpretation of results, and reviewed and revised the manuscript. GC contributed to interpretation of results and reviewed and revised the manuscript. FM contributed to interpretation of results and reviewed and revised the manuscript. EV conceptualized and designed the study, supervised the search and selection of studies, supervised data collection, assessed methodological quality, contributed to statistical analyses and interpretation of results, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00800/full#supplementary-material

REFERENCES

Baenziger, O., Jaggi, J. L., Mueller, A. C., Morales, C. G., Lipp, H. P., Lipp, A. E., et al. (1994). Cerebral blood flow in preterm infants affected by sex, mechanical ventilation, and intrauterine growth. *Pediatr. Neurol.* 11, 319–324. doi: 10.1016/0887-8994(94)90009-4

Behbodi, E., Villamor-Martínez, E., Degraeuwe, P. L., and Villamor, E. (2016). Chorioamnionitis appears not to be a risk factor for patent ductus arteriosus in preterm infants: a systematic review and meta-analysis. *Sci. Rep.* 6:37967. doi: 10.1038/srep37967

Biran, V., Bodiou, A.-M., Zana, E., Gaudin, A., Farnoux, C., Hovhannisyan, S., et al. (2011). Lésions acquises du cervelet chez le grand prématuré: prévalence, facteurs de risque et conséquences fonctionnelles. *Arch. Pediatr.* 18, 261–266. doi: 10.1016/j.arcped.2010.12.016

Brossard-Racine, M., Du Plessis, A. J., and Limperopoulos, C. (2015). Developmental cerebellar cognitive affective syndrome in ex-preterm survivors following cerebellar injury. *Cerebellum* 14, 151–164. doi: 10.1007/s12311-014-0597-9

Chau, V., Brant, R., Poskitt, K. J., Tam, E. W., Synnes, A., and Miller, S. P. (2012). Postnatal infection is associated with widespread abnormalities of brain development in premature newborns. *Pediatr. Res.* 71, 274. doi: 10.1038/pr.2011.40

Dobbing, J. (1974). The later growth of the brain and its vulnerability. *Pediatrics* 53, 2–6.

Du Plessis, A. J., Limperopoulos, C., and Volpe, J. J. (2018). "Cerebellar development," in *Volpe's Neurology of the Newborn* (Philadelphia, PA: Elsevier), 73–99.

Duerden, E. G., Brown-Lum, M., Chau, V., Poskitt, K. J., Grunau, R. E., Synnes, A., et al. (2013). Resuscitation intensity at birth is associated with changes in brain metabolic development in preterm neonates. *Neuroradiology* 55, 47–54. doi: 10.1007/s00234-013-1243-9

Dyet, L. E., Kennea, N., Counsell, S. J., Maalouf, E. F., Ajayi-Obe, M., Duggan, P. J., et al. (2006). Natural history of brain lesions in extremely preterm infants studied with serial magnetic resonance imaging from birth and neurodevelopmental assessment. *Pediatrics* 118, 536–548. doi: 10.1542/peds.2005-1866

Fumagalli, M., Bassi, L., Sirgiovanni, I., Mosca, F., Sannia, A., and Ramenghi, L. A. (2015). From germinal matrix to cerebellar haemorrhage. *J. Matern. Fetal Neonatal. Med.* 28, 2280–2285. doi: 10.3109/14767058.2013.796168

Fumagalli, M., Ramenghi, L. A., Righini, A., Groppo, M., Bassi, L., De Carli, A., et al. (2009). Cerebellar haemorrhages and pons development in extremely low birth weight infants. *Front. Biosci.* 1, 537–541. doi: 10.2741/e50

Gano, D., Ho, M.-L., Partridge, J. C., Glass, H. C., Xu, D., Barkovich, A. J., et al. (2016). Antenatal exposure to magnesium sulfate is associated with reduced cerebellar hemorrhage in preterm newborns. *J. Pediatr.* 178, 68–74. doi: 10.1016/j.jpeds.2016.06.053

Gemmell, L., Martin, L., Murphy, K., Modi, N., Håkansson, S., Reichman, B., et al. (2016). Hypertensive disorders of pregnancy and outcomes of preterm

infants of 24 to 28 weeks' gestation. *J. Perinatol.* 36, 1067. doi: 10.1038/jp.2016.133

Haines, K. M., Wang, W., and Pierson, C. R. (2013). Cerebellar hemorrhagic injury in premature infants occurs during a vulnerable developmental period and is associated with wider neuropathology. *Acta Neuropathol. Commun.* 1:69. doi: 10.1186/2051-5960-1-69

Handley, S. C., Passarella, M., Lee, H. C., and Lorch, S. A. (2018). Incidence trends and risk factor variation in severe intraventricular hemorrhage across a population based cohort. *J Pediatr.* 200, 24–29.e3. doi: 10.1016/j.jpeds.2018.04.020

Hartling, L., Liang, Y., and Lacaze-Masmonteil, T. (2012). Chorioamnionitis as a risk factor for bronchopulmonary dysplasia: a systematic review and meta-analysis. *Arch. Dis. Child. Fetal. Neonatal. Ed.* 97, F8–F17. doi: 10.1136/adc.2010.210187

Hortensius, L. M., Dijkshoorn, A. B., Ecury-Goossen, G. M., Steggerda, S. J., Hoebeek, F. E., Benders, M. J., et al. (2018). Neurodevelopmental consequences of preterm isolated cerebellar hemorrhage: a systematic review. *Pediatrics* 142:e20180609. doi: 10.1542/peds.2018-0609

Inder, T. E., Perlman, J. M., and Volpe, J. J. (2018). "Preterm intraventricular hemorrhage/posthemorrhagic hydrocephalus," in *Volpe's Neurology of the Newborn* (Philadelphia, PA: Elsevier), 637–698.

Johnson, S., Moore, T., and Marlow, N. (2014). Using the Bayley-III to assess neurodevelopmental delay: which cut-off should be used? *Pediatr Res.* 75:670. doi: 10.1038/pr.2014.10

Kidokoro, H., Anderson, P. J., Doyle, L. W., Woodward, L. J., Neil, J. J., and Inder, T. E. (2014). Brain injury and altered brain growth in preterm infants: predictors and prognosis. *Pediatrics* 134, e444–e453. doi: 10.1542/peds.2013-2336

Limperopoulos, C., Bassan, H., Gauvreau, K., Robertson, R. L., Sullivan, N. R., Benson, C. B., et al. (2007). Does cerebellar injury in premature infants contribute to the high prevalence of long-term cognitive, learning, and behavioral disability in survivors? *Pediatrics* 120, 584–593. doi: 10.1542/peds.2007-1041

Limperopoulos, C., Benson, C. B., Bassan, H., Disalvo, D. N., Kinnamon, D. D., Moore, M., et al. (2005a). Cerebellar hemorrhage in the preterm infant: ultrasonographic findings and risk factors. *Pediatrics* 116, 717–724. doi: 10.1542/peds.2005-0556

Limperopoulos, C., Chilingaryan, G., Sullivan, N., Guizard, N., Robertson, R. L., and Du Plessis, A. J. (2014). Injury to the premature cerebellum: outcome is related to remote cortical development. *Cereb. Cortex.* 24, 728–736. doi: 10.1093/cercor/bhs354

Limperopoulos, C., Du Plessis, A. J., and Volpe, J. J. (2018). "Cerebellar hemorrhage," in *Volpe's Neurology of the Newborn* (Philadelphia, PA: Elsevier), 623–636.

Limperopoulos, C., Gauvreau, K. K., O'leary, H., Moore, M., Bassan, H., Eichenwald, E. C., et al. (2008). Cerebral hemodynamic changes during intensive care of preterm infants. *Pediatrics* 122, e1006–e1013. doi: 10.1542/peds.2008-0768

Limperopoulos, C., Soul, J. S., Gauvreau, K., Huppi, P. S., Warfield, S. K., Bassan, H., et al. (2005b). Late gestation cerebellar growth is rapid and impeded by premature birth. *Pediatrics* 115, 688–695. doi: 10.1542/peds.2004-1169

Mccarthy, L. K., Donoghue, V., and Murphy, J. (2011). Ultrasonically detectable cerebellar haemorrhage in preterm infants. *Arch. Dis. Child. Fetal Neonatal. Ed.* 96, F281–F285. doi: 10.1136/adc.2010.183889

Mcpherson, C., Neil, J. J., Tjoeng, T. H., Pineda, R., and Inder, T. E. (2015). A pilot randomized trial of high-dose caffeine therapy in preterm infants. *Pediatr Res.* 78, 198. doi: 10.1038/pr.2015.72

Miracle, X., Di Renzo, G. C., Stark, A., Fanaroff, A., Carbonell-Estrany, X., and Saling, E. (2008). Guideline for the use of antenatal corticosteroids for fetal maturation. *J. Perinat. Med.* 36, 191–196. doi: 10.1515/JPM.2008.032

Mitra, S., Aune, D., Speer, C. P., and Saugstad, O. D. (2014). Chorioamnionitis as a risk factor for retinopathy of prematurity: a systematic review and meta-analysis. *Neonatology*. 105, 189–199. doi: 10.1159/000357556

Mohamed, M. A., and Aly, H. (2010). Male gender is associated with intraventricular hemorrhage. *Pediatrics. Peds.* 2008–3369. doi: 10.1542/peds.2008-3369

Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G., and Group, P. (2009). Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 6:e1000097. doi: 10.1371/journal.pmed.1000097

Moore, T., Johnson, S., Haider, S., Hennessy, E., and Marlow, N. (2012). Relationship between test scores using the second and third editions of the Bayley Scales in extremely preterm children. *J. Pediatr.* 160, 553–558. doi: 10.1016/j.jpeds.2011.09.047

Morsing, E., Maršál, K., and Ley, D. (2018). Reduced prevalence of severe intraventricular hemorrhage in very preterm infants delivered after maternal preeclampsia. *Neonatology* 114, 205–211. doi: 10.1159/000489039

Neubauer, V., Djurdjevic, T., Griesmaier, E., Biermayr, M., Gizewski, E. R., and Kiechl-Kohlendorfer, U. (2017). Routine magnetic resonance imaging at term-equivalent age detects brain injury in 25% of a contemporary cohort of very preterm infants. *PLoS ONE* 12:e0169442. doi: 10.1371/journal.pone.0169442

Nuñez, J. L., and Mccarthy, M. M. (2003). Sex differences and hormonal effects in a model of preterm infant brain injury. *Ann NY Acad Sci.* 1008, 281–284. doi: 10.1196/annals.1301.032

O'Driscoll, D. N., Mcgovern, M., Greene, C. M., and Molloy, E. J. (2018). Gender disparities in preterm neonatal outcomes. *Acta Paediatr.* 107, 1494–1499. doi: 10.1111/apa.14390

O'Shea, T. M., Kuban, K. C., Allred, E. N., Paneth, N., Pagano, M., Dammann, O., et al. (2008). Neonatal cranial ultrasound lesions and developmental delays at 2 years of age among extremely low gestational age children. *Pediatrics* 122:e662. doi: 10.1542/peds.2008-0594

Parodi, A., Rossi, A., Severino, M., Morana, G., Sannia, A., Calevo, M. G., et al. (2015). Accuracy of ultrasound in assessing cerebellar haemorrhages in very low birthweight babies. *Arch. Dis. Child. Fetal. Neonatal. Ed.* 100, F289–F292. doi: 10.1136/archdischild-2014-307176

Pierson, C. R., and Al Sufiani, F. (2016). Preterm birth and cerebellar neuropathology. *Semin. Fetal. Neonatal. Med.* 21, 305–311. doi: 10.1016/j.siny.2016.04.006

Sehgal, A., El-Naggar, W., Glanc, P., and Asztalos, E. (2009). Risk factors and ultrasonographic profile of posterior fossa haemorrhages in preterm infants. *J. Paediatr. Child Health.* 45, 215–218. doi: 10.1111/j.1440-1754.2008.01456.x

Steggerda, S., and Van Wezel-Meijler, G. (2016). Cranial ultrasonography of the immature cerebellum: role and limitations. *Semin. Fetal. Neonatal. Med.* 21, 295–304. doi: 10.1016/j.siny.2016.04.011

Steggerda, S. J., De Bruïne, F. T., Van Den Berg-Huysmans, A. A., Rijken, M., Leijser, L. M., Walther, F. J., et al. (2013). Small cerebellar hemorrhage in preterm infants: perinatal and postnatal factors and outcome. *Cerebellum* 12, 794–801. doi: 10.1007/s12311-013-0487-6

Steggerda, S. J., Leijser, L. M., Wiggers-De Bruïne, F. T., Van Der Grond, J., Walther, F. J., and Van Wezel-Meijler, G. (2009). Cerebellar injury in preterm infants: incidence and findings on US and MR images. *Radiology* 252, 190–199. doi: 10.1148/radiol.2521081525

Stolwijk, L. J., Keunen, K., De Vries, L. S., Groenendaal, F., Van Der Zee, D. C., Van Herwaarden, M. Y., et al. (2017). Neonatal surgery for noncardiac congenital anomalies: neonates at risk of brain injury. *J. Pediatr.* 182, 335-341.e331. doi: 10.1016/j.jpeds.2016.11.080

Strunk, T., Inder, T., Wang, X., Burgner, D., Mallard, C., and Levy, O. (2014). Infection-induced inflammation and cerebral injury in preterm infants. *Lancet Infect. Dis.* 14, 751–762. doi: 10.1016/S1473-3099(14)70710-8

Tam, E. W., Rosenbluth, G., Rogers, E. E., Ferriero, D. M., Glidden, D., Goldstein, R. B., et al. (2011). Cerebellar hemorrhage on magnetic resonance imaging in preterm newborns associated with abnormal neurologic outcome. *J. Pediatr.* 158, 245–250. doi: 10.1016/j.jpeds.2010.07.049

Villamor-Martinez, E., Cavallaro, G., Raffaeli, G., Mohammed Rahim, O. M. M., Gulden, S., Ghazi, A. M. T., et al. (2018a). Chorioamnionitis as a risk factor for retinopathy of prematurity: An updated systematic review and meta-analysis. *PLoS ONE*. 13:e0205838. doi: 10.1371/journal.pone.0205838

Villamor-Martinez, E., Fumagalli, M., Mohammed Rahim, O., Passera, S., Cavallaro, G., Degraeuwe, P., et al. (2018b). Chorioamnionitis is a risk factor for intraventricular hemorrhage in preterm infants: a systematic review and meta-analysis. *Front. Physiol.* 9:1253. doi: 10.3389/fphys.2018.01253

Vinukonda, G., Dummula, K., Malik, S., Hu, F., Thompson, C. I., Csiszar, A., et al. (2010). Effect of prenatal glucocorticoids on

cerebral vasculature of the developing brain. *Stroke.* 41, 1766–1773. doi: 10.1161/STROKEAHA.110.588400

Volpe, J. J. (2009). Cerebellum of the premature infant: rapidly developing, vulnerable, clinically important. *J. Child. Neurol.* 24, 1085–1104. doi: 10.1177/0883073809338067

Wan, X., Wang, W., Liu, J., and Tong, T. (2014). Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Med. Res. Methodol.* 14:135. doi: 10.1186/1471-2288-14-135

Wapner, R. J. (2013). Antenatal corticosteroids for periviable birth. *Semin. Perinatol.* 37, 410–413. doi: 10.1053/j.semperi.2013.06.024

Wells, G., Shea, B., O'connell, D., Peterson, J., Welch, V., Losos, M., et al. (2001). *The Newcastle-Ottawa Scale (NOS) for Assessing the Quality of Nonrandomised Studies in Meta-Analyses*. Available online at: http://www.ohri.ca/programs/clinical_epidemiology/oxford.Htm (accessed May 12, 2017).

Xu, H., Hu, F., Sado, Y., Ninomiya, Y., Borza, D. B., Ungvari, Z., et al. (2008). Maturational changes in laminin, fibronectin, collagen IV, and perlecan in germinal matrix, cortex, and white matter and effect of betamethasone. *J. Neurosci. Res.* 86, 1482–1500. doi: 10.1002/jnr.21618

Zayek, M., Benjamin, J., Maertens, P., Trimm, R., Lal, C., and Eyal, F. (2012). Cerebellar hemorrhage: a major morbidity in extremely preterm infants. *J. Perinatol.* 32, 699–704. doi: 10.1038/jp.2011.185

Conflict of Interest Statement: MF and FM co-authored one of the studies included in the review and provided additional data.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Villamor-Martinez, Fumagalli, Alomar, Passera, Cavallaro, Mosca and Villamor. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

SYSTEMATIC REVIEW
published: 26 July 2019
doi: 10.3389/fphys.2019.00961

Postnatal Nutrition to Improve Brain Development in the Preterm Infant: A Systematic Review From Bench to Bedside

Lisa M. Hortensius[1], Ruurd M. van Elburg[2,3], Cora H. Nijboer[4], Manon J. N. L. Benders[1,4] and Caroline G. M. de Theije[4]*

[1] Department of Neonatology, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands, [2] Emma Children's Hospital, Amsterdam UMC, University of Amsterdam, Amsterdam, Netherlands, [3] Danone Nutricia Research, Utrecht, Netherlands, [4] Laboratory of Neuroimmunology and Developmental Origins of Disease, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Max Berry,
University of Otago, New Zealand
Stephane Vladimir Sizonenko,
Geneva University Hospitals
(HUG), Switzerland

***Correspondence:**
Manon J. N. L. Benders
m.benders@umcutrecht.nl

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: 25 January 2019
Accepted: 11 July 2019
Published: 26 July 2019

Citation:
Hortensius LM, van Elburg RM, Nijboer CH, Benders MJNL and de Theije CGM (2019) Postnatal Nutrition to Improve Brain Development in the Preterm Infant: A Systematic Review From Bench to Bedside. Front. Physiol. 10:961. doi: 10.3389/fphys.2019.00961

Background: Preterm infants are at high risk for Encephalopathy of Prematurity and successive adverse neurodevelopmental outcome. Adequate nutrition is crucial for healthy brain development. Maternal breast milk is first choice of post-natal enteral nutrition for preterm infants. However, breast milk contains insufficient nutrient quantities to meet the greater nutritional needs of preterm infants, meaning that supplementation is recommended.

Aim: To provide an overview of current literature on potential nutritional interventions for improvement of neurodevelopmental outcome in preterm infants, by taking a bench to bedside approach from pre-clinical models of neonatal brain injury to randomized controlled clinical trials (RCTs) in preterm infants.

Methods: Separate clinical and pre-clinical searches were performed in Medline and Embase for English written papers published between 08/2008 and 08/2018 that studied a single nutritional component. Papers were included if one of the following components was studied: lipids, carbohydrates, proteins, vitamins, minerals, probiotics, prebiotics, oligosaccharides, fatty acids, or amino acids, with brain injury, brain development or neurodevelopmental outcome as outcome measure in preterm infants (gestational age <32 weeks and/or birth weight <1,500 g) or in animal models of neonatal brain injury.

Results: In total, 2,671 pre-clinical studies and 852 RCTs were screened, of which 24 pre-clinical and 22 RCTs were included in this review. In these trials supplementation with amino acids and protein, lipids, probiotics (only clinical), prebiotics (only clinical), vitamins, and minerals was studied. All included pre-clinical studies show positive effect of supplementation on brain injury and/or neurodevelopment. Although some nutrients, such as glutamine, show promising short term outcome in clinical studies, no evident long term effect of any supplemented nutrient was found. Main limitations were inclusion of studies no older than 10 years at time of search and studies that focused on single nutritional components only.

Conclusion: Even though many pre-clinical trials demonstrate promising effects of different nutritional interventions on reducing brain injury and/or improving neurodevelopmental outcome, these positive effects have so far not evidently been demonstrated in RCTs. More clinically relevant animal models and long term follow up after clinical trials are needed to move novel nutritional therapies from bench to bedside of preterm infants.

Keywords: nutrition, preterm infants, animal models, clinical, pre-clinical, brain injury, neurodevelopment

INTRODUCTION

Preterm birth is commonly defined as any birth before 37 completed weeks of gestation and has been estimated to account for 10% of all births (Liu et al., 2012). Over the past decade, advances in neonatal care have led to decreased mortality of preterm infants in Western society (Euro-Peristat Project with SCPE EUROCAT, 2013). Therefore, the main focus of neonatal care has changed from reducing mortality to reducing long-term morbidity. Brain injury and successive impaired neurodevelopmental outcome is a major morbidity in the preterm infant (Twilhaar et al., 2018c). Despite improvements in preventative strategies (e.g., prenatal steroids) and enhanced neuromonitoring, the rate of neurodevelopmental impairments remains high and is estimated to be 25% in extremely preterm infants born before 28 weeks of gestation (Glass et al., 2015).

During the third trimester of gestation, the brain undergoes a rapid trajectory of growth. Brain growth from 30 to 40 weeks of gestation is greatest in the cerebellum, which increases 258% in size (Kersbergen et al., 2016). The total brain volume is increased by 140% during these 10 weeks. Third trimester brain volumes have been shown to be reduced in preterm infants compared to healthy control fetuses, and growth trajectories were slower in cerebrum, cerebellum, brain stem and intracranial cavity (Bouyssi-Kobar et al., 2016). Injury to the preterm brain is commonly defined as "Encephalopathy of Prematurity" (EoP) and includes different pathologies. The most commonly observed pathology is cerebral white matter injury (WMI), which can present as diffuse or as punctate, cystic, or hemorrhagic lesions. In addition, also germinal matrix-intraventricular hemorrhages, and cerebellar disturbances are frequently observed in the preterm brain (van Tilborg et al., 2016). The pre- and post-natal conditions leading to EoP are typically associated with cerebral oxygen fluctuations and systemic inflammation (van Tilborg et al., 2016). These risk factors are thought to lead to an arrest in oligodendrocyte maturation, followed by a myelination failure of neuronal axons (van Tilborg et al., 2018b), resulting in reduced neuronal connectivity and brain volume. Both neuronal connectivity and brain volume are important predictors of neurodevelopmental outcome in the preterm infant.

As the rapidly growing brain at this stage in particular is vulnerable for nutrient insufficiencies (Georgieff, 2007), one may hypothesize that nutrient intake by preterm infants may predict brain growth and possibly neurodevelopmental outcomes. Intake quantities of selective and total nutrients during early life of preterm infants were shown to be positively associated with increased head circumference growth (Dabydeen et al., 2008), brain growth and basal nuclei volumes (Coviello et al., 2018; Schneider et al., 2018). In addition, nutrient intake was also positively correlated with higher fractional anisotropy (FA) values in selected white matter tracts (Schneider et al., 2018), such as the posterior limb of internal capsule (PLIC) (Coviello et al., 2018), and with greater axonal diameters in the corticospinal tract, indicating improved white matter integrity (Dabydeen et al., 2008). Moreover, cumulative protein intake was positively associated with higher cognitive and motor scores (Coviello et al., 2018). In a different study, it was demonstrated that first week protein and energy intakes were associated with improved developmental outcomes in extremely preterm born infants (Stephens et al., 2009). Together, these studies highlight the importance of adequate nutrition and the balance between protein, fat, and caloric content for early life brain development of the preterm infant.

Currently, "adequate" nutrition for the preterm infant is designed to "provide nutrients to approximate the rate of growth and composition of weight gain for a normal fetus of the same post-menstrual age and to maintain normal concentrations of blood and tissue nutrients" (American Academy of Pediatrics, 2009). The preferred source of nutrition for preterm infants is human milk from the infant's own mother. However, it has been long known that very/extremely preterm infants fed exclusively breast milk cannot match intrauterine growth patterns and may end up with extra uterine growth restriction. Therefore, human milk is fortified with a Human Milk Fortifier (HMF) to enhance protein, caloric, vitamin and mineral intake. It is important to note that extremely preterm infants are fed fortified human milk, regardless of the actual content of the human milk itself. From a Cochrane systematic review on the use of HMF for preterm nutrition it was concluded that HMF slightly increased in-hospital growth rates, but there was not enough evidence to suggest that feeding preterm infants with a standard amount of multi-nutrient fortified breast milk improves important developmental outcomes (Brown et al., 2016). This highlights the knowledge gap between what we currently feed the preterm infant and what has actually been proven to be effective on developmental outcome. The importance of this issue has

Abbreviations: AA, arachidonic acid; BSITD, Bayley Scales of Infant and Toddler Development; DHA, docosahexaenoic acid; EoP, Encephalopathy of Prematurity; EPA, eicosapentaenoic acid; FA, fractional anisotropy; HIE, hypoxic-ischemic encephalopathy; HMF, human milk fortifier; PLIC, posterior limb of the internal capsule; RCT, randomized controlled trial; WMI, white matter injury.

been acknowledged by the World Health Organization (WHO). In their recommendations on interventions to improve preterm birth outcomes (World Health Organization, 2015), they pose the following two questions as research priorities: (1) "What are the optimal feeding methods for preterm infants with birth weight <1,200 g?" and (2) "Is there a role for total parenteral nutrition in the management of preterm infants?"

In recent years, there has been increased interest in how to optimally feed the preterm infant, especially with respect to neurodevelopment. Randomized controlled trials (RCTs) have been focused on increasing protein, caloric, or fat intake and on supplementation with specific nutritional components. Also in pre-clinical studies, the potential mechanisms in which various specific nutritional supplements can protect or treat the neonatal brain from injury have been investigated in recent years. Therefore, this systematic review aimed to provide an overview of the current literature on potential nutritional interventions for the improvement of neurodevelopmental outcome in preterm infants, by taking a bench to bedside approach from pre-clinical models of neonatal brain injury to randomized clinical trials in preterm infants.

METHODS

Search

This systematic review was conducted according to PRISMA guidelines (Moher et al., 2009). To evaluate the clinical impact of nutritional interventions on brain development and neurodevelopmental outcome, Embase and Medline (Pubmed) were searched on August 15 2018 for RCTs published in the last 10 years. The search was divided into a pre-clinical search and a clinical search. For the pre-clinical search, studies were included if they met the following inclusion criteria: (1) neonatal brain injury induced by hypoxia, ischemia, inflammation and/or hyperoxia, induced during gestation or in the neonatal period (no later than post-natal day 10 (P10), which corresponds to near term human neurodevelopment) (Salmaso et al., 2014), (2) post-natal intervention with a nutritional component that started between birth and weaning (P21) with one of the following nutritional components: lipids, carbohydrates, proteins, vitamins, minerals, probiotics, prebiotics, oligosaccharides, fatty acids or amino acids, and (3) brain injury or neurodevelopment as outcome measure. Studies were excluded if (1) the article was written in a language other than English (2) the article was published over 10 years ago at time of search or if (3) more than one nutritional component was studied as the aim of this review was to study individual nutritional components. Embase and Medline were both searched with similar search strategies. For example, for Medline the following search was used:

(((((“Brain Injuries”[Mesh]) OR ((((“Brain”[Mesh]) OR brain[Title/Abstract])) AND ((damage[Title/Abstract]) OR injury[Title/Abstract]))))) AND ((“animal experimentation”[MeSH Terms] OR “models, animal”[MeSH Terms] OR “invertebrates”[MeSH Terms] OR “Animals”[Mesh:noexp] OR “animal population groups”[MeSH Terms] OR “Mice”[Mesh] OR “Rats”[Mesh] OR “Rodentia”[Mesh] OR mice[Title/Abstract] OR mouse[Title/Abstract] OR rodent[Title/Abstract] OR rodents[Title/Abstract] OR rat[Title/Abstract] OR rats[Title/Abstract] OR murine[tiab] OR “Papio”[Mesh] OR baboon[Title/Abstract] OR baboons[Title/Abstract] OR marmoset[tiab] OR marmosets[tiab] OR monkey[tiab] OR monkeys[tiab] OR primate[Title/Abstract] OR primates[Title/Abstract] OR “Primates”[Mesh] OR “Sheep, Domestic”[Mesh] OR sheep[Title/Abstract] OR lamb[Title/Abstract] OR lambs[Title/Abstract] OR pigs[tiab] OR pig[tiab] OR swine[tiab] OR swines[tiab] OR piglets[tiab] OR piglet[tiab] OR boar[tiab] OR boars[tiab] OR “guinea pigs”[tiab] OR “guinea pig”[tiab] OR rabbits[tiab] OR rabbit[tiab]))) AND (((“Infant, Low Birth Weight”[Mesh] OR “small for gestational age”[tiab] OR “small for date”[tiab] OR sga[tiab] OR “low birthweight”[tiab] OR vlbw[tiab] OR elbw[tiab] OR “Infant, Newborn”[Mesh] OR “Intensive Care, Neonatal”[Mesh] OR “Intensive Care Units, Neonatal”[Mesh] OR “Neonatal Nursing”[Mesh] OR infant[tiab] OR newborn*[tiab] OR neonat*[tiab] OR prematur*[tiab] OR preterm*[tiab] OR “Infant, Premature”[Mesh] OR “Infant, Very Low Birth Weight”[Mesh] OR “low birth weight”[tiab])))) AND ((((((((((((((((((((“Lipids”[Mesh]) OR lipid[Title/Abstract]) OR lipids[Title/Abstract])) OR ((((“Carbohydrates”[Mesh]) OR carbohydrate[Title/Abstract]) OR carbohydrates[Title/Abstract])) OR ((((“Proteins”[Mesh:NoExp]) OR protein[Title/Abstract]) OR proteins[Title/Abstract])) OR ((((“Vitamins”[Mesh]) OR vitamin[Title/Abstract]) OR vitamins[Title/Abstract])) OR ((((“Probiotics”[Mesh]) OR beneficial bacteria[Title/Abstract]) OR probiotics[Title/Abstract]) OR probiotic[Title/Abstract])) OR ((((“Prebiotics”[Mesh]) OR prebiotic[Title/Abstract]) OR prebiotics[Title/Abstract])) OR ((((“Minerals”[Mesh]) OR mineral[Title/Abstract]) OR minerals[Title/Abstract])) OR ((((“Oligosaccharides”[Mesh]) OR oligosaccharide[Title/Abstract]) OR oligosaccharides[Title/Abstract])) OR ((((“Amino Acids”[Mesh]) OR amino acid[Title/Abstract]) OR amino acids[Title/Abstract])) OR ((fatty acid[Title/Abstract]) OR fatty acids[Title/Abstract])) OR ((“Diet”[Mesh]) OR diet*[Title/Abstract])) OR ((“Enteral Nutrition”[Mesh]) OR enteral[Title/Abstract])) OR ((“Parenteral Nutrition”[Mesh:NoExp]) OR parenteral[Title/Abstract])) OR ((((“Micronutrients”[Mesh]) OR micronutrient[Title/Abstract]) OR micronutrients[Title/Abstract])) OR ((nutrient[Title/Abstract]) OR nutrients[Title/Abstract])) OR ((macronutrient[Title/Abstract]) OR macronutrients[Title/Abstract])) OR energy[Title/Abstract]) OR fortif*[Title/Abstract]) OR nutrition*[Title/Abstract])).*

For the clinical search, inclusion criteria were as follows: (1) preterm infants born before 32 weeks of gestation and/or birth weight below 1,500 g, (2) a post-natal nutritional intervention that was started between day of birth and 28 days post-natal and that contained one of the following nutritional components: lipids, carbohydrates, proteins, vitamins, minerals, probiotics, prebiotics, oligosaccharides, fatty acids, or amino acids, and (3) brain development, brain injury or neurodevelopmental outcome as outcome measure. As with the pre-clinical search, exclusion criteria were: (1) non-English written papers, (2) papers older than 10 years at time of the search, and (3) studies that researched more than one nutritional component. Embase and Medline were both searched with similar search strategies. For example, for Medline the following search was used:

((((((“Infant, Low Birth Weight”[Mesh] OR “small for gestational age”[tiab] OR “small for date”[tiab] OR sga[tiab] OR “low birthweight”[tiab] OR vlbw[tiab] OR elbw[tiab] OR prematur[tiab] OR preterm*[tiab] OR “Infant, Premature”[Mesh] OR “Infant, Very Low Birth Weight”[Mesh] OR “low birth weight”[tiab]))))) AND ((((((((((((((((((((((“Lipids”[Mesh]) OR lipid[Title/Abstract]) OR lipids[Title/Abstract])) OR (((“Carbohydrates”[Mesh]) OR carbohydrate[Title/Abstract]) OR carbohydrates[Title/Abstract])) OR (((“Proteins”[Mesh:NoExp]) OR protein[Title/Abstract]) OR proteins[Title/Abstract])) OR ((((“Vitamins”[Mesh]) OR vitamin[Title/Abstract]) OR vitamins[Title/Abstract])) OR (((((“Probiotics”[Mesh]) OR beneficial bacteria[Title/Abstract]) OR probiotics[Title/Abstract]) OR probiotic[Title/Abstract])) OR (((“Prebiotics”[Mesh]) OR prebiotic[Title/Abstract]) OR prebiotics[Title/Abstract])) OR (((“Minerals”[Mesh]) OR mineral[Title/Abstract]) OR minerals[Title/Abstract])) OR (((“Oligosaccharides”[Mesh]) OR oligosaccharide[Title/Abstract]) OR oligosaccharides[Title/Abstract])) OR (((“Amino Acids”[Mesh]) OR amino acid[Title/Abstract]) OR amino acids[Title/Abstract])) OR ((fatty acid[Title/Abstract]) OR fatty acids[Title/Abstract])) OR ((“Diet”[Mesh]) OR diet*[Title/Abstract])) OR ((“Enteral Nutrition”[Mesh]) OR enteral[Title/Abstract])) OR ((“Parenteral Nutrition”[Mesh:NoExp]) OR parenteral[Title/Abstract])) OR (((“Micronutrients”[Mesh]) OR micronutrient[Title/Abstract]) OR micronutrients[Title/Abstract])) OR ((nutrient[Title/Abstract]) OR nutrients[Title/Abstract])) OR ((macronutrient[Title/Abstract]) OR macronutrients[Title/Abstract])) OR energy[Title/Abstract]) OR fortif*[Title/Abstract]) OR nutrition*[Title/Abstract])))))) AND ((((“Brain”[Mesh]) OR brain[Title/Abstract])) OR (((“neurodevelopmental outcome”[Title/Abstract]) OR “neurodevelopmental outcomes”[Title/Abstract]) OR neurodevelopment[Title/Abstract]))) AND (((randomized controlled trial[pt] OR controlled clinical trial[pt] OR randomized[tiab] OR placebo[tiab] OR drug therapy[sh] OR randomly[tiab] OR trial[tiab] OR groups[tiab]))).*

Study Selection

Two authors (LH and CdT) reviewed all titles and abstracts independently. If an inconsistency occurred during abstract or full text analysis, consensus was reached in a meeting or a third author (RvE) was consulted. Quality of the included studies was assessed using the Cochrane Risk of Bias assessment tool (Higgins et al., 2011) and the adapted SYCLE's Risk of Bias tool (Hooijmans et al., 2014), for assessing risk of bias for the clinical and pre-clinical studies, respectively. Graphs for risk of bias for individual studies and across studies were made [Review Manager (RevMan) [computer program], 2014].

Data Collection and Data Items

Included studies were categorized in the results section based on the nutritional component.

From all pre-clinical papers, the following data were extracted (by CdT): nutritional component and amount used as intervention, duration of intervention, duration of follow up, test used to assess neurodevelopmental outcome and/or method to detect brain injury, type of brain injury (hypoxic, ischemic, inflammatory, and/or hyperoxic) and post-natal day of insult.

For all clinical studies, the following data were extracted (by LH): nutritional component and amount used as intervention, duration of intervention, duration of follow up, test used to assess neurodevelopmental outcome, number of patients, gestational age and birth weight of patients and neurodevelopmental outcome.

Principal Summary Measures

Our primary outcome measure was brain injury and/or neurodevelopmental outcome. For the pre-clinical search, brain injury was defined as either brain volume loss or myelination impairments. Secondary outcomes on brain injury include cell death, oligodendrocyte numbers, glial activation, and cytokine levels. Neurodevelopmental outcome was included in this review if assessed with a validated behavioral task.

For the clinical search, brain injury was defined as either brain volume loss, or brain injury defined by a brain injury scoring system as reported in the original paper. Neurodevelopmental outcome was included in this review if assessed with a validated neurodevelopmental outcome scale. No statistical analysis was done. Outcome was reported as described by the original authors and no authors were contacted.

RESULTS

Study Selection

Pre-clinical Trials

The pre-clinical search through Medline and Embase provided 1,855 and 847 results, respectively. After screening of title and abstract, 46 papers potentially met our in- and exclusion criteria and were screened as full text to assess eligibility, after which 24 were included in final analysis. **Figure 1** gives an overview of the screening process, including reason of exclusion.

Clinical Trials

The clinical search through Medline and Embase provided 526 and 361 results, respectively. After screening of title and abstract, 71 papers potentially met our in- and exclusion criteria and were screened as full text to assess eligibility, after which 22 were included in final analysis. **Figure 2** gives an overview of the screening process, including reason of exclusion.

Figures 3–6 show the results of the quality assessment for the pre-clinical and clinical studies. No studies were excluded as a result of quality assessment.

Study Characteristics

Pre-clinical Trials

The pre-clinical search resulted in the inclusion of 24 studies. Of those, eight evaluated proteins (including single amino acids), 10 studies evaluated lipids, two studies evaluated probiotic metabolites, two studies evaluated minerals and two studies evaluated vitamins. There were no studies that evaluated supplementation with carbohydrates, probiotics, or prebiotics. The clinical characteristics of the included studies are summarized in **Table 1**.

Clinical Trials

The clinical search resulted in the inclusion of 22 studies. Of those, 10 studies evaluated proteins (including single amino

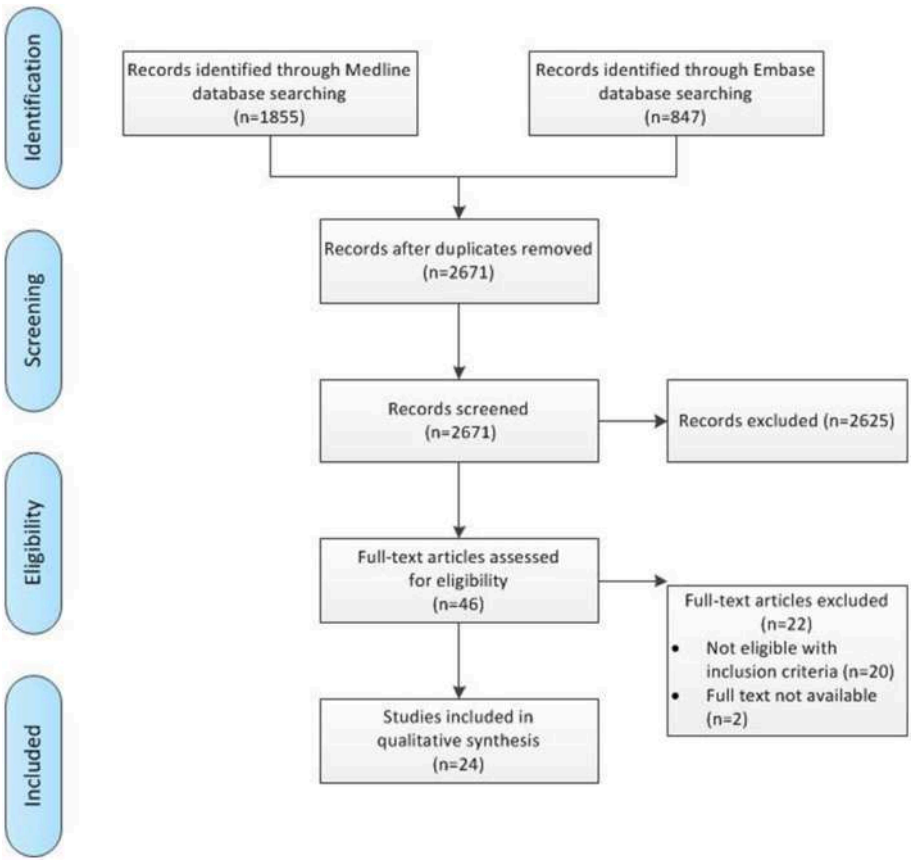


FIGURE 1 | Flowchart of pre-clinical search process.

acids), five studies evaluated lipids, three studies evaluated probiotics, two studies evaluated prebiotics, one study evaluated minerals and one study evaluated vitamins. There were no studies that evaluated only carbohydrate intake. The clinical characteristics of the included studies are summarized in **Table 2**.

Protein Intake (Including Individual Amino Acids)

Pre-clinical Trials

The effects of protein or amino acid supplementation on neonatal brain injury were described in eight pre-clinical studies. Of those studies, two studies investigated the effects of supplementation with lactoferrin (van de Looij et al., 2014; Ginet et al., 2016), a protein abundantly present in human milk. Furthermore, three studies were focused on amino acid-based biosynthetic products acetyl-l-carnitine (Xu et al., 2015; Tang et al., 2016) and taurine (Zhu et al., 2016) that are also present in human milk. Additionally, three pre-clinical studies focused on supplementation with the single amino acids glycine (Mori et al., 2017) or L-cysteine (Liu et al., 2017; Xin et al., 2018).

Lactoferrin is a glycoprotein produced by exocrine glands and released at high levels in human milk, especially colostrum (Nagasawa et al., 1972). van de Looij et al. (2014) investigated the effects of maternal lactoferrin supplementation in a rat model of preterm hypoxic-ischemic encephalopathy (HIE), induced at post-natal day 3 (P3, equivalent to 24–28 weeks gestational age in humans) that presents with impaired cortical development and white matter integrity (van de Looij et al., 2014). Lactoferrin (1 g/kg/day) was supplemented in the maternal diet from P0 throughout lactation and resulted in increased levels of lactoferrin in the stomach and serum of the offspring. MRI analysis revealed reduced cerebral edema hours after the insult and reduced cortical loss accompanied by improved diffusivity at post-natal day 25 in neonatal rats supplemented with lactoferrin. Also the impairments in white matter structure were partly restored upon lactoferrin supplementation, as indicated by improved diffusivity and FA values in the external capsule but not in the corpus callosum of injured rats supplemented with lactoferrin compared to control diet. The authors hypothesized that neuroprotective effects of lactoferrin may be driven by its anti-inflammatory properties, as indicated by a reduction of brain TNF-α and IL-6 expression, which resulted in reduced apoptotic cell death, as indicated by reduced caspase 3 activation at 1 day after injury in the lactoferrin-supplemented rats compared to controls. In a following study, the same research group repeated the lactoferrin intervention study in an inflammation-induced rat model of preterm brain injury,

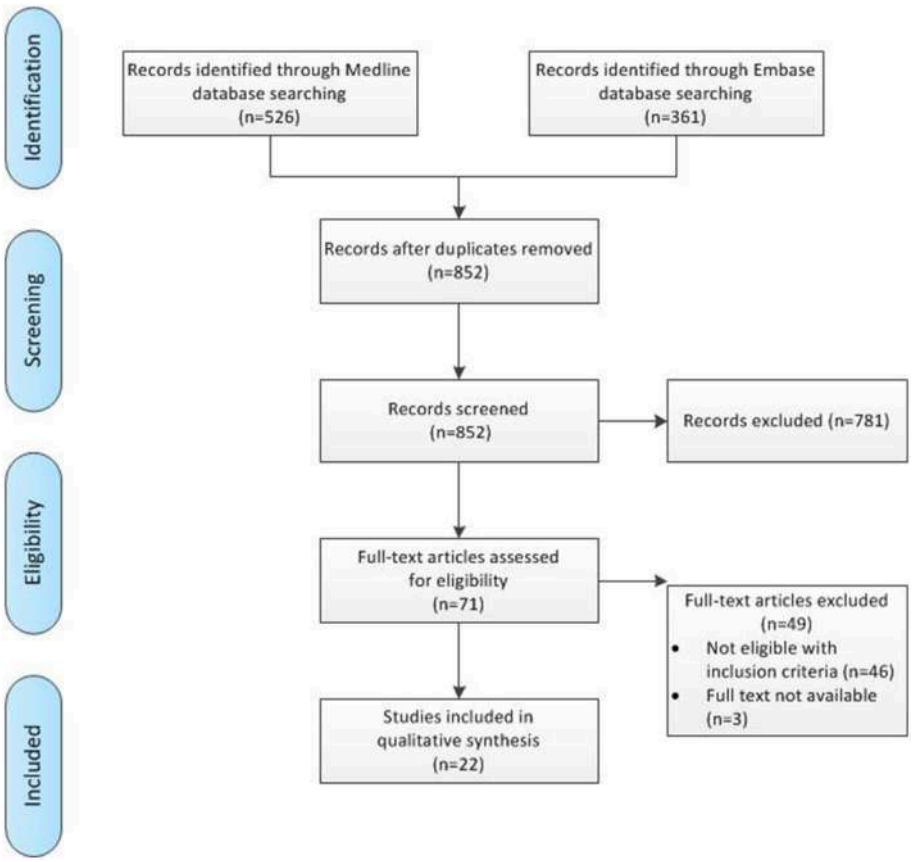


FIGURE 2 | Flowchart of clinical search process.

using injection of LPS in the subcortical white matter at P3 (Ginet et al., 2016). Lactoferrin supplementation during lactation again provided neuroprotection as shown by reduced ventriculomegaly, lesion size, and myelination deficits. Although lactoferrin supplementation did not reduce astrogliosis and pro-inflammatory cytokine expression, it did reduce microglia activation as compared to the control diet in the LPS-injured neonatal rat brain.

The biosynthetic product acetyl-l-carnitine is also a component of human milk produced from the amino acids lysine and methionine. After consumption, it rapidly enters the brain and its metabolite carnitine facilitates long chain fatty acid metabolism to produce energy. One group described in two studies the effects of acetyl-l-carnitine supplementation in a rat model of neonatal HIE induced at P7 (equivalent to near term in humans) (Xu et al., 2015; Tang et al., 2016). Pups were injected subcutaneously with 4 doses of 100 mg/kg acetyl-l-carnitine at 0, 4, 24, and 48 h post-HI. Treatment with acetyl-l-carnitine reduced lesion size and promoted oxidative cerebral energy production and minimized anaerobic glycolysis, if administered early after the HI insult (Xu et al., 2015). In the second study, acetyl-l-carnitine supplementation again reduced lesion size, and also improved simple reflexes and spatial memory but not social play, when compared to HIE rats that received vehicle (Tang et al., 2016).

Although strictly classified as a biosynthetic compound derived from cysteine and methionine, taurine is often regarded as the second most abundant amino acid in human milk (Rassin, 1981). After consumption, taurine accumulates in the brain in high concentrations (Rassin, 1981), where it has various functions, for example as a neurotransmitter and trophic factor (Wu and Prentice, 2010). One pre-clinical trial investigated the effects of taurine supplementation in a rat model of neonatal HIE induced at P7 (Zhu et al., 2016). Taurine was administered intraperitoneally (i.p.) every 12 h for 2 days post-HI at doses of 30–120 mg/kg. Taurine dose-dependently reduced lesion size, cell death, and oxidative stress in brains of neonatal HIE rats, as compared to vehicle.

In summary, supplementation with lactoferrin from the day of birth was neuroprotective in two models of preterm brain injury, as shown by improvement of neurological outcome on MRI, potentially though inhibition of neuroinflammation. In addition, peripheral administration of amino acid-based biosynthetic products acetyl-l-carnitine and taurine after the insult, reduced lesion size in a rat model of neonatal near term HIE.

FIGURE 3 | Risk of bias of individual pre-clinical studies.

The amino acid glycine acts as an important inhibitory neurotransmitter in the brain. The effects of glycine treatment were described in one study in a rat model of neonatal HIE induced at P7 (Mori et al., 2017). Intraperitoneal injections with 800 mg/kg glycine prior to the hypoxic insult induced a large but brief increase in glycine concentrations in the cerebrospinal fluid and subsequently reduced lesion size and neuronal cell death in neonatal HIE rats, when compared to vehicle injections. This neuroprotection by glycine was associated with reduced expression of TNF-α levels at 12 and 24 h post-HI and 3 days later, reduced neuroinflammation was measured by a lower numbers of astrocytes and microglia in the lesioned area.

One group reported in two pre-clinical studies the effects of l-cysteine treatment in a mouse model of neonatal HIE induced at P7 (Liu et al., 2017; Xin et al., 2018). L-cysteine is catalyzed in the brain to produce endogenous hydrogen sulfide (H_2S), which has been suggested to inhibit inflammation, oxidative stress and apoptosis. Neonatal mice were subjected to HI and given daily intraperitoneal injections with 2.5–5.0 mg/kg l-cysteine for 3 consecutive days, starting immediately post-HI. Treatment with l-cysteine attenuated brain edema, lesion size and neuronal cell death after neonatal HIE, when compared to vehicle (Liu et al., 2017; Xin et al., 2018). L-cysteine treatment also improved behavioral deficits, including neurological reflexes and spatial working memory, in mice with neonatal HIE. The neuroprotective effects of l-cysteine on functional and anatomical outcome in neonatal mice with HIE were associated with improved synapse formation and reduced oxidative stress and neuroinflammation, as measured by microglia and astrocyte infiltration. Furthermore, the treatment effect of l-cysteine was reversed when H_2S formation was inhibited *in vivo*, indicating that the neuroprotective mechanisms of l-cysteine depend on its function as an H_2S donor.

In summary, single amino acid supplementations with glycine (as a pre-treatment) and l-cysteine (as a post-treatment) in rodent models of neonatal near term HIE reduced brain injury and neuroinflammation, and the latter treatment also improved behavioral outcome.

Clinical Trials

Ten randomized controlled clinical trials (RCTs) described outcome after amino acid or protein supplementation (Amin et al., 2009; Blanco et al., 2012; de Kieviet et al., 2012, 2014; Burattini et al., 2013; van den Akker et al., 2014; Bellagamba et al., 2016; Balakrishnan et al., 2017; Dogra et al., 2017; Twilhaar et al., 2018a). In six trials different *quantities* of amino acids or protein were evaluated, but all trials had different target values for intervention or control group, different starting time of intervention and/or different length of intervention. Three studies (Blanco et al., 2012; Burattini et al., 2013; Balakrishnan et al., 2017) evaluated increased *parenteral* supplementation with amino acids, starting shortly after birth and targeting 4 g/kg/day within the first days of life, compared to control, aiming on 2, 2.5, or 3 g/kg/day within the first days of life. In total, these studies evaluated 242 infants around 18–24 months corrected age. No beneficial effect of high dose of *parenteral* amino acid supplementation on neurodevelopment was found. Burattini et al. (2013) found no difference in neurodevelopmental outcome or growth between the two groups ($n = 96$ in total). Balakrishnan et al. (2017) found no difference in neurodevelopmental outcome

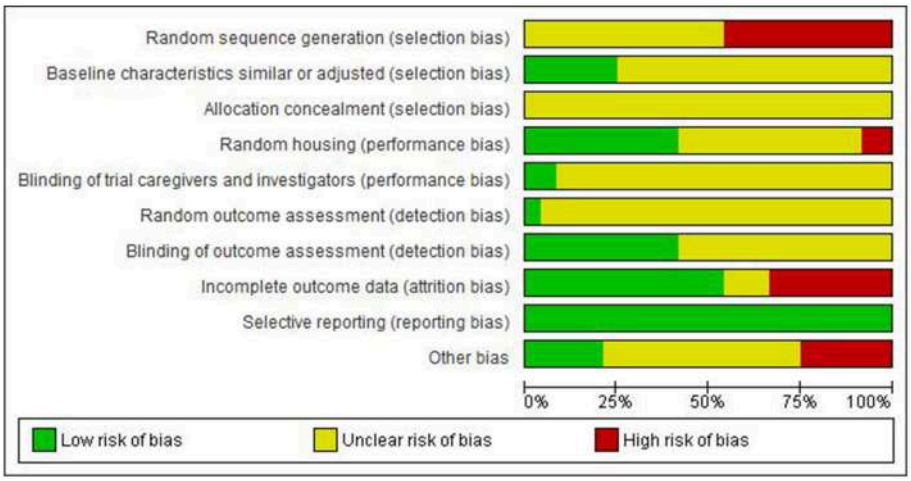


FIGURE 4 | Risk of bias across pre-clinical studies.

($n = 114$ in total), but did find lower mean weight, length and head circumference at discharge in infants that received high amino acid supplementation. Blanco et al. (2012) found that infants in the intervention group had significantly lower Mental Developmental Index (Bayley Scales of Infant and Toddler Development, second edition) at 18 months corrected age. At 24 months follow up, this difference was no longer present and no other differences in neurodevelopmental outcome between the two groups were observed ($n = 32$ in total). However, during the 24 months follow up period, infants in the intervention group showed significantly lower head circumference and overall growth. One study evaluated a different target level of amino acids. Bellagamba et al. (2016) evaluated enriched (3.5 g/kg/day parenteral and 4.6 g/kg/day enteral) vs. regular (2.5 g/kg/day parenteral and 3.6 g/kg/day enteral) supplementation from day of birth until a weight of 1,800 g was achieved. The intervention included both *parenteral* and *enteral* supplementation. At 2 years corrected age, no difference in neurodevelopmental outcome was seen ($n = 164$ in total). One study assessed the effect of early amino acid supplementation during the first 3 days of life. van den Akker et al. (2014) started *parenteral* supplementation (2.4 g/kg/day) within 2 h after birth in comparison to controls, who started 24–48 h after birth (1.2 g/kg/day, increased to 2.4 g/kg/day on day 3). At 2 years of age, no difference in outcome between both groups was seen ($n = 111$ in total). However, subgroup analysis revealed higher odds of normal outcome for boys treated with early amino acids (OR 6.17 [1.01–38.46]). In contrast, girls in the intervention group had a significantly lower Mental Developmental Index compared to girls in the control group. The paper by Dogra et al. (2017) was the only study evaluating exclusive *enteral* protein supplementation. Infant feedings were supplemented with regular (0.4 g/100 ml) or protein enriched (1.0 g/100 ml) HMF, which started at an enteral feeding volume of 100 ml/kg/day and ended at discharge. At term equivalent age, infants that received protein enriched HMF had significantly greater head circumferences. However, during the 2 year follow up period, no difference in growth nor mental or motor outcome was seen ($n = 92$ in total).

In summary, all studies combined investigated 609 infants and conflicting evidence was found. Most studies found no improvement of neurodevelopmental outcome after early and/or enriched (par)enteral amino acid supplementation or found an adverse effect on growth and head circumference. The single study that described exclusive enteral protein supplementation showed a beneficial effect on head circumference, but not on neurodevelopmental outcome.

Supplementation with a *single* amino acid was described in four papers. de Kieviet et al. (2012, 2014) and Twilhaar et al. (2018a) described in three papers long term follow up after the same RCT (GEEF study), investigating glutamine supplementation (de Kieviet et al., 2012, 2014; Twilhaar et al., 2018a). Infants received enteral glutamine supplementation (0.3 g/kg/day) or placebo between post-natal day 3 and day 30. The effect of glutamine on head circumference growth during the first year of life ($n = 65$ in total) (de Kieviet et al., 2014) and brain volumes at 8 years of age ($n = 52$ in total) (de Kieviet et al., 2012) was evaluated. Glutamine supplementation was associated with increased head circumference growth during the first year of life as well as with increased white matter, hippocampal and brain stem volumes at 8 years. Neurodevelopmental follow up at 13 years of age ($n = 61$ in total) showed comparable outcomes on all domains between glutamine and placebo groups, except for the visuospatial working memory forward span and the parent rated attention skills, which favored the control group (Twilhaar et al., 2018a).

In a different study, Amin et al. (2009) evaluated the effect of 261 mg/kg/day L-arginine compared to placebo during the first 28 days of life on neurodevelopmental outcome at 36 months corrected age ($n = 132$ in total). No significant difference was found in neurodevelopmental outcome between both groups.

In summary, enteral glutamine supplementation showed increased head circumference growth during the first year of

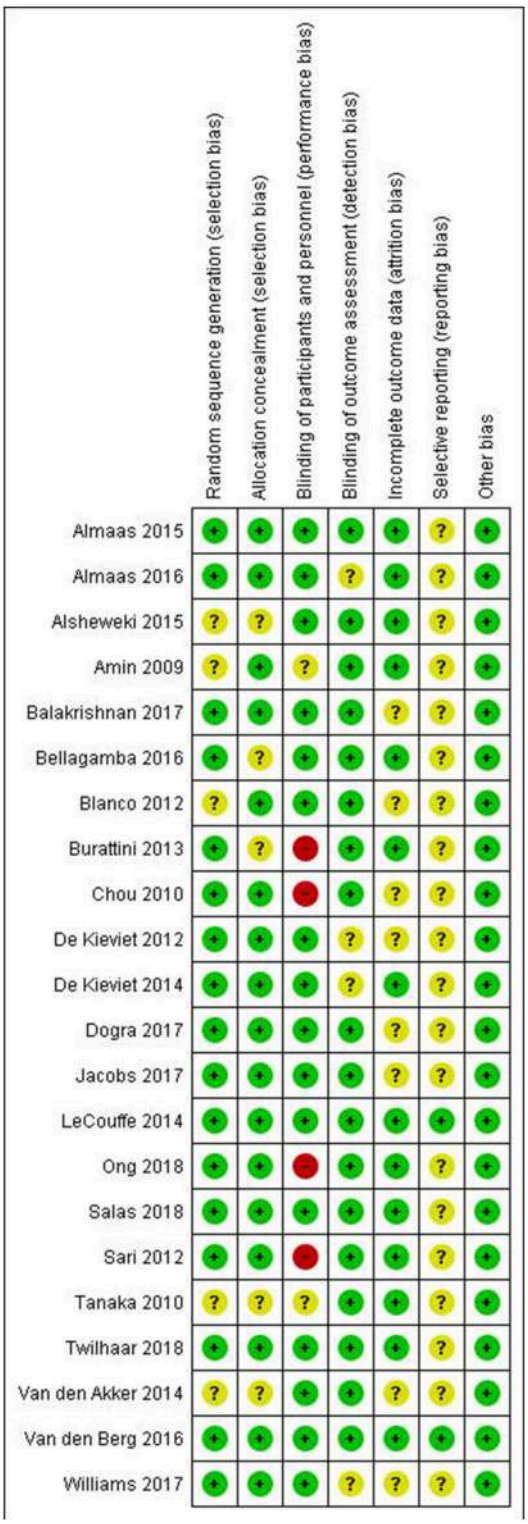


FIGURE 5 | Risk of bias of individual clinical studies.

life and increased brain volumes at 8 years. However, at 13 years of age, no positive effect of glutamine was found on neurodevelopmental outcome. Working memory and attention skills differed between the glutamine and control group, favoring the control group, but were within the normal range for both groups.

No advantage from supplementation with L-arginine was detected.

Lipid Intake (Including Fatty Acids)

Pre-clinical Trials

The effects of lipid supplementation on pre-clinical neonatal brain injury have been reported in 10 studies, all focused on docosahexaenoic acid (DHA) supplementation only (Berman et al., 2009, 2010, 2013; Williams et al., 2013; Mayurasakorn et al., 2016; Revuelta et al., 2016; Arteaga et al., 2017; Solberg et al., 2017; Buddington et al., 2018; Huun et al., 2018).

In a rat model for neonatal HIE induced at P7, an i.p. injection of 1–5 mg/kg DHA 4 h prior to the insult improved sensorimotor outcome and reduced lesion size (Berman et al., 2009). In a similar neonatal HIE model, 1 mg/kg DHA was administered i.p. at 10 min prior the HI insult, and prevented impairments in the auditory brain stem response and in myelination of the inferior colliculus, a principal midbrain nucleus of the auditory pathway (Revuelta et al., 2016). Neuron morphology and astrogliosis was also measured, but not statistically analyzed. In addition, the group reported in a similar experimental design that DHA pre-treatment reduced lesion size and mitochondrial cell damage at the hippocampal level, and rescued deficits in myelination in the external capsule and striatum and in spatial memory (Arteaga et al., 2017). In a rat model for neonatal inflammatory HIE using LPS injection prior to HI, pre-treatment with DHA improved sensorimotor outcome, but did not reduce lesion size (Berman et al., 2010).

In addition to DHA administration *prior* to the insult, also treatment *after* the insult has been investigated in rodent models of neonatal HIE. Williams et al. (2013) compared post-treatment with DHA or eicosapentaenoic acid (EPA) triglycerides in a mouse model for neonatal HIE induced at P10 (equivalent to near term in humans) (Williams et al., 2013). Triglyceride emulsions allow for slower release of n-3 fatty acids and therefore avoid high levels of n-3 fatty acids that may lead to brain and liver toxicity (Singh et al., 1989; Oliveira et al., 1997). Intraperitoneal treatment with 375 mg/kg of tri-DHA, but not tri-EPA, immediately after the HI insult reduced lesion size. Furthermore, a treatment delay for up to 2 h after the insult provided neuroprotection on histology level, but a further delay failed to reduce lesion size. In a subsequent study, the group demonstrated that neuroprotection by tri-DHA after neonatal HIE was associated with increased DHA content in the mitochondria and DHA-derived bioactive metabolites in the brain (Mayurasakorn et al., 2016). Tri-DHA treatment immediately post-HI reduced oxidative damage in the brain and improved long-term neurological outcomes. Also in combination with hypothermia, DHA supplementation reduced lesion size and improved sensorimotor outcome when compared to treatment with hypothermia only (Berman et al., 2013).

To improve clinical translation of DHA therapy for neonatal brain injury, Solberg et al. (2017) investigated the effects of DHA in a newborn piglet model of severe hypoxia-reoxygenation (Solberg et al., 2017). DHA was administered

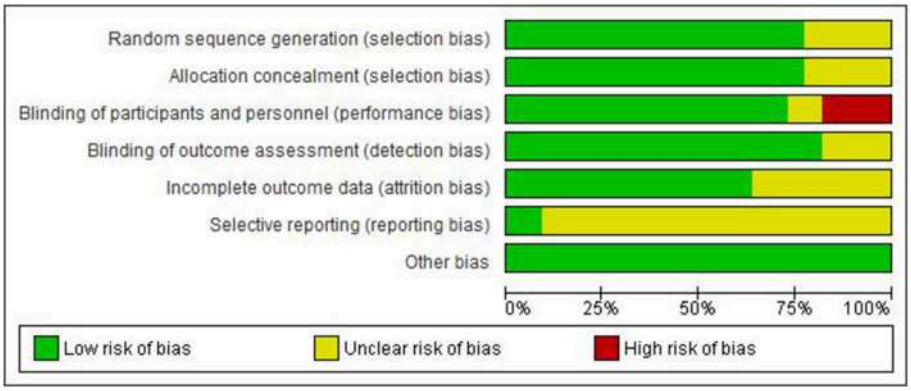


FIGURE 6 | Risk of bias across clinical studies.

i.v. at 5 mg/kg in newborn piglets at 4 h after subjection to hypoxia. Lipid peroxidation markers were significantly lower in the hippocampus and cortex of DHA-treated newborn piglets compared to the untreated control group measured at 9.5 h after treatment. These results indicate that DHA has anti-oxidative effects in the brain. Additionally, the group recently reported in a similar experimental design changes in oxidative stress in different brain regions in response to DHA in combination with hypothermia (Huun et al., 2018). In the white matter, regardless of hypothermia treatment, DHA reduced the levels of di-homo-isoprostanes, a product of adrenic acid oxidation. In the cortex, DHA significantly reduced levels of neurofuranes and di-homo-isoprostanes, only when hypothermia was not applied. In contrast to the first study of Solberg et al. (2017), no differences were observed in the hippocampus.

In addition, one study reported the effects of oral DHA supplementation in preterm-born piglets at 92% gestation (considered relevant to 32 week-old preterm infant) (Buddington et al., 2018). Preterm pigs were born by sterile caesarian section and provided with parenteral nutrition for 16–18 h. Thereafter, the source of nutrition was entirely milk replacer. Milk replacer was supplemented with phosphotidylserine (PS)–DHA to provide 58 mg/kg/day, or sunflower oil as the placebo. Based on rat experiments (Vaisman and Pelled, 2009), the authors argue that chronic administration of PS-DHA results in greater DHA accretion in the cortex compared with tri-DHA, indicating further improved DHA bioavailability. PS-DHA was supplemented until euthanization at term-equivalent age, i.e., 10 days after delivery. Supplementing preterm pigs with PS-DHA did not increase overall weight gain, but did significantly restore the decline in weight of the cerebellum at term-equivalent age up to the weight of term control pigs. PS-DHA supplementation also resulted in higher DTI indices in different brain regions, including the internal capsule, thalamus, hypothalamus, globus pallidus, hippocampus, and cortex. This indicates that a higher degree of myelination was present in brains of preterm pigs that were supplemented with PS-DHA as compared to placebo. Additionally, PS-DHA supplementation accelerated development of recognition memory and the auditory pathway as compared with placebo-treated preterm piglets.

In summary, these pre-clinical studies demonstrate that peripheral DHA supplementation effectively reduced lesion size and improved neurodevelopmental outcome in rodent models of neonatal HIE, when administered prior to of soon after the insult. In addition, DHA supplementation reduced lipid peroxidation markers in a piglet model of severe hypoxia-reoxygenation, indicating that DHA has anti-oxidative effects in the brain. In preterm-born piglets, enteral supplementation with DHA improved white matter integrity and cerebellar growth.

Clinical Trials

In total, five trials that described outcome after an intervention with lipids were included. Of those, one trial evaluated the effect of different amounts of lipids (Ong et al., 2018), three trials focused on supplementation with fatty acids (Almaas et al., 2015, 2016; Alshweki et al., 2015), and in one trial supplementation with sphingomyelin was used (Tanaka et al., 2013).

Ong et al. (2018) described the only included study in which different quantities of regular *parenteral* lipids were studied. They explored the impact of low (1 g/kg/day) and standard (3 g/kg/day) dose *parenteral* lipids (soybean oil, Intralipid) on growth and neurodevelopmental outcome until 2 years corrected age. No difference was found in neurodevelopmental outcome or growth up to 2 years of age between the groups ($n = 30$ in total) that received low or high dose soybean, with an exception for 12 months corrected age, at which a significant higher cognitive composite score was found in the patients with low dose soybean oil.

Supplementation with the fatty acids DHA and arachidonic acid (AA) was investigated in three included studies. In two papers, Almaas et al. (2015, 2016) described (1) cognitive ($n = 98$ in total) and brain volume outcome (Almaas et al., 2015) ($n = 81$ in total), and (2) Diffusion Tensor Imaging ($n = 82$ in total), and behavioral outcome (Almaas et al., 2016) ($n = 98$ in total) at 8 years of age in infants supplemented with enteral DHA and AA (ratio 1/1). Supplementation was initiated when the infant reached enteral feeding of 100 ml/kg/day and

TABLE 1 | Baseline characteristics of included pre-clinical studies.

Study	Nutritional component	Timing (and mode) of administration	Brain injury model	Primary outcome measure
van de Looij et al., 2014	Lactoferrin	Pre- and post-treatment (enteral, in maternal diet)	HI P3 rat	Lesion size, myelination
Ginet et al., 2016	Lactoferrin	Pre- and post-treatment (enteral, in maternal diet)	LPS i.c. P3 rat	Lesion size, myelination
Xu et al., 2015	Acetyl-l-carnitine	Post-treatment (s.c.)	HI P7 rat	Lesion size
Tang et al., 2016	Acetyl-l-carnitine	Post-treatment (s.c.)	HI P7 rat	Lesion size, behavior
Zhu et al., 2016	Taurine	Post-treatment (i.p.)	HI P7 rat	Lesion size
Mori et al., 2017	Glycine	Pre-treatment (i.p.)	HI P7 rat	Lesion size
Liu et al., 2017	L-cysteine	Post-treatment (i.p.)	HI P7 mouse	Lesion size, behavior
Xin et al., 2018	L-cysteine	Post-treatment (i.p.)	HI P7 mouse	Lesion size, behavior
Berman et al., 2009	DHA	Pre-treatment (i.p.)	HI P7 rat	Lesion size, behavior
Revuelta et al., 2016	DHA	Pre-treatment (i.p.)	HI P7 rat	Myelination
Arteaga et al., 2017	DHA	Pre-treatment (i.p.)	HI P7 rat	Lesion size, myelination, behavior
Berman et al., 2010	DHA	Pre-treatment (i.p.)	HI+ LPS P7 rat	Lesion size, behavior
Williams et al., 2013	tri-DHA tri-EPA	Post-treatment (i.p.)	HI P10 mouse	Lesion size
Mayurasakorn et al., 2016	tri-DHA tri-EPA	Post-treatment (i.p.)	HI P10 mouse	Lesion size, behavior
Berman et al., 2013	DHA	Post-treatment (i.p.)	HI P7 rat	Lesion size, behavior
Solberg et al., 2017	DHA	Post-treatment (i.v.)	HR newborn piglet	Lipid peroxidation
Huun et al., 2018	DHA	Post-treatment (i.v.)	HR newborn piglet	Lipid peroxidation
Buddington et al., 2018	PS-DHA	Post-treatment (enteral)	Preterm-born piglet	Brain weight, myelination
Jaworska et al., 2017	Na-butyrate	Post-treatment (i.p.)	HI P7 rat	Lesion size
Ziemka-Nalecz et al., 2017	Na-butyrate	Post-treatment (i.p.)	HI P7 rat	Behavior
Ramani et al., 2017	Vitamin A	Post-treatment (enteral)	Hyperoxia P2–14 mouse	Behavior
Miura et al., 2009	Vitamin C	Pre-treatment (i.p.)	HI P7 rat	Macroscopical lesion size
Koning et al., 2017	$MgSO_4$	Pre-treatment (i.p.)	HI P7 rat	Lesion size
Seyama et al., 2018	$MgSO_4$	Pre-treatment (i.p.)	HI P6 rat	Myelination

i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous; i.c., intracranial; HI, hypoxia-ischemia; HR, hypoxia-reoxygenation; P, postnatal day; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; tri, triglyceride; PS, phosphatidylserine; Na, sodium; $MgSO_4$, magnesiumsulphate.

consisted of 0.5 ml study oil on 100 ml milk, which contained 32 mg DHA and 31 mg AA or placebo. Intervention ended when the patient was discharged or when a total of 100 ml of study oil was given. At 8 years of age outcome on brain volumes, cortical volumes, surface areas, thickness and white matter tract development as well as cognitive and motor outcome was similar between the fatty acid-supplemented and placebo patients. Alshweki et al. (2015) investigated the effect of AA/DHA ratio on outcome. They provided infants with enriched AA formula (AA/DHA ratio 2/1) or regular formula (ratio 1/1) and attempted to maintain this ratio in infant feeding during the entire follow up period of 2 years ($n = 45$ in total). A group of 25 infants that received exclusively breast milk were used as control group. At 2 years of age, the infants that received enriched AA formula had a higher score on the Brunet Lézine scale (Scale of Psychomotor Development of Children) compared to infants that received standard infant formula. Additionally, compared to breastfed controls, AA-enriched formula infants had similar Brunet Lézine scales whereas regular formula infants had significantly lower scores.

Tanaka et al. (2013) focused on supplementation with sphingomyelin. Infants ($n = 24$ in total) received either sphingomyelin-fortified enteral feeding (sphingomyelin 20% of all phospholipids in milk) or standard enteral feeding (sphingomyelin 13% of all phospholipids in milk). At 3–18 months of age infants in the intervention group scored better on the preference rate on the Fagan test and the sustained-attention test and showed shorter latency of visual evoked potentials. No differences in mental or psychomotor development were found.

In summary, in the one study that we included on quantities of lipids, no difference between high and low dose soybean oil was found at 2 years of age ($n = 30$ in total). DHA and AA supplementation showed no effect on brain volumes, brain maturation, and neurodevelopmental outcome ($n = 98$ in total). High AA/DHA ratio (2/1) did show a positive effect on Brunet Lézine scale compared to regular AA/DHA ratio (1/1) ($n = 45$ in total). Sphingomyelin supplementation improved some neurodevelopmental outcome measures, but showed no effect on mental or psychomotor development.

TABLE 2 | Baseline characteristics of included clinical studies.

Study	Nutritional component	Enteral/parenteral	Inclusion criteria	Selected infants (*n*)	Primary study outcome	NDO or brain injury outcome measure
Blanco et al., 2012	Protein	Parenteral	>24 weeks and <1,000 g	32	Reduction of potassium	NDO 0–24 months
Burattini et al., 2013	Protein	Parenteral	<1,250 g	96	Body seize at 36 weeks PMA	NDO 24 months
Balakrishnan et al., 2017	Protein	Parenteral	<32 weeks and <1,250 g	114	NDO 18–24 months	NDO 18–24 months
van den Akker et al., 2014	Protein	Parenteral	<32 weeks and <1,500 g	111	Short term safety and efficacy	NDO 24 months
Bellagamba et al., 2016	Protein	Both	<1,250 g	164	Weight gain from birth−1,800 g	NDO 24 months
Dogra et al., 2017	Protein	Enteral	<32 weeks or <1,500 g	92	Head growth at 40 weeks PMA	NDO 12–18 months
de Kieviet et al., 2012	Glutamine	Enteral	<32 weeks or <1,500 g	65	Feeding tolerance	Brain volumes and white matter integrity at 8 years
de Kieviet et al., 2014	Glutamine	Enteral	<32 weeks or <1,500 g	52	Feeding tolerance	HC growth at 1 year and brain volumes at 8 years
Twilhaar et al., 2018a	Glutamine	Enteral	<32 weeks or <1,500 g	61	Feeding tolerance	NDO 13 years
Amin et al., 2009	L-arginine	Both	≤32 weeks or ≤1,250 g	132	Necrotizing enterocolitis	NDO 36 months
Ong et al., 2018	Lipids	Parenteral	≤29 weeks	30	Cholestasis	NDO 6–24 months
Almaas et al., 2015	DHA, AA	Enteral	<1,500 g	98	NDO 6 months	NDO, brain volumes and brain maturation at 8 years
Almaas et al., 2016	DHA, AA	Enteral	<1,500 g	98	NDO 6 months	Behavior and white matter integrity at 8 years
Alshweki et al., 2015	AA/DHA ratio	Enteral	25–32 weeks or <1,500 g	45	NDO 24 months	NDO 24 months
Tanaka et al., 2013	Sphingomyelin	Enteral	<1,500 g	24	NDO 0–18 months	NDO 0–18 months
Chou et al., 2010	Probiotics	Enteral	<1,500 g	301	Necrotizing enterocolitis	NDO 3 years
Jacobs et al., 2017	Probiotics	Enteral	<32 weeks and <1,500 g	735	Late onset sepsis	NDO 2–5 years
Sari et al., 2012	Probiotics	Enteral	<33 weeks or <1,500 g	174	Necrotizing enterocolitis	NDO 18–22 months
LeCouffe et al., 2014	Prebiotics	Enteral	<32 weeks or <1,500 g	93	Neonatal infections	NDO 0–12 months
van den Berg et al., 2016	Prebiotics	Enteral	<32 weeks or <1,500 g	77	Neonatal infections	NDO 24 months
Salas et al., 2018	Vitamin D	Enteral	23–27 weeks	91	Vitamin D concentration	NDO 22–26 months
Williams et al., 2017	Iodide	Both	<31 weeks	1,259	NDO 24 months	NDO 24 months

NDO, Neurodevelopmental outcome; PMA, Postmenstrual age; HC, Head circumference; DHA, Docosahexaenoic acid; AA, Arachidonic acid.

Probiotic Supplementation

Pre-clinical Trials

Although no pre-clinical studies were found on the use of probiotics to improve neurodevelopmental outcome after neonatal brain injury, one group investigated the effects of butyrate (Jaworska et al., 2017; Ziemka-Nalecz et al., 2017), a short-chain fatty acid synthesized by bacteria in the colon by fermenting otherwise non-digestible fiber. Butyrate enters the bloodstream and subsequently the brain where it serves as a histone deacetylase (HDAC) inhibitor, activates G protein-coupled receptors, and acts as an energy metabolite to produce ATP. In two publications, the authors demonstrated in rats with neonatal HIE induced at P7 that i.p administration of 300 mg/kg sodium butyrate for five consecutive days post-HI reduced lesion size and neuroinflammation, as measured by levels of IL-1β and chemokine CXCL10 and polarization of microglia from pro-inflammatory M1 to anti-inflammatory M2 (Jaworska et al., 2017; Ziemka-Nalecz et al., 2017). In addition, butyrate treatment enhanced the generation of newly formed neuroblasts and oligodendrocytes in the dentate gyrus of the hippocampus of neonatal HIE rats, as compared to vehicle treatment. This neuroregenerative effect of butyrate is potentially driven by enhanced endogenous production of BDNF in the ipsilateral hemisphere. However, butyrate treatment failed to improve neurobehavioral outcome, as measured by behavioral tasks for motor function, spatial memory and social communication.

Clinical Trials

Three trials were included that used different (combinations of) bacteria as probiotic supplementation (Chou et al., 2010; Sari et al., 2012; Jacobs et al., 2017). Chou et al. (2010) supplemented preterm infants ($n = 301$ in total) with Infloran, containing *Lactobacillus acidophilus* and *Bifidobacterium infantis,* or placebo from 1 week after birth until discharge. Follow up at 3 years showed no difference in neurodevelopmental outcome between both groups. Jacobs et al. (2017) included and randomized patients ($n = 735$ in total) in receiving a combination of *Bifidobacterium infantis*, *Streptococcus thermophiles,* and *Bifidobacterium lactis* or placebo, from 72 h after birth until discharge or term equivalent age. Outcome was assessed

between 2 and 5 years of age and no difference was found between both groups. Sari et al. (2012) evaluated the effect of supplementation of all feedings until discharge with *Lactobacillus Sporogenus* on neurodevelopmental outcome at 18–22 months corrected age ($n = 174$ in total). Again, no effect of supplementation on neurodevelopmental outcome was observed.

In total, all three studies combined evaluated 1,210 patients. In none of the studies, a significant effect of supplementation with probiotics on neurodevelopmental outcome was found.

Prebiotic Supplementation

Pre-clinical Trials

No pre-clinical studies were found on prebiotic supplementation for the treatment of neonatal brain injury.

Clinical Trials

Two papers described neurodevelopmental outcome after supplementation with prebiotics (LeCouffe et al., 2014; van den Berg et al., 2016). Both papers assessed the same cohort, but at different time points. LeCouffe et al. (2014) evaluated outcome in the first year of life ($n = 93$ in total), whereas van den Berg et al. (2016) described outcome at 2 years corrected age ($n = 77$ in total). Intervention consisted of small-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS) and pectin-derived acidic oligosaccharides (pAOS) compared to controls from day 3 until day 30 post-natal. During the entire follow up period of 2 years, no significant difference was found in neurodevelopmental outcome between the patients with and without prebiotics supplementation.

Vitamin Intake

Pre-clinical Trials

The effects of vitamins on neonatal brain injury were described in two pre-clinical studies (Miura et al., 2009; Ramani et al., 2017). The first study investigated whether a combination of vitamin A and its derivate retinoic acid attenuated hyperoxia-induced preterm brain injury in mice (Ramani et al., 2017). Chronic hyperoxia from P2–14 (equivalent to 24–40 weeks gestational age in humans) induced deficits in spatial memory and reductions in hippocampal volume and hippocampal synaptophysins. Enteral treatment with vitamin A (0.05 mmol/g) and retinoic acid (0.005 mmol/g) every other day during the hyperoxic period partially restored brain injury and behavioral outcome in mice subjected to hyperoxia. The authors suggested that neuroprotection by vitamin A plus retinoic acid was mediated via increased signaling of the mTOR pathway.

In the second study, the effect of vitamin C (i.e., ascorbic acid) was tested in a rat model of neonatal HIE induced at P7 (Miura et al., 2009). Treatment with 750 mg/kg of vitamin C i.p. prior to hypoxia reduced macroscopical brain injury and apoptotic and necrotic cell death in the cortex, hippocampus, caudate putamen, and thalamus of rats with neonatal HIE, as compared to vehicle. Microscopical analysis of lesion size was not examined in this study.

Clinical Trials

Only one RCT evaluated the effect of vitamin supplementation on neurodevelopmental outcome. Salas et al. (2018) studied the impact of two different doses of vitamin D (200 or 600 IU/day) compared to placebo from day 1 of enteral feeding until day 28 post-natal on neurodevelopmental outcome at 2 years of age ($n = 91$ in total). They found no significant difference in neurodevelopmental impairment (including cerebral palsy), cognitive impairment and language impairment between patients in the placebo group, the low dose supplementation group or the high dose supplementation group.

Mineral Intake

Pre-clinical Trials

The effects of magnesium sulfate ($MgSO_4$) supplementation were described in two pre-clinical studies from the same group (Koning et al., 2017; Seyama et al., 2018), using a rat model of neonatal HIE induced at P6 or P7. The first study in P7 rats demonstrated that the optimal dose of 1.1 mg/g $MgSO_4$ i.p. effectively reduced lesion size when given as a pre-treatment between 6 days and 12 h prior to the insult, but not at later timepoints (Koning et al., 2017). In addition, mRNAs and miRNAs involved in metabolism and mitochondrial function were altered and mitochondrial respiration was preserved upon $MgSO_4$ supplementation of neonatal HIE rats. $MgSO_4$ pretreatment attenuated HI-induced increases in ROS production and neuroinflammation, as measured by cytokines and chemokine levels. The second study investigated $MgSO_4$ pretreatment in a neonatal HIE rat model induced at P6 (equivalent to 29 weeks gestational age in humans) (Seyama et al., 2018). HI at this age generates selective decrease in myelination in the pericallosal white matter without overt gray matter loss, which more closely resembles non-cystic PVL. Pretreatment with MgSO4, 30 min before the hypoxic insult, attenuated hypomyelination of the ipsilateral pericallosal white matter, as compared to vehicle pre-treatment. This increase in myelination was associated with increased oligodendrocyte numbers *in vivo* and reduced pre-oligodendrocyte death *in vitro*. Therefore, the authors suggested that MgSO4 ameliorated the white matter damage by preventing cell death of pre-oligodendrocytes.

Clinical Trials

Williams et al. (2017) investigated neurodevelopmental outcome after iodide supplementation. Preterm infants ($n = 1259$ in total) were randomly assigned to receive either placebo (30 μg/kg/day sodium chloride) or iodide supplementation (30 μg/kg/day sodium iodide) from <42 h after birth until 34 weeks gestational age. Neurodevelopmental outcome at 2 years of age was not significantly different in the groups.

DISCUSSION

With this review we provided an overview of the currently available evidence for the effect of nutritional interventions on neurodevelopmental outcome from clinical RCTs in preterm infants and pre-clinical models of neonatal brain injury. Some clinical nutritional intervention studies showed beneficial short

term effects, such as increased head circumference growth, but overall, there is little clinical evidence for long term positive effects of nutritional supplementation on neurodevelopmental outcome in preterm infants. Some clinical studies even showed adverse short term effects of increased nutritional intake. On the contrary, all the included pre-clinical studies found positive effects of nutritional supplementation on brain injury and/or neurodevelopment.

The most promising clinical results were seen in RCTs focusing on supplementation with glutamine, that showed improved head circumference growth during the first year of life and increased brain volumes at 8 years of age. However, at 13 years of age neurodevelopmental outcome was similar between glutamine-supplemented infants and controls. Early and/or high parenteral amino acid supplementation resulted in inconsistent outcome, with no effect on neurodevelopment, but both neutral and negative effects on growth and head circumference. Enteral protein supplementation increased head circumference growth at term equivalent age, but showed no effect on neurodevelopment. Overall lipid intake or increased DHA and AA intake (ratio 1/1) did not improve neurodevelopmental or brain injury outcome, but increased AA/DHA ratio (2/1) normalized neurodevelopmental outcome to the level of breast fed term controls. No positive or negative effect of supplementation with probiotics, prebiotics, vitamin D, and iodide were found in clinical trials. In pre-clinical models, less brain injury and/or improved behavioral outcome was seen after supplementation with lactoferrin, acetyl-l-carnitine, taurine, glycine, l-cysteine, DHA, butyrate, vitamins A and C, and magnesium sulfate.

The clinical part of our systematic review is in line with findings from previously published systematic reviews or meta-analyses regarding neurodevelopment following nutritional intervention. Both Chan et al. (2016) and Schneider and Garcia-Rodenas (2017) included studies with heterogeneous interventions in their systematic reviews and/or meta-analysis that showed conflicting results with regard to (individual) nutritional components (Chan et al., 2016; Schneider and Garcia-Rodenas, 2017). To our knowledge, this is the first systematic review of nutritional interventions in pre-clinical models of neonatal brain injury.

Also in term infants supplementation with different nutritional components, such as probiotics, prebiotics and fatty acids, has been tested. Bertelsen et al. reviewed the evidence regarding probiotic and prebiotic supplementation in relation to development of the gut microbiome (Bertelsen et al., 2016). In that review, brain injury or neurodevelopment was not assessed. A systematic Cochrane review by Jasani et al. summarized the evidence of supplementation with fatty acids and their effect on neurodevelopment (among other things) (Jasani et al., 2017). The authors conclude that there is no beneficial effect or harm of supplementation of fatty acids in relation to neurodevelopmental outcome. Another interesting study population that this review did not specifically focus on is the intra-uterine growth restricted (IUGR) infant. These infants are, for different reasons, already *in utero* deprived of a sufficient amount of nutritional components to thrive adequately. Therefore, IUGR infants might benefit extra from nutritional interventions post-partum. Since the population of IUGR infants is heterogeneous in GA, it was beyond the scope of this review to include studies on term IUGR infants specifically. However, since we selected RCTs based on GA or birth weight, IUGR preterm infants were included in our search. A systematic review specifically focused on nutritional interventions in IUGR infants would be of great relevance to address brain injury in this population.

An interesting observation in this review is the difference between clinical and pre-clinical study outcomes. All pre-clinical studies described positive outcomes after nutritional supplementation, while almost no clinical studies found a positive result. This could be due to several factors. First of all, long term neurodevelopmental outcome in clinical studies is much more dependent on environmental factors compared to a pre-clinical study, in which the environment of the animals is strictly regulated. Long term follow up in preterm infants is performed one or more years after the intervention. In this time frame, environmental factors such as socioeconomic status, which is an important predictor for later neurodevelopment (Linsell et al., 2015), and post-discharge nutritional intake, cannot (or hardly) be regulated. It is therefore more difficult to detect an effect of a clinical intervention on long term outcome, especially when the intervention is given for a short period of time (usually during a few days or weeks after birth). Secondly, similar to the heterogenic environment, the population of preterm infants is much more heterogenic than a controlled animal population. Preterm infants could be suffering from all kinds of short and long term complications, such as sepsis, intraventricular hemorrhage, pulmonary or gut problems or they can have a relatively uneventful course. These complications can have a major impact on later development. For instance, bronchopulmonary dysplasia is an important risk factor for adverse neurodevelopment and academic performance (Twilhaar et al., 2018b,c). Furthermore, preterm infants can have different types of WMI, such as diffuse, cystic or hemorrhagic injury, which can in turn affect neurodevelopment differently. Additionally, nutritional intake of preterm infants can also be heterogenic, even in a controlled clinical trial. Preterm infants are often fed breast milk, but the content of preterm breast milk varies substantially, depending for example on maternal diet or post-natal day (Boyce et al., 2016; Mimouni et al., 2017). The quantity of nutrients in breast milk is often not measured nor taken into account in clinical nutritional intervention trials, which may lead to a clinically relevant difference in intake, effecting study outcome. Heterogeneity might be an important factor when interpreting the results of clinical studies and could also (partly) explain why there is a difference between clinical and pre-clinical results.

Thirdly, the main outcome focus of pre-clinical studies was brain injury. This is in contrast with clinical studies, in which neurodevelopment was the main outcome measure and only a few studies focused on brain injury parameters (for example brain volumes or white matter tract development). It is possible that clinical interventions demonstrate the same effects on brain development as pre-clinical interventions, but that this

does not translate into improved neurodevelopmental outcome. Furthermore, most pre-clinical studies were conducted in models for neonatal HIE as observed in the term born infant. The timing of the insults is crucial for type of neurological outcome. Most of the included studies in this review subjected rodents to hypoxic-ischemic brain injury at P7. At this moment in rodent brain development, the white matter is in a state of maturation similar to human white matter between 30 and 36 weeks GA (Semple et al., 2013; Salmaso et al., 2014), meaning that oligodendrocytes have mostly matured and the stage at which EoP can be induced has already passed. Therefore, hypoxia-ischemia at P7 induces mainly neuronal death, as seen in perinatal asphyxia and neonatal stroke, but not an arrest of oligodendrocyte maturation, as is now commonly thought to be the major contributor to the development of EoP. In addition to timing, also inclusion of an inflammatory component is thought to be key to the development of translational animal models of EoP. In recent years, rodent models of EoP have been developed (Zeng et al., 2018), including several multiple hit models that combined fetal inflammation with post-natal hypoxia (van Tilborg et al., 2018a) or multiple post-natal injections of cytokines (Rangon et al., 2018). Also larger animal models of EoP have been developed, mostly using preterm lambs (Li et al., 2016; Wassink et al., 2017; Gussenhoven et al., 2018), piglets (Andersen et al., 2016; Buddington et al., 2018), and baboons (Griffith et al., 2012). Apparently, nutritional intervention studies have so far been scarcely conducted in pre-clinical models for EoP, as we included only four studies in rodent models of preterm EoP (van de Looij et al., 2014; Ginet et al., 2016; Ramani et al., 2017; Seyama et al., 2018) and one study in late preterm piglets (Buddington et al., 2018). Therefore, to move forward with the development of nutritional therapies for preterm infants, there is an urgent need for the use of these clinically relevant pre-clinical EoP models, which can hopefully improve the implementation of pre-clinical nutritional therapies in clinical practice in future.

From a statistical point of view, the absence of beneficial effects in clinical studies could also be due to the fact that almost none of the RCTs was powered to detect differences in neurodevelopmental outcome or brain injury. In these studies, long term effect of supplementation was a secondary or explorative outcome. It is therefore possible that effects of supplementation were present, but could not be detected. This is in contrast with the pre-clinical studies, in which brain injury and/or development was the primary study outcome. Furthermore, it is possible that the lack of neutral or negative results in the pre-clinical studies is due to publication bias.

Finally, the sensitivity of the clinical outcome measures could also be an issue in failure to detect positive effects. Because of their risk of adverse neurodevelopmental outcome, preterm infants are usually regularly seen for follow up in the clinic. One of the main issues in interpretation of the results of this systematic review is the value of this neurodevelopmental follow up. It remains difficult to predict neurodevelopmental outcome in extremely preterm children, even with the use of standardized outcome scales. The most commonly used outcome scale nowadays is the Bayley Scales of Infant and Toddler Development (BSITD), which has a first, second and third edition and consists, among other things, of a mental and motor scale. The BSITD is used to predict development at a later stage of life. However, a systematic review and meta-analysis by Luttikhuizen dos Santos et al. (2013) showed that the predictive value of the BSITD is limited (Luttikhuizen dos Santos et al., 2013). The mental score explains 37% of later cognitive functioning, while the motor score predicts only 12% of later motor functioning. Even though these percentages are low, they are used in clinical practice as well as in evaluating outcome of clinical trials. To get real insight into the effects of an early intervention on later functioning, longer follow up, perhaps even into adulthood, is needed. Furthermore, the goal of follow up is to monitor development and intervene when necessary. When delay in development is present or imminent, action is taken, such as physiotherapy or school support, to optimize the infant's change of normal development. This can influence the results of clinical trials evaluating outcome. This is in contrast with pre-clinical studies, when the natural course of outcome of the animals is followed, which will give a more reliable understanding of the effect of the intervention.

Our systematic review has some limitations. First of all, only studies that were published no longer than 10 years ago at the time of search were included and therefore older studies providing evidence regarding nutritional interventions have been missed. Considering the change in clinical pathophysiology of preterm brain injury from cystic periventricular leukomalacia to more diffuse forms of WMI in the past decades and the continuously improving clinical care, we focused on studies of the last 10 years, to have a relevant study population for the currently observed pathophysiology of EoP in preterm infants. Secondly, our review focused on brain injury and neurodevelopmental outcome after a nutritional intervention, but in some studies body and/or head circumference growth were also mentioned as an outcome measure. Especially head circumference growth is associated with neurodevelopmental outcome (Peterson et al., 2006; Franz et al., 2009). We therefore also mention the results of body and head circumference growth in this review. However, we have to take into account that we did not use growth or head circumference in the search strategy. Studies that solely focused on growth and not mentioned neurodevelopmental outcome can be missed with our search and the results on growth should therefore be interpreted with caution. Thirdly, as nutritional intervention studies have hardly been conducted in models for EoP, we included pre-clinical intervention studies done in all models for neonatal brain injury induced by hypoxia, ischemia, hyperoxia, and/or inflammation. Neonatal HIE is primarily characterized by gray matter injury, while in most types of EoP the white matter is mainly affected. This is an important difference in pathology that should be taken into account when testing nutritional interventions. Although the models also have similarities in pathophysiology on which nutrition has been shown to have effects, such as neuroinflammation and axonal injury, the interpretation of the pre-clinical data observed in neonatal models for HIE should be interpreted with caution. Finally,

only studies that evaluated a single nutritional component were included. We thereby excluded multiple studies that looked at combined interventions and that could have given meaningful results. We chose this approach to be able to interpret our results directly related to the single nutrient that was supplemented.

CONCLUSION

Even though many pre-clinical trials show beneficial effects of different nutritional interventions on brain injury and/or neurodevelopmental outcome, these positive effects have so far not clearly been demonstrated in RCTs. To move novel nutritional therapies for encephalopathy of prematurity from the bench to the bedside of preterm infants, there is an urgent need to investigate nutritional therapies for preterm infants in more translational and clinically relevant animal models. This review emphasizes the need for more consistent long term follow-up of preterm infants included in clinical trials and the development of better methods to evaluate neurodevelopmental outcome of this vulnerable population.

AUTHOR CONTRIBUTIONS

LH and CdT performed the searches, screened and selected papers, drafted the initial manuscript, and revised the manuscript. RvE supervised article selection and reviewed the manuscript critically for intellectual content. CN and MB critically reviewed the manuscript for intellectual content. All authors approved the final version of the manuscript and take full responsibility for the content.

FUNDING

This study was part of the "Utrecht Center for Food and Health—research program Specialized Nutrition," funded by the Dutch Ministry of Economic Affairs, Utrecht Province, and the municipality of Utrecht.

REFERENCES

Almaas, A. N., Tamnes, C. K., Nakstad, B., Henriksen, C., Grydeland, H., Walhovd, K. B., et al. (2016). Diffusion tensor imaging and behavior in premature infants at 8 years of age, a randomized controlled trial with long-chain polyunsaturated fatty acids. *Early Hum. Dev.* 95, 41–46. doi: 10.1016/j.earlhumdev.2016.01.021

Almaas, A. N., Tamnes, C. K., Nakstad, B., Henriksen, C., Walhovd, K. B., Fjell, A. M., et al. (2015). Long-chain polyunsaturated fatty acids and cognition in VLBW infants at 8 years: an RCT. *Pediatrics* 135, 972–980. doi: 10.1542/peds.2014-4094

Alshweki, A., Muñuzuri, A. P., Baña, A. M., de Castro, M. J., Andrade, F., Aldamiz-Echevarría, L., et al. (2015). Effects of different arachidonic acid supplementation on psychomotor development in very preterm infants; a randomized controlled trial. *Nutr. J.* 14:101. doi: 10.1186/s12937-015-0091-3

American Academy of Pediatrics (2009). "Committee on nutrition: nutritional needs of the preterm infant," in *Pediatric Nutrition Handbook, 6th Edn.* ed R. Kleinman (Elk Grove Village, IL: American Academy of Pediatrics), 83.

Amin, H. J., Soraisham, A. S., and Sauve, R. S. (2009). Neurodevelopmental outcomes of premature infants treated with l-arginine for prevention of necrotising enterocolitis. *J. Paediatr. Child. Health* 45, 219–223. doi: 10.1111/j.1440-1754.2008.01458.x

Andersen, A. D., Sangild, P. T., Munch, S. L., van der Beek, E. M., Renes, I. B., Ginneken, C. v., et al. (2016). Delayed growth, motor function and learning in preterm pigs during early postnatal life. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 310, R481–R492. doi: 10.1152/ajpregu.00349.2015

Arteaga, O., Revuelta, M., Urigüen, L., Martínez-Millán, L., Hilario, E., and Álvarez, A. (2017). Docosahexaenoic acid reduces cerebral damage and ameliorates long-term cognitive impairments caused by neonatal hypoxia-ischemia in rats. *Mol. Neurobiol.* 54, 7137–7155. doi: 10.1007/s12035-016-0221-8

Balakrishnan, M., Jennings, A., Przystac, L., Phornphutkul, C., Tucker, R., Vohr, B., et al. (2017). Growth and neurodevelopmental outcomes of early, high-dose parenteral amino acid intake in very low birth weight infants: a randomized controlled trial. *JPEN J. Parenter. Enteral. Nutr.* 42, 597–606. doi: 10.1177/0148607117696330

Bellagamba, M. P., Carmenati, E., D'Ascenzo, R., Malatesta, M., Spagnoli, C., Biagetti, C., et al. (2016). One extra gram of protein to preterm infants from birth to 1800 g: a single-blinded randomized clinical trial. *J. Pediatr. Gastroenterol. Nutr.* 62, 879–884. doi: 10.1097/MPG.0000000000000989

Berman, D. R., Liu, Y. Q., Barks, J., and Mozurkewich, E. (2010). Docosahexaenoic acid confers neuroprotection in a rat model of perinatal hypoxia-ischemia potentiated by *Escherichia coli* lipopolysaccharide-induced systemic inflammation. *Am. J. Obstet. Gynecol.* 202, 469 e461–466. doi: 10.1016/j.ajog.2010.01.076

Berman, D. R., Mozurkewich, E., Liu, Y., and Barks, J. (2009). Docosahexaenoic acid pretreatment confers neuroprotection in a rat model of perinatal cerebral hypoxia-ischemia. *Am. J. Obstet. Gynecol.* 200, 305.e1–e6. doi: 10.1016/j.ajog.2009.01.020

Berman, D. R., Mozurkewich, E., Liu, Y., Shangguan, Y., Barks, J. D., and Silverstein, F. S. (2013). Docosahexaenoic acid augments hypothermic neuroprotection in a neonatal rat asphyxia model. *Neonatology* 104, 71–78. doi: 10.1159/000351011

Bertelsen, R. J., Jensen, E. T., and Ringel-Kulka, T. (2016). Use of probiotics and prebiotics in infant feeding. *Best Pract. Res. Clin. Gastroenterol.* 30, 39–48. doi: 10.1016/j.bpg.2016.01.001

Blanco, C. L., Gong, A. K., Schoolfield, J., Green, B. K., Daniels, W., Liechty, E. A., et al. (2012). Impact of early and high amino acid supplementation on ELBW infants at 2 years. *J. Pediatr. Gastroenterol. Nutr.* 54, 601–607. doi: 10.1097/MPG.0b013e31824887a0

Bouyssi-Kobar, M., du Plessis, A. J., McCarter, R., Brossard-Racine, M., Murnick, J., Tinkleman, L., et al. (2016). Third trimester brain growth in preterm infants compared with in utero healthy fetuses. *Pediatrics* 138:e20161640. doi: 10.1542/peds.2016-1640

Boyce, C., Watson, M., Lazidis, G., Reeve, S., Dods, K., Simmer, K., et al. (2016). Preterm human milk composition: a systematic literature review. *Br. J. Nutr.* 116, 1033–1045. doi: 10.1017/S0007114516003007

Brown, J. V., Embleton, N. D., Harding, J. E., and McGuire, W. (2016). Multi-nutrient fortification of human milk for preterm infants. *Cochrane Database Syst. Rev.* 8:CD000343. doi: 10.1002/14651858.CD000343.pub3

Buddington, R. K., Chizhikov, V. V., Iskusnykh, I. Y., Sable, H. J., Sable, J. J., Holloway, Z. R., et al. (2018). A phosphatidylserine source of docosahexanoic acid improves neurodevelopment and survival of preterm pigs. *Nutrients* 10:637. doi: 10.3390/nu10050637

Burattini, I., Bellagamba, M. P., Spagnoli, C., D'Ascenzo, R., Mazzoni, N., Peretti, A., et al. (2013). Targeting 2.5 versus 4 g/kg/day of amino acids for extremely low birth weight infants: a randomized clinical trial. *J. Pediatr.* 163, 1278–1282 e1271. doi: 10.1016/j.jpeds.2013.06.075

Chan, S. H., Johnson, M. J., Leaf, A. A., and Vollmer, B. (2016). Nutrition and neurodevelopmental outcomes in preterm infants: a systematic review. *Acta Paediatr.* 105, 587–599. doi: 10.1111/apa.13344

Chou, I. C., Kuo, H. T., Chang, J. S., Wu, S. F., Chiu, H. Y., Su, B. H., et al. (2010). Lack of effects of oral probiotics on growth and neurodevelopmental outcomes in preterm very low birth weight infants. *J. Pediatr.* 156, 393–396. doi: 10.1016/j.jpeds.2009.09.051

Coviello, C., Keunen, K., Kersbergen, K. J., Groenendaal, F., Leemans, A., Peels, B., et al. (2018). Effects of early nutrition and growth on brain volumes, white matter microstructure, and neurodevelopmental outcome in preterm newborns. *Pediatr. Res.* 83, 102–110. doi: 10.1038/pr.2017.227

Dabydeen, L., Thomas, J. E., Aston, T. J., Hartley, H., Sinha, S. K., and Eyre, J. A. (2008). High-energy and -protein diet increases brain and corticospinal tract growth in term and preterm infants after perinatal brain injury. *Pediatrics* 121, 148–156. doi: 10.1542/peds.2007-1267

de Kieviet, J. F., Oosterlaan, J., Vermeulen, R. J., Pouwels, P. J., Lafeber, H. N., and van Elburg, R. M. (2012). Effects of glutamine on brain development in very preterm children at school age. *Pediatrics* 130, e1121–e1127. doi: 10.1542/peds.2012-0928

de Kieviet, J. F., Vuijk, P. J., van den Berg, A., Lafeber, H. N., Oosterlaan, J., and van Elburg, R. M. (2014). Glutamine effects on brain growth in very preterm children in the first year of life. *Clin. Nutr.* 33, 69–74. doi: 10.1016/j.clnu.2013.03.019

Dogra, S., Thakur, A., Garg, P., and Kler, N. (2017). Effect of differential enteral protein on growth and neurodevelopment in infants <1500 g: a randomized controlled trial. *J. Pediatr. Gastroenterol. Nutr.* 64, e126–e132. doi: 10.1097/MPG.0000000000001451

Euro-Peristat Project with SCPE and EUROCAT (2013). *European Perinatal Health Report. The Health and Care of Pregnant Women and Babies in Europe in 2010*. Available online at: www.europeristat.com (accessed July 18, 2019).

Franz, A. R., Pohlandt, F., Bode, H., Mihatsch, W. A., Sander, S., Kron, M., et al. (2009). Intrauterine, early neonatal, and postdischarge growth and neurodevelopmental outcome at 5.4 years in extremely preterm infants after intensive neonatal nutritional support. *Pediatrics* 123, e101–e109. doi: 10.1542/peds.2008-1352

Georgieff, M. K. (2007). Nutrition and the developing brain: nutrient priorities and measurement. *Am. J. Clin. Nutr.* 85, 614S—620S. doi: 10.1093/ajcn/85.2.614S

Ginet, V., van de Looij, Y., Petrenko, V., Toulotte, A., Kiss, J., Hüppi, P. S., et al. (2016). Lactoferrin during lactation reduces lipopolysaccharide-induced brain injury. *Biofactors* 42, 323–336. doi: 10.1002/biof.1278

Glass, H. C., Costarino, A. T., Stayer, S. A., Brett, C. M., Cladis, F., and Davis, P. J (2015). Outcomes for extremely premature infants. *Anesth Analg.* 120, 1337–1351. doi: 10.1213/ANE.0000000000000705

Griffith, J. L., Shimony, J. S., Cousins, S. A., Rees, S. E., McCurnin, D. C., Inder, T. E., et al. (2012). MR imaging correlates of white-matter pathology in a preterm baboon model. *Pediatr. Res.* 71, 185–191. doi: 10.1038/pr.2011.33

Gussenhoven, R., Westerlaken, R. J. J., Ophelders, D. R. M. G., Jobe, A. H., Kemp, M. W., Kallapur, S. G., et al. (2018). Chorioamnionitis, neuroinflammation, and injury: timing is key in the preterm ovine fetus. *J. Neuroinflamm.* 15:113. doi: 10.1186/s12974-018-1149-x

Higgins, J. P. T., Altman, D. G., and Sterne, J. A. C. (2011). "Chapter 8: assessing risk of bias in included studies," in *Cochrane Handbook for Systematic Reviews of Interventions,* eds J. P. T. Higgins and S. Green (The Cochrane Collaboration). Available online at: www.handbook.cochrane.org

Hooijmans, C. R., Rovers, M. M., de Vries, R. B., Leenaars, M., Ritskes-Hoitinga, M., and Langendam, M. W. (2014). SYRCLE's risk of bias tool for animal studies. *BMC Med. Res. Methodol.* 14:43. doi: 10.1186/1471-2288-14-43

Huun, M. U., Garberg, H. T., Buonocore, G., Longini, M., Belvisi, E., Bazzini, F., et al. (2018). Regional differences of hypothermia on oxidative stress following hypoxia-ischemia: a study of DHA and hypothermia on brain lipid peroxidation in newborn piglets. *J. Perinat. Med.* 47, 82–89. doi: 10.1515/jpm-2017-0355

Jacobs, S. E., Hickey, L., Donath, S., Opie, G. F., Anderson, P. J., Garland, S. M., et al. (2017). Probiotics, prematurity and neurodevelopment: follow-up of a randomised trial. *BMJ Paediatr. Open* 1:e000176. doi: 10.1136/bmjpo-2017-000176

Jasani, B., Simmer, K., Patole, S. K., and Rao, S. C. (2017). Long chain polyunsaturated fatty acid supplementation in infants born at term. *Cochrane Database Syst. Rev.* 3:CD000376. doi: 10.1002/14651858.CD000376.pub4

Jaworska, J., Ziemka-Nalecz, M., Sypecka, J., and Zalewska, T. (2017). The potential neuroprotective role of a histone deacetylase inhibitor, sodium butyrate, after neonatal hypoxia-ischemia. *J. Neuroinflamm.* 14:34. doi: 10.1186/s12974-017-0807-8

Kersbergen, K. J., Makropoulos, A., Aljabar, P., Groenendaal, F., de Vries, L. S., Counsell, S. J., et al. (2016). Longitudinal regional brain development and clinical risk factors in extremely preterm infants. *J Pediatr.* 178, 93–100.e6. doi: 10.1016/j.jpeds.2016.08.024

Koning, G., Leverin, A. L., Nair, S., Schwendimann, L., Ek, J., Carlsson, Y., et al. (2017). Magnesium induces preconditioning of the neonatal brain via profound mitochondrial protection. *J. Cereb. Blood Flow Metab.* 39, 1038–1055. doi: 10.1177/0271678X17746132

LeCouffe, N. E., Westerbeek, E. A., van Schie, P. E., Schaaf, V. A., Lafeber, H. N., and van Elburg, R. M. (2014). Neurodevelopmental outcome during the first year of life in preterm infants after supplementation of a prebiotic mixture in the neonatal period: a follow-up study. *Neuropediatrics* 45, 22–29. doi: 10.1055/s-0033-1349227

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., et al. (2016). Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells. *Exp. Neurol.* 283(Pt A), 179–187. doi: 10.1016/j.expneurol.2016.06.017

Linsell, L., Malouf, R., Morris, J., Kurinczuk, J. J., and Marlow, N. (2015). Prognostic factors for poor cognitive development in children born very preterm or with very low birth weight: a systematic review. *JAMA Pediatr.* 169, 1162–1172. doi: 10.1001/jamapediatrics.2015.2175

Liu, L., Johnson, H. L., Cousens, S., Perin, J., Scott, S., Lawn, J. E., et al. (2012). Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet* 379, 2151–2161. doi: 10.1016/S0140-6736(12)60560-1

Liu, S., Xin, D., Wang, L., Zhang, T., Bai, X., Li, T., et al. (2017). Therapeutic effects of L-Cysteine in newborn mice subjected to hypoxia-ischemia brain injury via the CBS/H2S system: role of oxidative stress and endoplasmic reticulum stress. *Redox Biol.* 13, 528–540. doi: 10.1016/j.redox.2017.06.007

Luttikhuizen dos Santos, E. S., de Kieviet, J. F., Königs, M., van Elburg, R. M., and Oosterlaan, J. (2013). Predictive value of the Bayley scales of infant development on development of very preterm/very low birth weight children: a meta-analysis. *Early Hum. Dev.* 89, 487–496. doi: 10.1016/j.earlhumdev.2013.03.008

Mayurasakorn, K., Niatsetskaya, Z. V., Sosunov, S. A., Williams, J. J., Zirpoli, H., Vlasakov, I., et al. (2016). DHA but not EPA emulsions preserve neurological and mitochondrial function after brain hypoxia-ischemia in neonatal mice. *PLoS ONE* 11:e0160870. doi: 10.1371/journal.pone.0160870

Mimouni, F. B., Lubetzky, R., Yochpaz, S., and Mandel, D. (2017). Preterm human milk macronutrient and energy composition: a systematic review and meta-analysis. *Clin. Perinatol.* 44, 165–172. doi: 10.1016/j.clp.2016.11.010

Miura, S., Ishida-Nakajima, W., Ishida, A., Kawamura, M., Ohmura, A., Oguma, R., et al. (2009). Ascorbic acid protects the newborn rat brain from hypoxic-ischemia. *Brain Dev.* 31, 307–317. doi: 10.1016/j.braindev.2008.06.010

Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G., and Group, P. (2009). Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* 6:e1000097. doi: 10.1371/journal.pmed.1000097

Mori, H., Momosaki, K., Kido, J., Naramura, T., Tanaka, K., Matsumoto, S., et al. (2017). Amelioration by glycine of brain damage in neonatal rat brain following hypoxia-ischemia. *Pediatr. In.* 59, 321–327. doi: 10.1111/ped.13164

Nagasawa, T., Kiyosawa, I., and Kuwahara, K. (1972). Amounts of lactoferrin in human colostrum and milk. *J. Dairy Sci.* 55, 1651–1659. doi: 10.3168/jds.S0022-0302(72)85741-2

Oliveira, F. L., Rumsey, S. C., Schlotzer, E., Hansen, I., Carpentier, Y. A., and Deckelbaum, R. J. (1997). Triglyceride hydrolysis of soy oil vs fish oil emulsions. *JPEN J. Parenter. Enteral. Nutr.* 21, 224–229. doi: 10.1177/014860719702100422

Ong, M. L., Purdy, I. B., Levit, O. L., Robinson, D. T., Grogan, T., Flores, M., et al. (2018). Two-year neurodevelopment and growth outcomes for preterm neonates who received low-dose intravenous soybean oil. *JPEN J. Parenter. Enteral. Nutr.* 42, 352–360. doi: 10.1177/0148607116674482

Peterson, J., Taylor, H. G., Minich, N., Klein, N., and Hack, M. (2006). Subnormal head circumference in very low birth weight children: neonatal correlates and school-age consequences. *Early Hum. Dev.* 82, 325–334. doi: 10.1016/j.earlhumdev.2005.09.014

Ramani, M., van Groen, T., Kadish, I., Ambalavanan, N., and McMahon, L. L. (2017). Vitamin, A., and retinoic acid combination attenuates neonatal hyperoxia-induced neurobehavioral impairment in adult mice. *Neurobiol. Learn. Mem.* 141, 209–216. doi: 10.1016/j.nlm.2017.04.013

Rangon, C. M., Schang, A. L., Van Steenwinckel, J., Schwendimann, L., Lebon, S., Fu, T., et al. (2018). Myelination induction by a histamine H3 receptor antagonist in a mouse model of preterm white matter injury. *Brain Behav. Immun.* 74, 265–276. doi: 10.1016/j.bbi.2018.09.017

Rassin, D. K. (1981). The function of taurine in the central nervous system. *Adv. Biochem. Psychopharmacol.* 29, 127–134.

Review Manager (RevMan) [computer program] (2014). *Version 5.3*. Copgenhagen: The Nordic Cochrane Centre The Cochrane Collaboration.

Revuelta, M., Arteaga, O., Montalvo, H., Alvarez, A., Hilario, E., and Martinez-Ibargüen, A. (2016). Antioxidant treatments recover the alteration of auditory-evoked potentials and reduce morphological damage in the inferior colliculus after perinatal asphyxia in rat. *Brain Pathol.* 26, 186–198. doi: 10.1111/bpa.12272

Salas, A. A., Woodfin, T., Phillips, V., Peralta-Carcelen, M., Carlo, W. A., and Ambalavanan, N. (2018). Dose-response effects of early vitamin D supplementation on neurodevelopmental and respiratory outcomes of extremely preterm infants at 2 years of age: a randomized trial. *Neonatology* 113, 256–262. doi: 10.1159/000484399

Salmaso, N., Jablonska, B., Scafidi, J., Vaccarino, F. M., and Gallo, V. (2014). Neurobiology of premature brain injury. *Nat. Neurosci.* 17, 341–346. doi: 10.1038/nn.3604

Sari, F. N., Eras, Z., Dizdar, E. A., Erdeve, O., Oguz, S. S., Uras, N., et al. (2012). Do oral probiotics affect growth and neurodevelopmental outcomes in very low-birth-weight preterm infants? *Am. J. Perinatol.* 29, 579–586. doi: 10.1055/s-0032-1311981

Schneider, J., Fischer Fumeaux, C. J., Duerden, E. G., Guo, T., Foong, J., Graz, M. B., et al. (2018). Nutrient intake in the first two weeks of life and brain growth in preterm neonates. *Pediatrics* 141:e20172169. doi: 10.1542/peds.2017-2169

Schneider, N., and Garcia-Rodenas, C. L. (2017). Early nutritional interventions for brain and cognitive development in preterm infants: a review of the literature. *Nutrients* 9:E187. doi: 10.3390/nu9030187

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., and Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. *Prog. Neurobiol.* 106–107, 1–16. doi: 10.1016/j.pneurobio.2013.04.001

Seyama, T., Kamei, Y., Iriyama, T., Imada, S., Ichinose, M., Toshimitsu, M., et al. (2018). Pretreatment with magnesium sulfate attenuates white matter damage by preventing cell death of developing oligodendrocytes. *J. Obstet. Gynaecol. Res.* 44, 601–607. doi: 10.1111/jog.13568

Singh, A. K., Yoshida, Y., Garvin, A. J., and Singh, I. (1989). Effect of fatty acids and their derivatives on mitochondrial structures. *J. Exp. Pathol.* 4, 9–15.

Solberg, R., Longini, M., Proietti, F., Perrone, S., Felici, C., Porta, A., et al. (2017). DHA reduces oxidative stress after perinatal asphyxia: a study in newborn piglets. *Neonatology* 112, 1–8. doi: 10.1159/000454982

Stephens, B. E., Walden, R. V., Gargus, R. A., Tucker, R., McKinley, L., Mance, M., et al. (2009). First-week protein and energy intakes are associated with 18-month developmental outcomes in extremely low birth weight infants. *Pediatrics* 123, 1337–1343. doi: 10.1542/peds.2008-0211

Tanaka, K., Hosozawa, M., Kudo, N., Yoshikawa, N., Hisata, K., Shoji, H., et al. (2013). The pilot study: sphingomyelin-fortified milk has a positive association with the neurobehavioural development of very low birth weight infants during infancy, randomized control trial. *Brain Dev.* 35, 45–52. doi: 10.1016/j.braindev.2012.03.004

Tang, S., Xu, S., Lu, X., Gullapalli, R. P., McKenna, M. C., and Waddell, J. (2016). Neuroprotective effects of Acetyl-L-Carnitine on neonatal hypoxia ischemia-induced brain injury in rats. *Dev. Neurosci.* 38, 384–396. doi: 10.1159/000455041

Twilhaar, E. S., de Kieviet, J. F., Aarnoudse-Moens, C. S., van Elburg, R. M., and Oosterlaan, J. (2018b). Academic performance of children born preterm: a meta-analysis and meta-regression. *Arch. Dis. Child. Fetal. Neonatal. Ed.* 103, F322–F330. doi: 10.1136/archdischild-2017-312916

Twilhaar, E. S., de Kieviet, J. F., Oosterlaan, J., and van Elburg, R. M. (2018a). A randomised trial of enteral glutamine supplementation for very preterm children showed no beneficial or adverse long-term neurodevelopmental outcomes. *Acta Paediatr.* 107, 593–599. doi: 10.1111/apa.14167

Twilhaar, E. S., Wade, R. M., de Kieviet, J. F., van Goudoever, J. B., van Elburg, R. M., and Oosterlaan, J. (2018c). Cognitive outcomes of children born extremely or very preterm since the 1990s and associated risk factors: a meta-analysis and meta-regression. *JAMA Pediatr.* 172, 361–367. doi: 10.1001/jamapediatrics.2017.5323

Vaisman, N., and Pelled, D. (2009). n-3 phosphatidylserine attenuated scopolamine-induced amnesia in middle-aged rats. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33, 952–959. doi: 10.1016/j.pnpbp.2009.04.021

van de Looij, Y., Ginet, V., Chatagner, A., Toulotte, A., Somm, E., Hüppi, P. S., et al. (2014). Lactoferrin during lactation protects the immature hypoxic-ischemic rat brain. *Ann. Clin. Transl. Neurol.* 1, 955–967. doi: 10.1002/acn3.138

van den Akker, C. H., te Braake, F. W., Weisglas-Kuperus, N., and van Goudoever, J. B. (2014). Observational outcome results following a randomized controlled trial of early amino acid administration in preterm infants. *J. Pediatr. Gastroenterol. Nutr.* 59, 714–719. doi: 10.1097/MPG.0000000000000549

van Tilborg, E., Achterberg, E. J. M., van Kammen, C. M., van der Toorn, A., Groenendaal, F., Dijkhuizen, R. M., et al. (2018a). Combined fetal inflammation and postnatal hypoxia causes myelin deficits and autism-like behavior in a rat model of diffuse white matter injury. *Glia* 66, 78–93. doi: 10.1002/glia.23216

van Tilborg, E., de Theije, C. G. M., van Hal, M., Wagenaar, N., de Vries, L. S., Benders, M. J., et al. (2018b). Origin and dynamics of oligodendrocytes in the developing brain: implications for perinatal white matter injury. *Glia* 66, 221–238. doi: 10.1002/glia.23256

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., et al. (2016). Impaired oligodendrocyte maturation in preterm infants: potential therapeutic targets. *Prog. Neurobiol.* 136, 28–49. doi: 10.1016/j.pneurobio.2015.11.002

van den Berg, J. P., Westerbeek, E. A., Bröring-Starre, T., Garssen, J., and van Elburg, R. M. (2016). Neurodevelopment of preterm infants at 24 months after neonatal supplementation of a prebiotic mix: a randomized trial. *J. Pediatr. Gastroenterol. Nutr.* 63, 270–276. doi: 10.1097/MPG.0000000000001148

Wassink, G., Davidson, J. O., Dhillon, S. K., Fraser, M., Galinsky, R., Bennet, L., et al. (2017). Partial white and grey matter protection with prolonged infusion of recombinant human erythropoietin after asphyxia in preterm fetal sheep. *J. Cereb. Blood Flow Metab.* 37, 1080–1094. doi: 10.1177/0271678X16650455

Williams, F. L. R., Ogston, S., Hume, R., Watson, J., Stanbury, K., Willatts, P., et al. (2017). Supplemental iodide for preterm infants and developmental outcomes at 2 years: an, R. C. T. *Pediatrics* 139:e20163703. doi: 10.1542/peds.2016-3703

Williams, J. J., Mayurasakorn, K., Vannucci, S. J., Mastropietro, C., Bazan, N. G., Ten, V. S., et al. (2013). N-3 fatty acid rich triglyceride emulsions are neuroprotective after cerebral hypoxic-ischemic injury in neonatal mice. *PLoS ONE* 8:e56233. doi: 10.1371/journal.pone.0056233

World Health Organization (2015). *WHO Recommendations on Interventions to Improve Preterm Birth Outcomes.* Geneva: World Health Organization.

Wu, J. Y., and Prentice, H. (2010). Role of taurine in the central nervous system. *J Biomed Sci.* 17 Suppl 1:S1. doi: 10.1186/1423-0127-17-S1-S1

Xin, D., Chu, X., Bai, X., Ma, W., Yuan, H., Qiu, J., et al. (2018). l-Cysteine suppresses hypoxia-ischemia injury in neonatal mice by reducing glial activation, promoting autophagic flux and mediating synaptic modification via H2S formation. *Brain Behav. Immun.* 73, 222–234. doi: 10.1016/j.bbi.2018.05.007

Xu, S., Waddell, J., Zhu, W., Shi, D., Marshall, A. D., McKenna, M. C., et al. (2015). In vivo longitudinal proton magnetic resonance spectroscopy on neonatal hypoxic-ischemic rat brain injury: Neuroprotective effects of acetyl-L-carnitine. *Magn. Reson. Med.* 74, 1530–1542. doi: 10.1002/mrm.25537

Zeng, Y., Wang, H., Zhang, L., Tang, J., Shi, J., Xiao, D., et al. (2018). The optimal choices of animal models of white matter injury. *Rev. Neurosci.* 30, 245–259. doi: 10.1515/revneuro-2018-0044

Zhu, X. Y., Ma, P. S., Wu, W., Zhou, R., Hao, Y. J., Niu, Y., et al. (2016). Neuroprotective actions of taurine on hypoxic-ischemic brain damage in neonatal rats. *Brain Res. Bull.* 124, 295–305. doi: 10.1016/j.brainresbull.2016.06.010

Ziemka-Nalecz, M., Jaworska, J., Sypecka, J., Polowy, R., Filipkowski, R. K., and Zalewska, T. (2017). Sodium butyrate, a histone deacetylase inhibitor, exhibits neuroprotective/neurogenic effects in a rat model of neonatal hypoxia-ischemia. *Mo. Neurobiol.* 54, 5300–5318. doi: 10.1007/s12035-016-0049-2

Conflict of Interest Statement: RvE is employed by Danone, Nutricia Research.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Hortensius, van Elburg, Nijboer, Benders and de Theije. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 30 July 2019
doi: 10.3389/fphys.2019.00955

Interneuron Development Is Disrupted in Preterm Brains With Diffuse White Matter Injury: Observations in Mouse and Human

Helen B. Stolp[1,2], Bobbi Fleiss[2,3,4], Yoko Arai[3†], Veena Supramaniam[2], Regina Vontell[2,5], Sebastian Birtles[2], Abi G. Yates[2,6], Ana A. Baburamani[2], Claire Thornton[1,2], Mary Rutherford[2], A. David Edwards[2] and Pierre Gressens[2,3]*

[1]Department for Comparative Biomedical Sciences, Royal Veterinary College, London, United Kingdom, [2]Department of Perinatal Imaging & Health, Centre for the Developing Brain, School of Biomedical Engineering and Imaging Science, King's College London, London, United Kingdom, [3]Université de Paris, NeuroDiderot, Inserm, Paris, France, [4]School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC, Australia, [5]Department of Neurology, University of Miami, Miller School of Medicine, Miami, FL, United States, [6]Department of Pharmacology, University of Oxford, Oxford, United Kingdom

OPEN ACCESS

Edited by:
Justin Dean,
The University of Auckland,
New Zealand

Reviewed by:
Rachel Anne Hill,
Monash University,
Australia
Alistair Jan Gunn,
The University of Auckland,
New Zealand

***Correspondence:**
Helen B. Stolp
hstolp@rvc.ac.uk

†Present address:
Yoko Arai,
BrainEver, Paris, France

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: 25 January 2019
Accepted: 09 July 2019
Published: 30 July 2019

Citation:
Stolp HB, Fleiss B, Arai Y, Supramaniam V, Vontell R, Birtles S, Yates AG, Baburamani AA, Thornton C, Rutherford M, Edwards AD and Gressens P (2019) Interneuron Development Is Disrupted in Preterm Brains With Diffuse White Matter Injury: Observations in Mouse and Human. Front. Physiol. 10:955. doi: 10.3389/fphys.2019.00955

Preterm brain injury, occurring in approximately 30% of infants born <32 weeks gestational age, is associated with an increased risk of neurodevelopmental disorders, such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD). The mechanism of gray matter injury in preterm born children is unclear and likely to be multifactorial; however, inflammation, a high predictor of poor outcome in preterm infants, has been associated with disrupted interneuron maturation in a number of animal models. Interneurons are important for regulating normal brain development, and disruption in interneuron development, and the downstream effects of this, has been implicated in the etiology of neurodevelopmental disorders. Here, we utilize postmortem tissue from human preterm cases with or without diffuse white matter injury (WMI; PMA range: 23^{+2} to 28^{+1} for non-WMI group, 26^{+6} to 30^{+0} for WMI group, $p = 0.002$) and a model of inflammation-induced preterm diffuse white matter injury (i.p. IL-1β, b.d., 10 μg/kg/injection in male CD1 mice from P1–5). Data from human preterm infants show deficits in interneuron numbers in the cortex and delayed growth of neuronal arbors at this early stage of development. In the mouse, significant reduction in the number of parvalbumin-positive interneurons was observed from postnatal day (P) 10. This decrease in parvalbumin neuron number was largely rectified by P40, though there was a significantly smaller number of parvalbumin positive cells associated with perineuronal nets in the upper cortical layers. Together, these data suggest that inflammation in the preterm brain may be a contributor to injury of specific interneuron in the cortical gray matter. This may represent a potential target for postnatal therapy to reduce the incidence and/or severity of neurodevelopmental disorders in preterm infants.

Keywords: parvalbumin, perineuronal nets, neuroinflammation, mouse, human

INTRODUCTION

While preterm birth has a multifactorial etiology, it is widely recognized as being precipitated by pro-inflammatory events (reviewed by Hagberg et al., 2015), and these vulnerable infants are at risk of further exposure to inflammation and infection. As such, in preterm born infants, the severity and duration of inflammation highly correlates with long-term outcome (Kuban et al., 2015). Inflammation is also a risk factor in the development of neurodevelopmental disorders (Hagberg et al., 2012; Jiang et al., 2018), such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), childhood epilepsy and disorders of learning, and cognition and emotional development. Given the associations, it is possibly unsurprising that up to 30% of preterm born infants are diagnosed with a neurodevelopmental disorders in childhood (Marlow et al., 2005; Wood et al., 2005; Johnson et al., 2010; Franz et al., 2018). Neurodevelopmental disorders have a substantial effect on the quality of life of affected individuals and their families, but we have limited options to improve the brain health of these infants. The development of therapies is hampered by the fact that the neuropathology of developmental disorders is as mixed as the diagnosis, varying both between and within specific clusters of disorders.

Specifically in preterm born infants, patterns of white matter injury, and increasingly of a gray matter injury, are recognized and associated with poor outcome. In contemporary cohorts of preterm infants, white matter injury includes periventricular leukomalacia in the most severe cases and more commonly diffuse white matter injury (Counsell et al., 2003; Back et al., 2007; Buser et al., 2010). The scale of white matter injury correlates with the severity of the outcome for preterm born infants (Counsell et al., 2008; Keunen et al., 2017; Tusor et al., 2017). Diffuse white matter injury has been successfully modeled in mice and sheep by recapitulating the exposure to early-life inflammation seen in preterm born infants (Mallard et al., 2003; Stolp et al., 2005; Dean et al., 2009; Favrais et al., 2011), though chronic hypoxia can result in similar pathology (Back et al., 2006), supporting a multifactorial etiology. Improvements in imaging modalities have made it possible to study the ultrastructure of the gray matter. Concomitantly, there has been a reduction in the most severe forms of white matter injury allowing the nature of subtle gray matter deficits to be probed in more detail. The microstructural pathology in the gray matter of preterm infants is still an on-going study (Ajayi-Obe et al., 2000; Boardman et al., 2006; Ball et al., 2013; Batalle et al., 2019), but it is clearly linked with the later development of cognitive disorders (Kersbergen et al., 2016; Lean et al., 2017). However, inflammation, synaptic dysfunction, and altered gamma-aminobutyric acid (GABA) signaling are frequently identified as underlying causes and mechanisms of injury in neurodevelopmental disorders (Pardo and Eberhart, 2007; Pinto et al., 2010; Deidda et al., 2014; Coelewij and Curtis, 2018; Dark et al., 2018). This suggests that synaptic dysfunction and altered GABA signaling are valid candidates to mediate the neurodevelopmental disorders associated with encephalopathy of prematurity.

Interneurons make up approximately 20–30% of cells within the cortex (slightly higher in primates than mice; Jones, 2009) and provide inhibitory balance for the excitatory pyramidal neurons by connecting cortical layers and cortical regions *via* interactions with glia, the vasculature, and other neurons (Cossart, 2011; Marin, 2012, 2013; Barber et al., 2018). Interneurons are primarily born within the medial and lateral ganglionic eminence during embryonic development and migrate to the cortex during the early postnatal period, where they mature over the first few weeks of life in mice (Cossart, 2011), equivalent to early childhood in humans. This maturation process includes differential expression of subtype markers (see below), maturation of dendritic arbors, and synpases, as well as changes in synaptic activity, and contacts, and transient networks (Butt et al., 2005, 2017; Cossart, 2011; Marques-Smith et al., 2016). There is a complex array of interneuron subtypes based on their morphological, electrophysiological, and molecular signatures (Kelsom and Lu, 2013). Major populations of interneurons in the cortex include those immunopositive for somatostatin (SST), calbindin (CalB), calretinin (CalR), and parvalbumin (PV). Changes in interneurons are termed an interneuronopathy, and deficits in each of these cellular subtypes have been associated with neurodevelopmental disorders (Marin, 2012), including those prevalent in preterm born children. There is currently little known about the effect of prematurity on cortical interneurons, although a recent study by Panda et al. (2018) has shown a general decrease in GABAergic (glutamate decarboxylase, GAD, positive) interneurons in the cortex, primarily driven by a decrease in parvalbumin-positive interneurons in the upper (layers II–IV), and to a lesser degree, lower (layers V and VI) cortex (Panda et al., 2018). However, this study focused on the effect of early or later prematurity and did not specifically study cases of encephalopathy of prematurity compared with controls. Changes in parvalbumin-positive interneurons in particular are commonly reported in studies of patients with neurodevelopmental disorders or in associated animal models (Kataoka et al., 2010; Gandal et al., 2012; Bitanihirwe and Woo, 2014; Barnes et al., 2015; Filice et al., 2016; Hashemi et al., 2018; Vogt et al., 2018). Parvalbumin knockout mice have ASD-like behavioral symptoms (Wohr et al., 2015). Additionally, knockout of metabotropic glutamate receptors (mGluR5) in parvalbumin interneurons results in specific memory deficits, altered sensory motor gating, and increased compulsive-like behaviors (Barnes et al., 2015). Similarly, maternal immune activation, a common experimental model for ASD, reduces parvalbumin inhibitory activity on pyramidal neurons, resulting in defects in attentional shifting (Canetta et al., 2016). Interneurons, including parvalbumin-positive interneurons, have a specialized area of extracellular matrix (chondroitin sulfate proteoglycans) surrounding them, called a perineuronal net. It has been shown in the past years that this net forms and enlarges during development, representing a marker

of a functionally mature neuron. The perineuronal net plays a role in regulating plasticity, and deficits are associated with epilepsy induced plasticity (Galtrey and Fawcett, 2007).

Here, we aim to assess whether there is a subtype specific disruption in interneuron maturation in our population of preterm infants with diffuse white matter injury compared with age-matched controls. Further to this, in mice exposed to IL-1β-induced inflammation, which produces diffuse white matter injury (Favrais et al., 2011; Krishnan et al., 2017), we will assess the long-term trajectory of interneuron development and the consequences for wider cortical maturation.

MATERIALS AND METHODS

Human Postmortem Tissue

Written informed parental consent was acquired according to the National Health Services (NHS) UK guidelines and study ethics were obtained from the National Research Ethics Services (West London), UK (ethics number: 07/H0707/139; Postmortem Magnetic Imaging Study of the Developing Brain). Thirteen extremely preterm postmortem brains (<30 weeks gestational age, 1 female/12 male) of vaginally delivered neonates were used in this study and obtained from the Perinatal Pathology Department, Imperial Health Care Trust, London, UK. The primary cause of death for each case was assessed by a pathologist. Brain tissue blocks from these cases had a postmenstrual age (PMA) range from 23^{+2} to 30^{+0} weeks, calculated by GA (at birth), plus age at death (PMA range for each cohort: 23^{+2} to 28^{+1} for non-WMI cases, 26^{+6} to 30^{+0} for WMI cases, $p = 0.002$). The details of each case are summarized in **Table 1**. Amniotic fluid infections were identified in most cases; however, no cases had identifiable vascular thrombosis or leptomeningitis. From postmortem examination, brains were assessed macroscopically and microscopically. Seven cases showed no significant brain pathology, these were used as non-neuropathologic controls (no WMI cases). Six brains had evidence of diffuse (non-cystic) white matter injury (WMI cases) including white matter gliosis and focal lesions.

As previously reported (Supramaniam et al., 2013; Vontell et al., 2013) after postmortem, whole brains were fixed with 4% formalin for 5–7 weeks, depending on size. The whole brains were sliced by a pathologist, and tissue blocks were processed on a Bright Tissue Processor (Bright Instrument Co. Ltd.). Paraffin-embedded tissue blocks of the frontal lobe at the level of the caudate (i.e., anterior to Ammon's Horn) were sectioned at 6 μm using a Leica RM2245 microtome (Leica Microsystems Ltd.).

Animal Model

All animal procedures were approved by the UK Home Office according to the regulations in the Animal (Scientific Procedures) Act (2012), and the King's College London (KCL) Animal Welfare and Ethical Review Board (AWERB;

TABLE 1 | Summary of clinical information of human postmortem cases.

Group	Sex	GA at birth (weeks)	Postnatal age	PMA at death (weeks)	Clinical context	Neuropathology
No white matter injury						
1	M	23 + 2	5 min	23 + 2	Ass IUGR, oligohydramnios, congestive heart failure	
2	M	23 + 6	9 h 51 min	23 + 6	Ass IUGR (twin)	Leptomeninges congested with focal hemorrhages
3	F	24 + 1	4 h 40 min	24 + 1	Extreme prematurity, congestive heart failure	
4	M	24 + 2	IUD/stillbirth	24 + 2	Constricted umbilical cord, congestive heart failure	
5	M	25 + 3	21 h 7 min	25 + 3	Ass IUGR (twin)	Odematous brain with transtentorial herniation of the unci
7	M	26 + 2	43 h	26 + 3	TTT, Ass IUGR, pulmonary hemorrhage	
8	M	28 + 1	<1 h	28 + 1	Oligohydramnios, lung hypoplasia, congestive heart failure	
Non-cystic white matter injury						
1	M	26 + 5	1 days 7 h 52 min	26 + 6	Ass IUGR, oligohydramnios, congestive heart failure	PVWM injury/pathology in PVWM at angles of the lateral ventricle
2	M	24 + 6	16 days 19 h 10 min	27 + 0	AAAFI, PPROM, acute necrotizing pneumonia	PVWM injury/pathology in PVWM at angles of the lateral ventricle
3	M	24 + 0	5 weeks 1 day 8 h 25 min	29 + 1	AAAFI, retroplacental hemorrhage	PVWM injury/pathology in PVWM at angles of the lateral ventricle
4	M	29 + 3	11 h 17 min	29 + 3	TTT, AAAFI, hydrops fetalis	GM/IVH
5	M	27 + 5	12 days	29 + 4	Ass IUGR, necrotizing entercolitis, uterine constraint/oligohydramnios	PVWM injury/pathology in PVWM at angles of the lateral ventricle
6	M	26 + 0	1 min	30 + 0	Necrotizing enterocilitis	Patchy WM gliosis

Ass IUGR, asymmetric interauterine growth restriction; AAAFI, acute ascending amniotic fluid infectin; GM, germinal matrix; IVH, intraventricular hemorrhage; PPROM, preterm premature rupture of the membranes; PVMW, periventricular white matter; TTT, twin-twin transfusion; WM, white matter.

PPL 70/8376). Inflammation-associated brain injury of the preterm born infant was modeled in mice by exposing them to systemic inflammation from postnatal day 1 (P1) through to P5; P0 is the day of birth, as previously reported (Favrais et al., 2011). P1–P5 is approximately equivalent to the period of 23–32 weeks gestation for brain development in the human pregnancy, based on a mixture of myelination and cortical development processes (Clancy et al., 2001, 2007; Semple et al., 2013). Pregnant CD-1 mice were purchased from Charles Rivers and transferred to the KCL Biological Services Unit (BSU) at embryonic day (E) 16 of pregnancy. Animals were housed separately in individually ventilated cages with food and water available *ad libitum*, in a temperature controlled environment with a 12 h light-dark cycle. This injection paradigm only produces consistent and reproducible diffuse white matter injury in male mice (Favrais et al., 2011), compared with female mice. Therefore, following birth, female pups were culled by cervical dislocation, and male pups were randomly divided into litters for saline or IL-1β treatment (typically numbering four to seven pups per "new" litter). Each pup received a 5 μl intraperitoneal (i.p.) injection twice daily from P1 to P4 and a final injection in the morning at P5 (Favrais et al., 2011). IL-1β (R&D Systems) was diluted in saline to a working concentration of 8 ng/μl (for a final dose of 10 μg/kg/injection). Animals remained housed in litter groups until weaning (P21), when they were group housed (3–4/cage). At P5 (6 h post final injection), P10 (approximate time of six layered cortex formation), P40 (when interneuron markers are mature), and P60 (early adulthood) randomly selected pups from each litter were killed by terminal anesthesia (i.p. pentobarbitone overdose) and perfused with saline followed by cold 4% paraformaldehyde. The brain was dissected out of the skull and immersion fixed in Bouin's solution (Sigma). Fixed tissue was washed and dehydrated through graded alcohol (progressing from 50 to 100%) and embedded in paraffin wax (Sigma, UK). Coronal 6-μm thick consecutive sections were cut and placed on microscope slides, with three sections at an interval of approximately 250 μm per slide.

Immunohistochemistry and Microscopy

As previously reported (Vontell et al., 2013), postmortem human sections underwent routine paraffin removal and rehydration, then were placed in 3% hydrogen peroxide to quench endogenous peroxidase activity, and immersed in preheated 10 mM citric acid with 0.1% Tween-20 (VWR International Ltd.) for 30 min and cooled at room temperature for 20 min. Sections were blocked with 5% normal goat or horse serum (as appropriate, based on the secondary antibody host species) and primary antibodies were incubated overnight at 4°C; concentrations below. The next day, biotinylated secondary antibodies (1:200, Vector Laboratories) goat anti-rabbit, goat anti-rat, or horse anti-mouse were incubated for 1 h at room temperature, then with avidin-biotin complex (ABC, 1:200, Vector Laboratories, UK) for 1 h. The reactions were visualized with 3,3′-diamino-benzidine (DAB; Sigma-Aldrich Company) for 10 min. Sections were then dehydrated, cleared in xylene, and cover-slipped. Primary antibodies used to identify all neurons in the developing human cortex were mouse anti-HuC/HuD (1:500, Life Technologies) and interneuron markers; mouse anti-calretinin (CalR; 1:100, Millipore), mouse anti-calbindin D-28 (CalB; 1:100, Sigma), rabbit anti-parvalbumin (PV; 1:500, Abcam), rat anti-somatostatin (SST; 1:50, Abcam), rabbit polyclonal neuropeptide Y (NPY; 1:5,000, Abcam). Routine H&E staining was also performed on this cohort of brain samples to assess for gross neuropathologies.

Mouse tissue was processed as described above and stained as previously reported (Stolp et al., 2011). Mouse anti-CTIP2 (1:400; Abcam) was used to identify neurons in the developing cortical plate in the mouse (Stolp et al., 2011; Garcez et al., 2018). Interneuron populations were stained with one of rabbit anti-Parvalbumin (PV, 1:200, Abcam), rabbit anti-Calretinin (CalR, 1:200, Swant), rat anti-Somatostatin (SST, 1:100, Abcam), rabbit anti-Neuropeptide Y (NPY, 1:100, Abcam), mouse anti-Calbindin (CalB, 1:100, Sigma), mouse anti-Reelin (1:500, Calbiochem), rabbit anti-Vasoactive Intestinal Peptide (VIP, 1:100, Abcam). Perineuronal nets were identified using biotinylated *Wisteria floribunda* Lectin (WFL, 1:200, Vector). Sections were incubated in the appropriate biotinylated or fluorescently-tagged secondary antibody: biotinylated goat anti-rabbit or mouse (1:200, Vector); donkey anti-mouse-488; donkey anti-rabbit-488/546 (1:400, Invitrogen) or streptavidin-488 (1:400, Invitrogen). All antibodies and lectins were diluted in 1% donkey serum in phosphate-buffered saline with 1% Tween-20. Sections with fluorescent secondaries were incubated with DAPI for 5 min (4′,6-diamidino-2-phenylindole, 1:1,000, Sigma-Aldrich) and mounted with ProlongGold (Thermo Fisher Scientific).

A subset of brains were processed for Golgi staining using the FD Rapid GolgiStain Kit (FD Neurotechnologies Inc.), following the manufacturer's instructions. Briefly, brain tissue was washed in dH_2O immediately following collection and then incubated in impregnation solution for 2 weeks. After this period, the samples were incubated in Solution C for 3 days before tissue sections were cut with a Vibratome (Leica) at 100 μm thickness, mounted in slides, and dehydrated through ethanol and xylene before mounting with DPX (Sigma).

Image Analysis and Statistics

Human tissue sections were visualized with bright-field microscopy, using a light microscope (Leica DM6000B, Leica Microsystems Ltd.), CCD color video camera (Leica CTR6000, Leica, UK), equipped with a motorized stage for automated sampling (MicroBrightfield Inc.). Cell number was assessed using optical fractionator stereological software (Stereo Investigator v8.27, MicroBrightfield Inc.). Contours were drawn around the frontal cortex magnified by a 5× objective (0.0426 mm^2), with an average area for each contour of 1 mm^2. Counting was performed at the magnification provided by the 40× objective, from multiple counting sites throughout the contour, to allow unbiased sampling of the frontal cortex and an estimate of cell density. The number of counting

sites was varied for each cell type, due to differences in density (determined from preliminary assessments of cell number); overall, an average of 35–45 counting sites were used for each contour (Vontell et al., 2013, 2015). Three contours were assessed for each brain region of interest (cortex and subcortical white matter, therefore, approx. 3 mm^2 assessed in total for each) and averaged to produce the estimated cell density for each region. Neurite lengths were assessed in positively stained somatostatin and neuropeptide Y neurons from images obtained with a ×40 objective. An average of seven cells were assessed per case, using ImageJ software (Schneider et al., 2012).

Mouse tissue sections were imaged with a Leica SP5 confocal microscope (P5, P10, and P40 fluorescently stained mouse tissue) or Leica DM4000 upright microscope (P40 mouse tissue). For cell counts, upper (II–IV) and lower (V and VI) layers were delineated as a region of interest for each image, and the immunoreactive cells counted manually, by a blinded observer, using the ImageJ cell counter tool. For the parvalbumin/perineuronal net counts, multiple markers were used so that single- and double-labeled neurons could be counted together in a single region of interest. Images were taken from the area of the somatosensory cortex identified by the presence of the barrel cortex, and measurements were averaged from both the medial (M1) and lateral (S1BF) cortex (Paxinos and Franklin, 2012), from three sections per brain. Therefore, data were averaged from three to six images per brain (tissue was excluded if it was damaged or where the staining had excessively high background or was non-specific), and from three to six brains per treatment and age (P5: n = 3–6 both groups; P10: n = 5 saline, n = 6 IL-1β; P40: n = 3 saline, n = 5 IL-1β).

Cell counts are presented as mean ± standard deviation (SD). Statistical analysis was performed using Prism 7 (GraphPad). Postmortem human samples were analyzed with an unpaired t-test. Grouped data from P5 tissue were analyzed using a two-way ANOVA for treatment and cell population, with *post hoc* analysis performed with Sidak's multiple comparison test. For P10 and P40 data, grouped data from each cell population were analyzed using a two-way ANOVA for treatment and layer, with *post hoc* analysis performed with Sidak's multiple comparison test. A p <0.05 was considered statistically significant.

Golgi stained neurons, with consistent stain impregnation and isolation from neighboring stained cells, were imaged with a Leica DM6000B microscope for analysis. Stacked images in the z-axis were obtained at ×40 magnification, with a 1 μm interval. Dendritic branching and intersections were quantified and analyzed by Sholl analysis (Gutierrez and Davies, 2007), using ImageJ image processing program. Image stacks were compressed in one 2D image, and the center of the soma was marked for reference. Concentric circles, with radii increasing by 9.06 μm (50 pixels) per circle, were set from the soma center. Multiple neurons (10–15 from multiple slices across the brain) were analyzed from three brains per treatment, and results are presented as the average number (± SD) of dendritic intersections per concentric circle, for each treatment group. Sholl profiles were statistically compared using a two-way ANOVA for treatment and distance from soma.

For spine analysis, dendrites were isolated in images of neuronal cells collected for Sholl analysis. Dendrites chosen were of different lengths, with a substantial portion in the plane of focus, and were representative of spine density and morphology of unselected dendrites. Spine density of selected dendrites was analyzed using Image-Pro Premier image analysis software (MediaCybernectics). Data were blinded, and images were categorized as having a high or low synaptic frequency (41 from saline treated brains, 57 from IL-1β treated) based on the number of synaptic protrusions along the length of the dendrite, and the spaces, if any, between the protrusions (Smrt and Zhao, 2010; Phillips and Pozzo-Miller, 2015). This categorical data were statistically compared using the Fisher exact test, with a p <0.05 considered statistically significant.

RESULTS

Preterm Born Infants With Diffuse White Matter Injury Had Reduced Number of Calretinin Interneurons in the Cortex and Altered Arborization of Other Interneuron Populations

Stereological assessment of cell number in the frontal cortex of the developing human brain showed no change in the total number of neurons, identified by HuC/HuD immunoreactivity, with 53,104 ± 11,009 immunopositive cells/mm^2 found in the control brains (n = 5), and 52,120 ± 6,327 cells/mm^2 in the cortex of the white matter injury cases (n = 4). In contrast, there was a significant decrease in the cortical calretinin-immunopositive cells in the white matter injury cases, compared with preterm infants without white matter injury (from 1,084 ± 96 to 663 ± 327 cells/mm^2, p = 0.043, **Figures 1A–D**). Calbindin- and parvalbumin-positive cells were observed in low numbers in both cases, insufficient for determining statistical significant changes. Somatostatin and neuropeptide Y immunopositive interneurons also occurred much less frequently than calretinin positive cells and were not found in the cortex at this stage of development. However, the cells immunopositive for these markers were found in the subcortical white matter, and these were analyzed for neurite length and branching. There were no statistical differences in the number of cells in either interneuron (SST or NPY) subpopulation between preterm infants with or without white matter injury (n = 5 for both). The branching of immunopositive cells was assessed with a modified Sholl Analysis, and there was a significant decrease in the arborization of neurons in both of these interneuron classes. Somatostatin cells in white matter injury cases had shorter leading neurite length (33.2 ± 8.7 μm, n = 6, compared with 56.7 ± 8.5 μm in the no WMI cases, n = 6; p < 0.001) and fewer branches (7.4 ± 1.4 compared with 4.6 ± 0.7, p = 0.001). Neuropeptide Y immunopositive neurons also had shorter neurites (51.5 ± 5.2 μm compared with 72.6 ± 13.7 μm in no-WMI group, n = 5–6; p = 0.01) and fewer branches

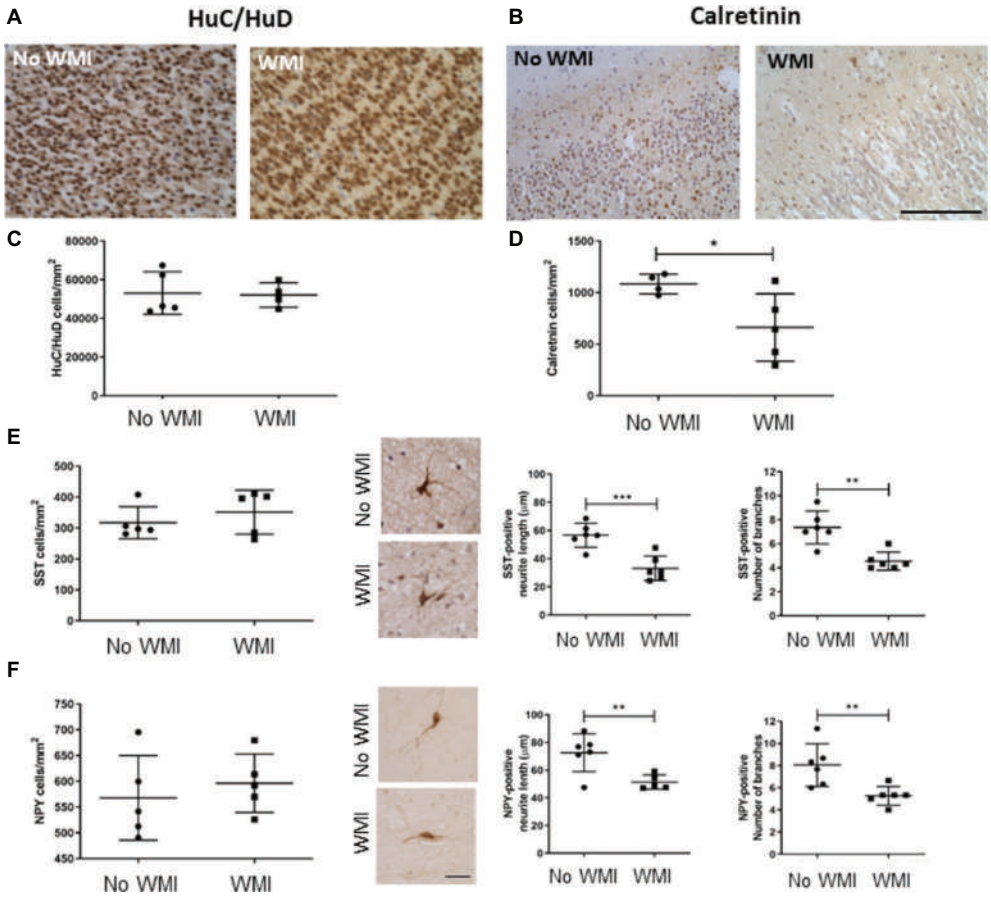


FIGURE 1 | Interneuron development in the cortex and subcortical white matter of the damaged preterm brain is altered compared with age-matched cases without white matter injury. Cell counts were performed in cortical and subcortical white matter in the frontal lobe, anterior to Ammon's Horn. Total cortical neuronal number was assessed counting HuC/HuD positive neurons **(A,C)**. A significant decrease in Calretinin (CalR)-positive interneurons was observed in the cortex of preterm brains with non-cystic white matter injury (WMI) compared with no WMI controls **(B,D)**. No significant difference in Somatostatin (SST) or Neuropeptide Y (NPY)-positive interneurons was observed in the subcortical white matter. However, SST and NPY labeled interneurons from non-cystic white matter injury showed shorter neurites, with fewer branches **(E,F)**. Data presented as mean ± SD; scale bar = 100 µm **(A–D)**, 25 µm **(E,F)**; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; SST, somatostatin; NPY, neuropeptide Y; WMI, white matter injury.

(5.3 ± 0.85 WMI, compared with 8.1 ± 1.9, $p = 0.009$) in white matter injury cases (**Figures 1E,F**).

The Developmental Trajectory of a Number of Interneuron Populations Is Disturbed in a Mouse Model of Preterm Birth

In mice with IL-1β-induced inflammation, at the end of the inflammatory exposure (P5), there was no gross alteration in the cortical layering (**Figure 2A**, DAPI) or cell density. In addition, no statistically significant change in CTIP2 immunoreactive neuron number per cortical area was observed (saline: 204.3 ± 37.4 cells, IL-1β: 162.7 ± 29.8 cells, $p < 0.086$, **Figure 2B**), suggesting no overall change in the development of the early cortical neurons with injury. There was a significant decrease in the number of reelin-positive (saline: 64.2 ± 5.1 cells, IL-1β: 51.7 ± 5.7 cells, $p = 0.047$) and calretinin-positive (saline: 55.7 ± 9.7 cells, IL-1β: 31.3 ± 12.6 cells, $p < 0.001$) neurons in the IL-1β treated animals compared with the saline-treated control (**Figure 2C**). In comparison to these reelin- and calretinin-positive cells, other interneuron populations were present in a much lower number (**Figures 2A,C**), as would be expected at this early stage of brain development, as the majority of interneuron markers do not fully develop until the second or third postnatal week in the mouse (Cossart, 2011). In general, at this stage of development, the interneuron populations were present in higher numbers in the lower cortical layers (layer V and VI) than in the upper layers (II, III, and IV). Specific analysis of interneuron populations (PV, CalB, SST, and NPY) in the upper layers showed no difference between treatment groups (**Figure 2D**). In the lower layers, two-way ANOVA analysis showed a significant treatment effect ($p < 0.043$), reflecting a general increase in the same interneuron populations in the IL-1β group (**Figure 2E**). However, no significant difference was found for individual populations following correction for multiple comparisons.

At P10 (**Figure 3**), the somatostatin, neuropeptide Y, calretinin, and calbindin populations were present in greater,

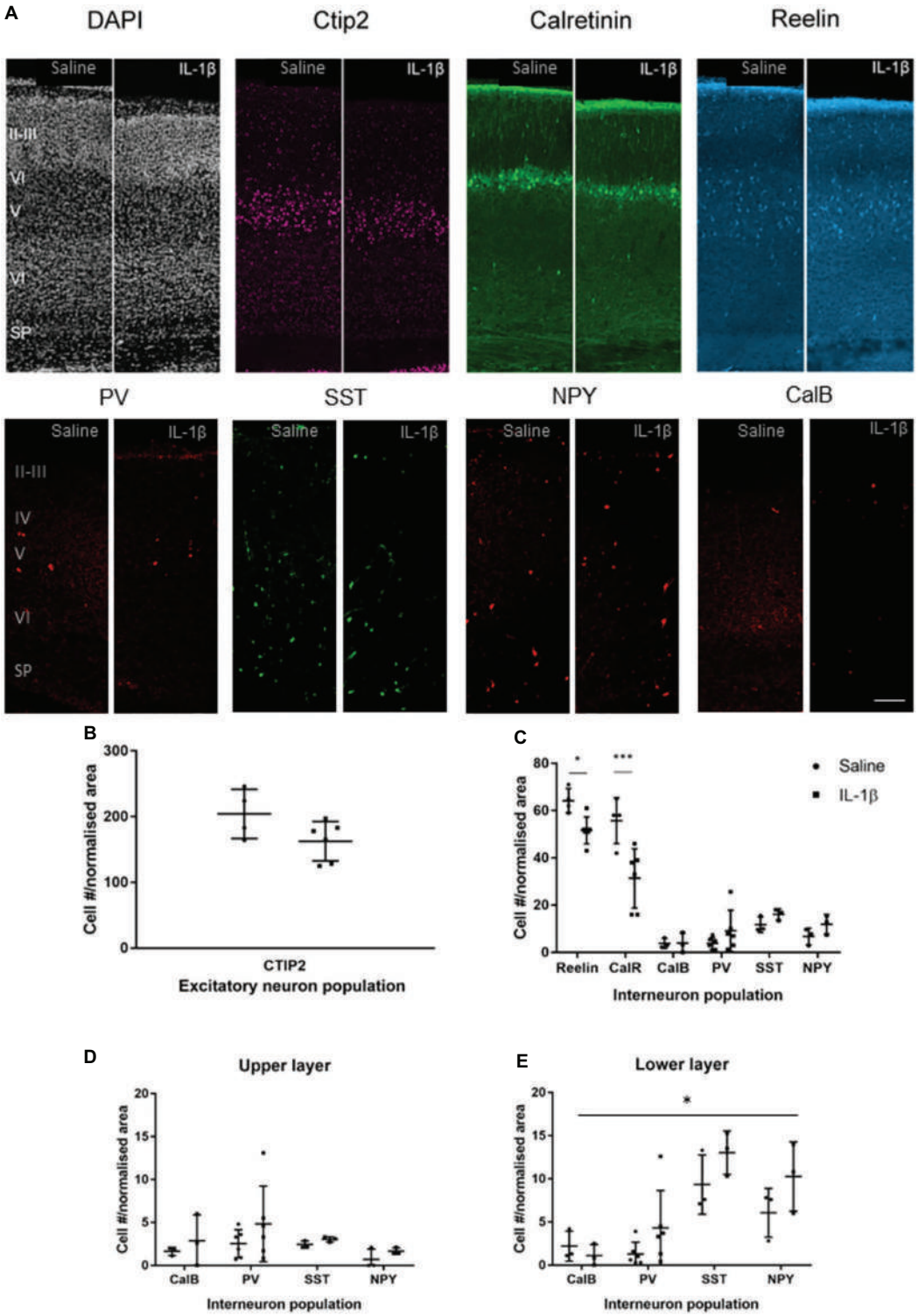


FIGURE 2 | P5 mice with inflammation-induced brain damage also show altered interneuron development across the cortex. Cortical development was assessed based on immunohistochemistry for general microstructure (DAPI), developing cortical neurons (CTIP2) and interneuron subpopulations at P5 **(A,B)**. A significant decrease in Reelin and Calretinin (CalR)-positive neurons was observed in the whole cortex **(C)**, but no change in CTIP2 positive pyramidal neurons **(B)**. Assessment of the less prevalent interneuron populations according to upper (II–IV) or lower (V, VI) cortical layer distribution showed a small but significant increase the majority of interneuron populations in the lower layers (treatment effect, $p = 0.04$, two-way ANOVA) and no significant change in the upper layers **(D,E)**. Data presented as mean ± SD; scale bar = 100 μm. $^{*}p < 0.05$, $^{***}p < 0.001$. CalR, calretinin; PV, parvalbumin; SST, somatostatin; NPY, Neuropeptide Y; CalB, calbindin.

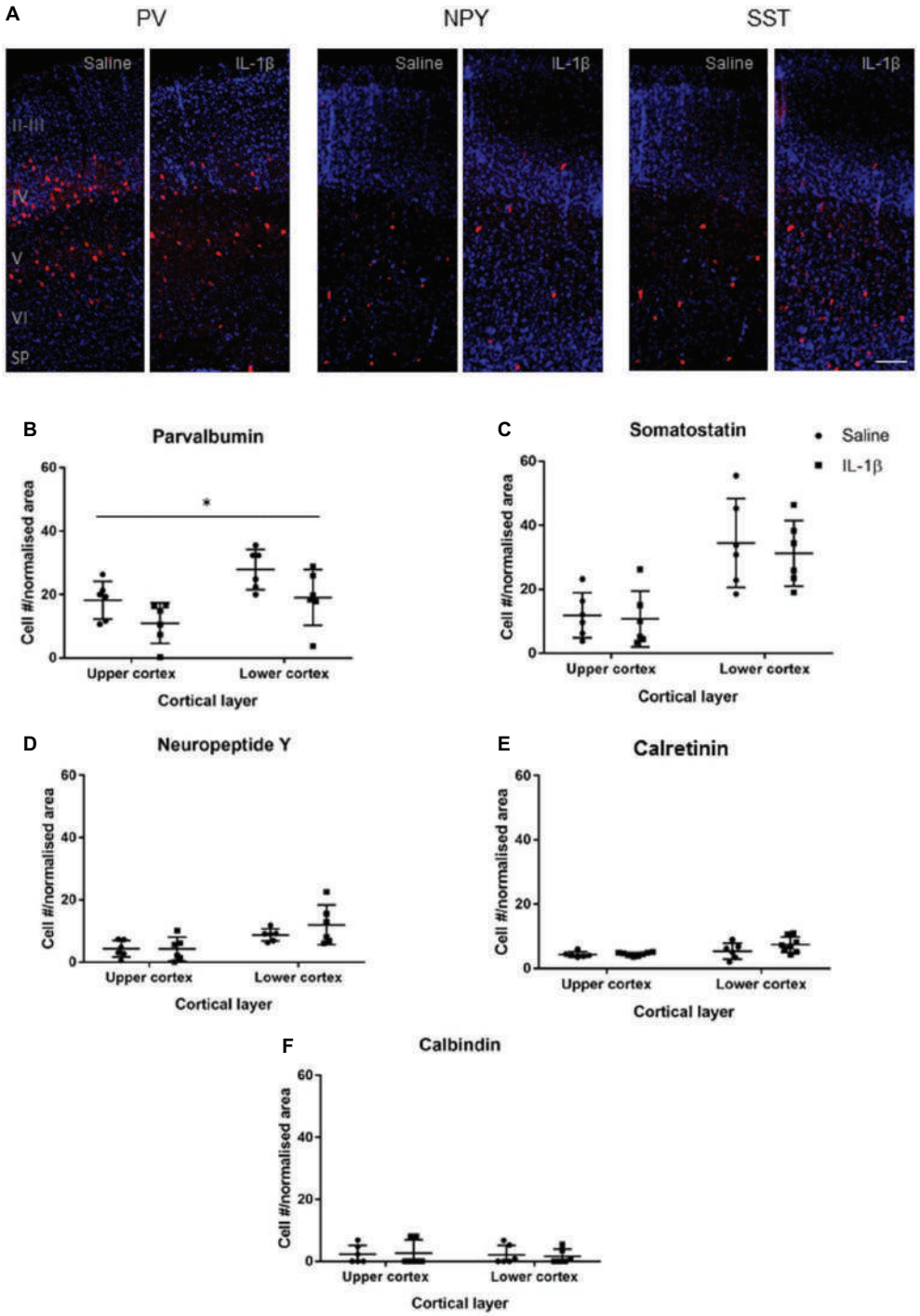


FIGURE 3 | Developmental pattern of interneuron injury changes by P10 in mice with inflammation-induced brain damage. Interneuron populations were assessed again at P10 by immunohistochemistry **(A)** and quantified **(B–E)** for number and distribution through cortical layers. There was a significant decrease in the number of parvalbumin (PV)-positive interneurons across the cortex (**B**; treatment effect, $p = 0.01$, two-way ANOVA), but no change for somatostatin (SST), neuropeptide Y (NPY), calretinin (CalR), or calbindin (CalB, **C–F**). Data presented as mean ± SD; scale bar = 100 μm. *$p < 0.05$.

though still relatively low, numbers, and there was no significant difference in their presence, or distribution through the cortex between the saline and IL-1β treated groups. Parvalbumin-positive cells were found in significantly greater numbers in layer IV, V, and VI ($p = 0.005$). There was an overall decrease in the number of parvalbumin-positive neurons ($p < 0.014$), with no significant layer effect (**Figure 3B**).

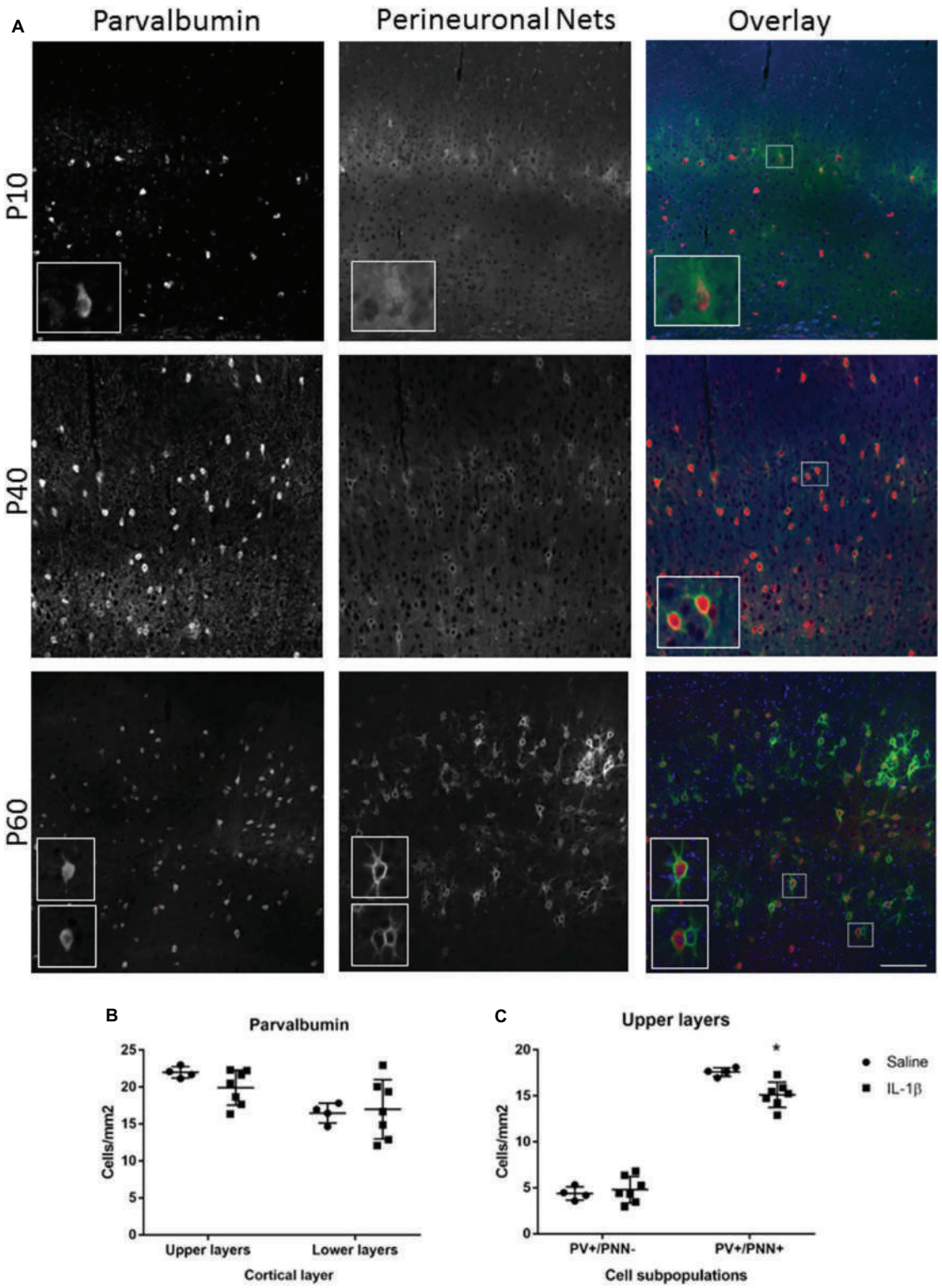


FIGURE 4 | Long-term changes in parvalbumin-positive interneurons and their perineuronal nets extend to P40 in mice with inflammation-induced brain damage. The beginning of perineuronal net formation could be seen in the cortex, primarily in layer IV, from as early as P10 (**A**, top row). The aggregation of the perineuronal nets and their association with the parvalbumin-positive interneurons became more pronounced through development (**A**, lower rows). In the IL-1β challenged mice, there was no gross alteration in perineuronal net formation or in the number of cortical parvalbumin neurons **(B)**. However, there was a significant decrease in the number of PV^{+}/PNN^{+} neurons in the upper layers of the cortex **(C)**. Data presented as mean ± SD; scale bar = 100 μm. $^{*}p < 0.05$. PV, parvalbumin); PNN, perineuronal nets.

Parvalbumin Interneurons Show Long-Term Disruption in Neuronal Number, Arborization, and Association With Perineuronal Nets

To determine whether perturbations in parvalbumin number present at P10 in mice following IL-1β-induced inflammation persisted beyond this early developing period, the number of these neurons was assessed at a later stage of development. At P40, there was a trend toward a decrease in parvalbumin cells in the upper layers of the cortex but not the lower layers (**Figures 4A,B**). The maturation state of parvalbumin interneurons was assessed by the presence of perineuronal nets, which start to form in a loose association with the parvalbumin neurons in the barrel cortex from P10 in the mouse (**Figure 4A**). Clear association of the nets with parvalbumin-positive interneurons is visible by P40, which mature further as development continues (P60; **Figure 4A**; Ueno et al., 2017). When explored in more detail, a statistically significant decrease in parvalbumin-positive neurons that had fully formed perineuronal nets (17.6 ± 0.2 cells/mm^2 in saline, 15.1 ± 0.5 cells/mm^2 in IL-1β, p = 0.008) was seen specifically in the upper layer of the IL-1β treated animals (**Figure 4C**). At this age, there was a relatively high proportion of parvalbumin neurons without perineuronal nets (25% in saline), but there was no change in this subpopulation of neurons with treatment (32% in IL-1β group, p = 0.8, **Figure 4C**). Likewise, in brains from both saline and IL-1β treated animals, a small number of perineuronal nets were observed without PV staining (1.5 ± 0.3 cells/mm^2 in saline and 1.2 ± 0.2 cells/mm^2 in IL-1β), but the proportions were very low and there was no significant difference between groups. In comparison with these small but significant changes in parvalbumin interneurons, there remained no significant difference in the number of either neuropeptide Y, calretinin, or VIP-positive neurons in the cortex between saline-and IL-1β-treated animals (**Figures 5A–C**).

Long-Term Changes in Arborization and Spine Density Are Broadly Observed in Areas of the Cortex With Disrupted Parvalbumin Interneuron Development

Golgi staining was performed to visualize the individual neurons within the cortex, to allow Sholl and spine analysis to be performed. No significant effect of IL-1β treatment was observed on the overall dendritic morphology of neurons at P40 (**Figure 5D**), but there was a statistically significant shift in the number of dendrites showing a low frequency of spines, with 35% of the spines from Golgi stained cells from IL-1β treated animals showing a low spine frequency compared with 15% in saline-treated tissue (p = 0.007, **Figures 5E,F**).

DISCUSSION

We have revealed a general decrease in the number of GABAergic interneurons in the cortex of preterm born human infants with diffuse white matter injury, compared with age-matched controls, and similarly in mice with diffuse white matter injury following IL-1β-induced inflammation. This interneuron deficit (reduced numbers and reduced morphological complexity) occurred in the absence of severe injury, as there were no changes in the numbers of neurons in the cortex in the clinical (human) or preclinical (mouse) study. Also, subtypes of interneurons were differentially affected over development indicating population specific vulnerabilities. The early disruption of interneuron populations in our clinical sample is supported by our preclinical evidence that not only did these changes persist but were found together with changes in the excitatory-inhibitory cell balance, spine density, and the density of perineuronal nets. Altogether, these data support the hypothesis that inflammation associated with preterm birth may alter the excitatory-inhibitory balance in the brain. This effect would partly explain the link between exposure to inflammation during development and poor neurodevelopmental outcomes in these infants that lead to life-long functional impairment. However, Canetta et al. (2016) have shown that maternal immune activation at the very beginning of neurogenesis (E9 in the mouse) can also result in parvalbumin-specific pathology, possibly suggesting a particular susceptibility of this cell population to disturbances in normal development. More work will be required to explore this concept.

Interneurons are produced within the medial ganglionic eminence throughout development and migrate to all cortical layers (Butt et al., 2005, 2017). Interneuron fate is genetically coded and follows a clear pattern of migration and maturation, related to the regulatory transcription factors imposed in the ganglionic eminence at the time of cell production (reviewed by Marin, 2012; Kelsom and Lu, 2013). It is postulated that the developmental period during which an injury or perturbation occurs will influence the cell population affected. Injury would also interact with genetic risks, which may affect when phenotypes caused by altered genetic code manifest, explaining the variety of neurodevelopmental disorders associated with similar phenotypes. Animal models allow the progression of injury to be assessed, in comparison with the snapshot of information typically obtained from clinical studies. The utility of the mice with IL-1β-induced inflammation and subsequent diffusion white matter injury used here is that the injury is broadly comparable in nature to that seen in our patient samples. Specifically, this model displays the hallmarks of injury observed in contemporary preterm born infants: hypomyelination, axonopathy, oligodendrocyte dysmaturation and also, reported here and previously, no gross cortical injury evidenced by no change in cortical neuronal number (Favrais et al., 2011; Schang et al., 2014; Krishnan et al., 2017; Rangon et al., 2018). In this model, females have a less severe and more transient phenotype, which is why they were not used in this study (unpublished data), suggesting a bias seen in the animal model similar to that observed in the human population (Marlow et al., 2005). However, it is of course impossible to recapitulate the complex processes leading to injury in the human completely with any model, and we would acknowledge that the differences in the magnitude of the observed changes probably lie in multifactorial injury to the infants.

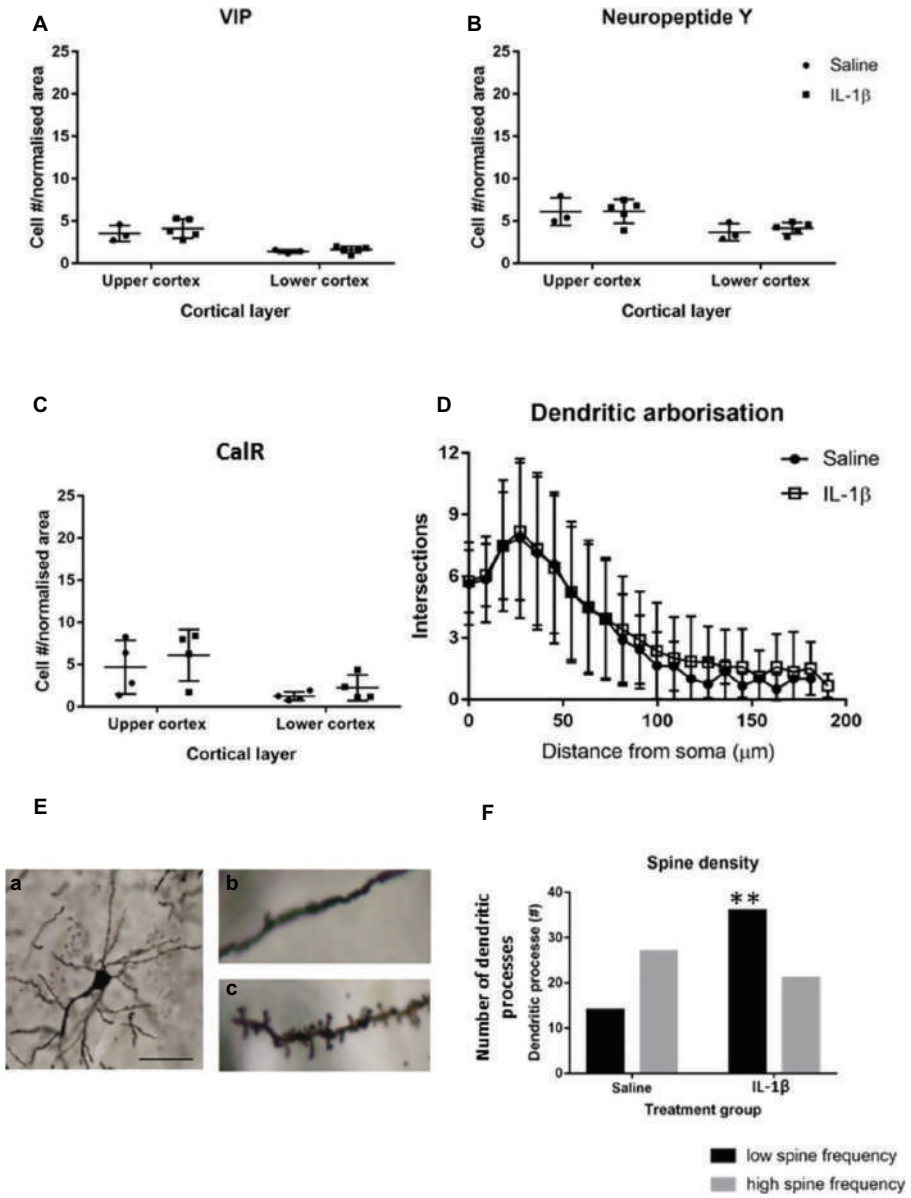


FIGURE 5 | By P40 the majority of interneuron pathology has recovered, but there are long-term changes in dendritic spines associated with the early inflammation-induced brain damage. No difference was seen in the number of neuropeptide Y (NPY), calretinin (CalR), or VIP-positive neurons **(A–C)** at P40. Golgi staining was performed to assess the general morphological development of cortical neurons at this stage. **(E)** Image (**a**) is an example of a well stained cortical neuron used for spine analysis and images (**b,c**) are representative images of spine density on dendrites in the brains of IL-1β and saline treated mice, respectively. There was no significant difference in the dendritic arborisation **(D)** but there was a significant reduction in spine frequency on the dendritic processes in the IL-1β challenged mice **(E,F)**. Data presented as mean ± SD; scale bar = 60 μm for **Ea**, 10 μm for **Eb,c**. $^{**}p < 0.01$.

In this animal model, at the time when the brain is still being subjected to an inflammatory challenge (Krishnan et al., 2017), interneurons appear slightly increased in the cortex of injured animals compared with controls. It is worth noting that most interneuron populations are not properly established in the cortex at this stage (Cossart, 2011), reflected in lower numbers than were found in the adult analysis. As development progresses, and after the injurious stimuli has resolved (at P10, Krishnan et al., 2017), a mild parvalbumin-specific neuronal reduction becomes apparent. Parvalbumin interneurons make up approximately 40% of GABAergic cortical interneurons, which, compared with the less frequent interneuron subpopulations, may make the detection of statistical changes in the cell population easier. Parvalbumin interneurons are fast-spiking interneurons with basket or chandelier morphology that make synaptic connections on proximal dendrites and the soma of target neurons (reviewed by Marin, 2012; Kelsom and Lu, 2013). The cells produce oscillations in the gamma range (30–80 Hz), which is associated with cognitive processes such as memory and attention

(Bartos et al., 2007; Canetta et al., 2016). This function may partly explain why parvalbumin knockout mice have an ASD-like phenotype: repetitive behaviors, impaired social interaction and communication, as well as reduced startle response and increased risk of seizure (Wohr et al., 2015).

The severity of injury will also have an effect on outcome, with more severe injury producing larger, more widespread changes. Previous studies of gray matter injury have been performed in human preterm infants with cystic white matter injury, in which there is extensive cell death and reactive gliosis. However, in our human post-mortem cases of diffuse white matter injury, there was no overall change to neuronal number. Possibly unsurprisingly then, compared with our data of limited and cell type-specific changes, studies of gray matter injury in severe cystic cases show more widespread gray matter injury (Andiman et al., 2010; Kinney et al., 2012). As such, our data are an important contribution to understanding how interneuron deficits may impact on neurodevelopmental disorders for contemporaneous cohorts of preterm born infants. The diffuse white matter injury studied here is the predominant form of injury seen in preterm born infants, and cystic cases account for only approximately 5% of neuropathology, based on MRI and ultrasound imaging studies in preterm cohorts (Hamrick et al., 2004; reviewed by Back, 2017).

It must be noted that preterm birth occurs due to a complex, and often undetermined etiology and can be concomitant with other clinical findings, e.g., intrauterine growth restriction (IUGR), which in themselves are also predictive of reduced brain development (Rees et al., 2008; O'Shea et al., 2009; Korzeniewski et al., 2017). The fact that many infants in our cases (both WMI and non-WMI groups) have IUGR may well be a confounder in the injury observed. While the mouse data clearly links interneuron (and white matter) pathology with inflammation, it is likely that there are many contributing factors in preterm brain injury, of which inflammation is only one. This, and the different developmental timetables of mouse and human brains, may also explain the different interneuronopathies identified in this study. Adding to that complexity is the varying age range between the preterm white matter injury and non-white matter injury cohorts examined in this study. From a developmental point of view, the greater PMA of WMI group compared with control should be associated with more visible interneurons in the cortex (through processes of migration and maturation). As a result, we suggest that the significant decrease in interneuron numbers in this group is, if anything, an underestimate of the interneuron injury in preterms with diffuse WMI. However, the longer survival times of these infants does increase the difficulty of defining the etiology of the interneuron injury, as many features of the *ex utero* environment may contribute to the injury severity. For instance, Panda et al. (2018) suggest that deficits in maternal estrogen is a major contributor to interneuron injury. That being said, in the data from the mouse model, when exposure to maternal estrogen (and other factors of the external environment) is constant, there is still a reduction in interneurons.

The significant early decreases seen for interneurons in general in this study are similar to findings recently reported by Panda et al. (2018), where early prematurity in humans was associated with a reduction in parvalbumin-positive interneurons, primarily in the upper cortical layers, and an increase in somatostatin-positive interneurons also in the upper cortex. There was also an overall decrease in glutamate decarboxylase (GAD)-positive neurons, attributed primarily to the changes in the parvalbumin subpopulation. However, a direct comparison with Panda and colleagues is difficult as their cases were compared based on the degree of prematurity rather than on their diagnosis (Panda et al., 2018). This resulted in a comparison of early born infants that had long survival times (up to 3–4 weeks) with later born preterm infants who had survival times of only 2–3 days. The role of postnatal care in interneuron development has been shown in a model of preterm delivery in the baboon, where 14 days of positive pressure ventilation lead a reduction in the numbers of calretinin-positive cells in the visual cortex (Verney et al., 2010). Although this and other confounding factors are almost impossible to remove from a human postmortem study, we are confident that our cases provide evidence more specifically related to the effects of prematurity on interneuron development without the specific effect of postnatal care.

To further assess interneuron number, we investigated their migration through the subcortical white matter in our clinical cases. Reduced morphological complexity was observed for somatostatin and neuropeptide Y immunopositive neurons within the subcortical white matter in this study. As these neurons are still migrating, changes in their morphology may reflect a disruption in migration that will contribute to later reduced interneurons in the cortex, another process associated with neurodevelopmental disorders (Marin, 2013). It is possible that a delay in migration could explain initial reductions in interneuron number in the cortex that lessen or normalize as development continues. Mild models of perinatal injury have recently shown altered neuronal arborization in the sheep subplate (McClendon et al., 2017) and cortex (Dean et al., 2013), though these were not specific to interneurons. These changes in arborization are generally interpreted as a delayed maturation, consistent with diffusion tensor imaging data showing altered diffusion characteristics in the cortex of preterm infants (Ball et al., 2013). These most likely reflect reduced cortical complexity and contribute to a delay in local connection formation (Batalle et al., 2019), which will have downstream effects on subsequent brain development. The mechanism of delayed maturation is, as yet, unclear but could be due to delayed interneuron migration to the correct position within the cortex (as discussed above). Alternatively, this may be due to intrinsic changes that have occurred within the cells as a result of the injury or due to a wider disruption of the network and integration of neurons within it. More work is required to elucidate this process.

Perineuronal nets are a dynamic structure of extracellular matrix proteins that develop around a subpopulation of neurons in the brain. Parvalbumin interneurons are one of the cell types most commonly associated with perineuronal nets, and it has been suggested that these nets modulate the connections between cells and the plasticity to form new connections (Shen, 2018).

The presence of perineuronal nets around parvalbumin interneurons regulates Otx2 levels and critical periods of synaptic plasticity between these interneurons and their local networks (Beurdeley et al., 2012). It is unclear in many reports of parvalbumin neuronal loss in clinical cases or animal models of injury/neurodevelopmental disorders whether the deficit is actually in the number of parvalbumin interneurons or in the expression of the parvalbumin protein within the neurons. In a SHANK3 knockout model of ASD, a significant loss of parvalbumin staining was observed in the cortex, but no disruption in the perineuronal nets, implying the presence of "parvalbumin-neurons" but not of the parvalbumin protein (Filice et al., 2016). This phenotype in both SHANK3 and parvalbumin knockout mice suggests that loss of parvalbumin protein is associated with increased inhibition, whereas loss of the parvalbumin interneurons as a whole is associated with decreased inhibition (Wohr et al., 2015; Filice et al., 2016).

It should be noted that a number of models of hypoxic-ischemic perinatal injury have also shown changes in parvalbumin interneurons and, in some cases, their perineuronal nets. In the recent study by Fowke et al. (2018), a substantial loss of interneurons was observed in the cortex, and there was an associated disruption of the perineuronal nets within 7 days of the injury, particularly in layer 6 of the cortex. Loss of calbindin and parvalbumin immunopositive neurons from the striatum has also been reported after perinatal asphyxia (Van de Berg et al., 2003). However, as mentioned above, due to the much greater severity of this model of brain injury, compared with the inflammation-injury model used here, the mechanism of damage is likely to be quite different. In the context of severe damage, it is unclear how much the interneuron damage alone contributes to the neuropathology and behavioral deficits observed in cases of human hypoxic-ischemic encephalopathy.

Early changes in calretinin-positive interneurons were also seen in this study, both in human and mouse tissue; however, in mouse, they did not persist after the initial injury period. Calretinin-positive neurons are present in the cortex from as early as 12 weeks of gestation in the human brain (Al-Jaberi et al., 2015), possibly explaining their early response to injury. A reduction in calretinin-positive neurons was also observed in the visual cortex (but not other cortical regions) in a preterm baboon exposed to ventilator support (Verney et al., 2010) but not in the caudate following prenatal cerebral ischemia in sheep (McClendon et al., 2014). However, it has recently been shown that there is a significant decrease in size and number of calretinin-positive neurons in the caudate nucleus in the brains of autistic patients (Adorjan et al., 2017). A previous study of adult males with autism also showed a decrease in calretinin-positive interneurons specifically localized in the dentate gyrus of the hippocampus and more widespread changes in parvalbumin-positive interneuron numbers (Lawrence et al., 2010). Of note, Canetta et al. (2016) clearly show no change in calretinin, or somatostatin, interneurons in their model of maternal immune activation-induced brain injury. This suggests that calretinin and parvalbumin injury may occur *via* different mechanisms, or that early, mild changes in calretinin interneurons are normalized as development progresses.

The altered frequency of synaptic boutons on the dendrites of Golgi stained neurons in the cortex was an interesting finding. Favuzzi et al. (2017) have shown that altering the integrity of perineuronal nets by the deletion of one component, in this case brevican, alters the electrophysical properties of interneurons, and subsequently the number of synapses of parvalbumin interneurons onto pyramidal neurons. While in the present study, there was not a significant loss of perineuronal nets at P40, the time when this altered synaptic distribution was observed, there was a small but significant decrease in the number of cells positive for both parvalbumin and perineuronal nets. This may suggest an on-going reduction in inhibitory control in the cortex and a related change in the pyramidal neuron function. Electrophysiological studies will need to be performed to confirm if this is the case. However, it is important to note that long-term behavioral changes have been observed in this mouse model (Favrais et al., 2011) where animals treated with IL-1β from P1 to P5 fail to recognize novel or displaced objects in memory tests. Brevican deletion in the adult also showed impaired working and short-term memory (Favuzzi et al., 2017) supporting the assumption that the reduction in parvalbumin interneurons and perineuronal nets observed in this study may contribute to a disordered brain function.

In conclusion, this study provides clinically important information on the effects of preterm birth to decrease parvalbumin neurons and their perineuronal nets, disrupt the excitatory-inhibitory cell balance, and reduce spine density in the cortex. Altogether, these suggest that a decrease in cortical inhibition may result from preterm birth and its associated exposures to inflammatory injuries, though the potential contribution of other factors in this complex clinical situation cannot be ruled out. The pathology can be consistently recognized from P10 in the mouse, approximately equivalent to term in humans. This suggests that it may be possible for pharmacological tools that modulate cortical excitability to correct the abnormal developmental trajectory of the brain. This would open therapeutic avenues for reducing the long-term burden of neurodevelopmental sequelae occurring in many millions of infants every year due to brain injury associated with preterm birth.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of National Health Services (NHS) UK guidelines with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the National Research Ethics Services (West London), UK (ethics number: 07/H0707/139; Post-mortem Magnetic Imaging Study of the Developing Brain). This study was carried out in accordance with the recommendations of UK Home Office according to the regulations in the Animal (Scientific Procedures) Act (2012). The protocol was approved by the King's College London (KCL) Animal Welfare and Ethical Review Board, PPL 70/8376.

AUTHOR CONTRIBUTIONS

HS, BF, YA, and PG contributed to conceptualization. HS, YA, SB, VS, RV, and AY contributed to formal analysis and investigation. CT, MR, AE, and PG contributed to resources. HS, BF, and AB contributed to data curation and visualization. HS contributed to writing – original draft preparation. All authors contributed to writing – review and editing. HS, CT, BF, MR, AE, and PG contributed to supervision. HS and PG contributed to project administration. HS, PG, CT, MR, and AE contributed to funding acquisition.

FUNDING

The authors' research is supported by the Medical Research Council [MR/K006355/1], the Wellcome/EPSRC Centre for Medical Engineering at King's College London [WT 203148/Z/16/Z], Inserm, Université Paris Diderot, "Investissement d'Avenir -ANR-11-INBS-0011-"NeurATRIS, Fondation Grace de Monaco, Fondation Roger de Spoelberch, PremUP, Cerebral Palsy Alliance, and Fondation des Gueules Cassées. The authors acknowledge financial support from the Department of Health *via* the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. The supporting bodies played no role in any aspect of study design, analysis, interpretation, or decision to publish this data.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the families that consented for this study and the UK Medical Research Council for the use of human tissue samples.

REFERENCES

Adorjan, I., Ahmed, B., Feher, V., Torso, M., Krug, K., Esiri, M., et al. (2017). Calretinin interneuron density in the caudate nucleus is lower in autism spectrum disorder. *Brain* 140, 2028–2040. doi: 10.1093/brain/awx131

Ajayi-Obe, M., Saeed, N., Cowan, F. M., Rutherford, M. A., and Edwards, A. D. (2000). Reduced development of cerebral cortex in extremely preterm infants. *Lancet* 356, 1162–1163. doi: 10.1016/S0140-6736(00)02761-6

Al-Jaberi, N., Lindsay, S., Sarma, S., Bayatti, N., and Clowry, G. J. (2015). The early fetal development of human neocortical GABAergic interneurons. *Cereb. Cortex* 25, 631–645. doi: 10.1093/cercor/bht254

Andiman, S. E., Haynes, R. L., Trachtenberg, F. L., Billiards, S. S., Folkerth, R. D., Volpe, J. J., et al. (2010). The cerebral cortex overlying periventricular leukomalacia: analysis of pyramidal neurons. *Brain Pathol.* 20, 803–814. doi: 10.1111/j.1750-3639.2010.00380.x

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathol.* 134, 331–349. doi: 10.1007/s00401-017-1718-6

Back, S. A., Craig, A., Luo, N. L., Ren, J., Akundi, R. S., Ribeiro, I., et al. (2006). Protective effects of caffeine on chronic hypoxia-induced perinatal white matter injury. *Ann. Neurol.* 60, 696–705. doi: 10.1002/ana.21008

Back, S. A., Riddle, A., and McClure, M. M. (2007). Maturation-dependent vulnerability of perinatal white matter in premature birth. *Stroke* 38(Suppl. 2), 724–730. doi: 10.1161/01.STR.0000254729.27386.05

Ball, G., Srinivasan, L., Aljabar, P., Counsell, S. J., Durighel, G., Hajnal, J. V., et al. (2013). Development of cortical microstructure in the preterm human brain. *Proc. Natl. Acad. Sci. USA* 110, 9541–9546. doi: 10.1073/pnas.1301652110

Barber, M., Andrews, W. D., Memi, F., Gardener, P., Ciantar, D., Tata, M., et al. (2018). Vascular-derived Vegfa promotes cortical interneuron migration and proximity to the vasculature in the developing forebrain. *Cereb. Cortex* 28, 2577–2593. doi: 10.1093/cercor/bhy082

Barnes, S. A., Pinto-Duarte, A., Kappe, A., Zembrzycki, A., Metzler, A., Mukamel, E. A., et al. (2015). Disruption of mGluR5 in parvalbumin-positive interneurons induces core features of neurodevelopmental disorders. *Mol. Psychiatry* 20, 1161–1172. doi: 10.1038/mp.2015.113

Bartos, M., Vida, I., and Jonas, P. (2007). Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks. *Nat. Rev. Neurosci.* 8, 45–56. doi: 10.1038/nrn2044

Batalle, D., O'Muircheartaigh, J., Makropoulos, A., Kelly, C. J., Dimitrova, R., Hughes, E. J., et al. (2019). Different patterns of cortical maturation before and after 38 weeks gestational age demonstrated by diffusion MRI *in vivo*. *NeuroImage* 185, 764–775. doi: 10.1016/j.neuroimage.2018.05.046

Beurdeley, M., Spatazza, J., Lee, H. H., Sugiyama, S., Bernard, C., Di Nardo, A. A., et al. (2012). Otx2 binding to perineuronal nets persistently regulates plasticity in the mature visual cortex. *J. Neurosci.* 32, 9429–9437. doi: 10.1523/JNEUROSCI.0394-12.2012

Bitanihirwe, B. K., and Woo, T. U. (2014). Perineuronal nets and schizophrenia: the importance of neuronal coatings. *Neurosci. Biobehav. Rev.* 45, 85–99. doi: 10.1016/j.neubiorev.2014.03.018

Boardman, J. P., Counsell, S. J., Rueckert, D., Kapellou, O., Bhatia, K. K., Aljabar, P., et al. (2006). Abnormal deep grey matter development following preterm birth detected using deformation-based morphometry. *NeuroImage* 32, 70–78. doi: 10.1016/j.neuroimage.2006.03.029

Buser, J. R., Segovia, K. N., Dean, J. M., Nelson, K., Beardsley, D., Gong, X., et al. (2010). Timing of appearance of late oligodendrocyte progenitors coincides with enhanced susceptibility of preterm rabbit cerebral white matter to hypoxia-ischemia. *J. Cereb. Blood Flow Metab.* 30, 1053–1065. doi: 10.1038/jcbfm.2009.286

Butt, S. J., Fuccillo, M., Nery, S., Noctor, S., Kriegstein, A., Corbin, J. G., et al. (2005). The temporal and spatial origins of cortical interneurons predict their physiological subtype. *Neuron* 48, 591–604. doi: 10.1016/j.neuron.2005.09.034

Butt, S. J., Stacey, J. A., Teramoto, Y., and Vagnoni, C. (2017). A role for GABAergic interneuron diversity in circuit development and plasticity of the neonatal cerebral cortex. *Curr. Opin. Neurobiol.* 43, 149–155. doi: 10.1016/j.conb.2017.03.011

Canetta, S., Bolkan, S., Padilla-Coreano, N., Song, L. J., Sahn, R., Harrison, N. L., et al. (2016). Maternal immune activation leads to selective functional deficits in offspring parvalbumin interneurons. *Mol. Psychiatry* 21, 956–968. doi: 10.1038/mp.2015.222

Clancy, B., Darlington, R. B., and Finlay, B. L. (2001). Translating developmental time across mammalian species. *Neuroscience* 105, 7–17. doi: 10.1016/S0306-4522(01)00171-3

Clancy, B., Finlay, B. L., Darlington, R. B., and Anand, K. J. (2007). Extrapolating brain development from experimental species to humans. *Neurotoxicology* 28, 931–937. doi: 10.1016/j.neuro.2007.01.014

Coelewij, L., and Curtis, D. (2018). Mini-review: update on the genetics of schizophrenia. *Ann. Hum. Genet.* 82, 239–243. doi: 10.1111/ahg.12259

Cossart, R. (2011). The maturation of cortical interneuron diversity: how multiple developmental journeys shape the emergence of proper network function. *Curr. Opin. Neurobiol.* 21, 160–168. doi: 10.1016/j.conb.2010.10.003

Counsell, S. J., Allsop, J. M., Harrison, M. C., Larkman, D. J., Kennea, N. L., Kapellou, O., et al. (2003). Diffusion-weighted imaging of the brain in preterm infants with focal and diffuse white matter abnormality. *NeoReviews* 112, 1–7. doi: 10.1542/peds.112.1.1

Counsell, S. J., Edwards, A. D., Chew, A. T., Anjari, M., Dyet, L. E., Srinivasan, L., et al. (2008). Specific relations between neurodevelopmental abilities and white matter microstructure in children born preterm. *Brain* 131, 3201–3208. doi: 10.1093/brain/awn268

Dark, C., Homman-Ludiye, J., and Bryson-Richardson, R. J. (2018). The role of ADHD associated genes in neurodevelopment. *Dev. Biol.* 438, 69–83. doi: 10.1016/j.ydbio.2018.03.023

Dean, J. M., Farrag, D., Zahkouk, S. A., El Zawahry, E. Y., Hagberg, H., Kjellmer, I., et al. (2009). Cerebellar white matter injury following systemic endotoxemia in preterm fetal sheep. *Neuroscience* 160, 606–615. doi: 10.1016/j.neuroscience.2009.02.071

Dean, J. M., McClendon, E., Hansen, K., Azimi-Zonooz, A., Chen, K., Riddle, A., et al. (2013). Prenatal cerebral ischemia disrupts MRI-defined cortical microstructure through disturbances in neuronal arborization. *Sci. Transl. Med.* 5:168ra167. doi: 10.1126/scitranslmed.3004669

Deidda, G., Bozarth, I. F., and Cancedda, L. (2014). Modulation of GABAergic transmission in development and neurodevelopmental disorders: investigating physiology and pathology to gain therapeutic perspectives. *Front. Cell. Neurosci.* 8:119. doi: 10.3389/fncel.2014.00119

Favrais, G., van de Looij, Y., Fleiss, B., Ramanantsoa, N., Bonnin, P., Stoltenburg-Didinger, G., et al. (2011). Systemic inflammation disrupts the developmental program of white matter. *Ann. Neurol.* 70, 550–565. doi: 10.1002/ana.22489

Favuzzi, E., Marques-Smith, A., Deogracias, R., Winterflood, C. M., Sanchez-Aguilera, A., Mantoan, L., et al. (2017). Activity-dependent gating of parvalbumin interneuron function by the perineuronal net protein brevican. *Neuron* 95, 639.e10–655.e10. doi: 10.1016/j.neuron.2017.06.028

Filice, F., Vorckel, K. J., Sungur, A. O., Wohr, M., and Schwaller, B. (2016). Reduction in parvalbumin expression not loss of the parvalbumin-expressing GABA interneuron subpopulation in genetic parvalbumin and shank mouse models of autism. *Mol. Brain* 9:10. doi: 10.1186/s13041-016-0192-8

Fowke, T. M., Galinsky, R., Davidson, J. O., Wassink, G., Karunasinghe, R. N., Prasad, J. D., et al. (2018). Loss of interneurons and disruption of perineuronal nets in the cerebral cortex following hypoxia-ischaemia in near-term fetal sheep. *Sci. Rep.* 8:17686. doi: 10.1038/s41598-018-36083-y

Franz, A. P., Bolat, G. U., Bolat, H., Matijasevich, A., Santos, I. S., Silveira, R. C., et al. (2018). Attention-deficit/hyperactivity disorder and very preterm/very low birth weight: a meta-analysis. *Pediatrics* 141:e20171645. doi: 10.1542/peds.2017-1645

Galtrey, C. M., and Fawcett, J. W. (2007). The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. *Brain Res. Rev.* 54, 1–18. doi: 10.1016/j.brainresrev.2006.09.006

Gandal, M. J., Nesbitt, A. M., McCurdy, R. M., and Alter, M. D. (2012). Measuring the maturity of the fast-spiking interneuron transcriptional program in autism, schizophrenia, and bipolar disorder. *PLoS One* 7:e41215. doi: 10.1371/journal.pone.0041215

Garcez, P. P., Stolp, H. B., Sravanam, S., Christoff, R. R., Ferreira, J., Dias, A. A., et al. (2018). Zika virus impairs the development of blood vessels in a mouse model of congenital infection. *Sci. Rep.* 8:12774. doi: 10.1038/s41598-018-31149-3

Gutierrez, H., and Davies, A. M. (2007). A fast and accurate procedure for deriving the Sholl profile in quantitative studies of neuronal morphology. *J. Neurosci. Methods* 163, 24–30. doi: 10.1016/j.jneumeth.2007.02.002

Hagberg, H., Gressens, P., and Mallard, C. (2012). Inflammation during fetal and neonatal life: implications for neurologic and neuropsychiatric disease in children and adults. *Ann. Neurol.* 71, 444–457. doi: 10.1002/ana.22620

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., et al. (2015). The role of inflammation in perinatal brain injury. *Nat. Rev. Neurol.* 11, 192–208. doi: 10.1038/nrneurol.2015.13

Hamrick, S. E., Miller, S. P., Leonard, C., Glidden, D. V., Goldstein, R., Ramaswamy, V., et al. (2004). Trends in severe brain injury and neurodevelopmental outcome in premature newborn infants: the role of cystic periventricular leukomalacia. *J. Pediatr.* 145, 593–599. doi: 10.1016/j.jpeds.2004.05.042

Hashemi, E., Ariza, J., Rogers, H., Noctor, S. C., and Martinez-Cerdeno, V. (2018). The number of parvalbumin-expressing interneurons is decreased in the prefrontal cortex in autism. *Cereb. Cortex* 28, 1931–1943. doi: 10.1093/cercor/bhw021

Jiang, N. M., Cowan, M., Moonah, S. N., and Petri, W. A. Jr. (2018). The impact of systemic inflammation on neurodevelopment. *Trends Mol. Med.* 24, 794–804. doi: 10.1016/j.molmed.2018.06.008

Johnson, S., Hollis, C., Kochhar, P., Hennessy, E., Wolke, D., and Marlow, N. (2010). Psychiatric disorders in extremely preterm children: longitudinal finding at age 11 years in the EPICure study. *J. Am. Acad. Child Adolesc. Psychiatry* 49, 453.e1–463.e1. doi: 10.1016/j.jaac.2010.02.002

Jones, E. G. (2009). The origins of cortical interneurons: mouse versus monkey and human. *Cereb. Cortex* 19, 1953–1956. doi: 10.1093/cercor/bhp088

Kataoka, Y., Kalanithi, P. S., Grantz, H., Schwartz, M. L., Saper, C., Leckman, J. F., et al. (2010). Decreased number of parvalbumin and cholinergic interneurons in the striatum of individuals with Tourette syndrome. *J. Comp. Neurol.* 518, 277–291. doi: 10.1002/cne.22206

Kelsom, C., and Lu, W. (2013). Development and specification of GABAergic cortical interneurons. *Cell Biosci.* 3:19. doi: 10.1186/2045-3701-3-19

Kersbergen, K. J., Leroy, F., Isgum, I., Groenendaal, F., de Vries, L. S., Claessens, N. H. P., et al. (2016). Relation between clinical risk factors, early cortical changes, and neurodevelopmental outcome in preterm infants. *NeuroImage* 142, 301–310. doi: 10.1016/j.neuroimage.2016.07.010

Keunen, K., Benders, M. J., Leemans, A., Fieret-Van Stam, P. C., Scholtens, L. H., Viergever, M. A., et al. (2017). White matter maturation in the neonatal brain is predictive of school age cognitive capacities in children born very preterm. *Dev. Med. Child Neurol.* 59, 939–946. doi: 10.1111/dmcn.13487

Kinney, H. C., Haynes, R. L., Xu, G., Andiman, S. E., Folkerth, R. D., Sleeper, L. A., et al. (2012). Neuron deficit in the white matter and subplate in periventricular leukomalacia. *Ann. Neurol.* 71, 397–406. doi: 10.1002/ana.22612

Korzeniewski, S. J., Allred, E. N., Joseph, R. M., Heeren, T., Kuban, K. C. K., O'Shea, T. M., et al. (2017). Neurodevelopment at age 10 years of children born <28 weeks with fetal growth restriction. *Pediatrics* 140:e20170697. doi: 10.1542/peds.2017-0697

Krishnan, M. L., Van Steenwinckel, J., Schang, A. L., Yan, J., Arnadottir, J., Le Charpentier, T., et al. (2017). Integrative genomics of microglia implicates DLG4 (PSD95) in the white matter development of preterm infants. *Nat. Commun.* 8, 428–439. doi: 10.1038/s41467-017-00422-w

Kuban, K. C., O'Shea, T. M., Allred, E. N., Fichorova, R. N., Heeren, T., Paneth, N., et al. (2015). The breadth and type of systemic inflammation and the risk of adverse neurological outcomes in extremely low gestation newborns. *Pediatr. Neurol.* 52, 42–48. doi: 10.1016/j.pediatrneurol.2014.10.005

Lawrence, Y. A., Kemper, T. L., Bauman, M. L., and Blatt, G. J. (2010). Parvalbumin-, calbindin-, and calretinin-immunoreactive hippocampal interneuron density in autism. *Acta Neurol. Scand.* 121, 99–108. doi: 10.1111/j.1600-0404.2009.01234.x

Lean, R. E., Melzer, T. R., Bora, S., Watts, R., and Woodward, L. J. (2017). Attention and regional Gray matter development in very preterm children at age 12 years. *J. Int. Neuropsychol. Soc.* 23, 539–550. doi: 10.1017/S1355617717000388

Mallard, C., Welin, A. K., Peebles, D., Hagberg, H., and Kjellmer, I. (2003). White matter injury following systemic endotoxemia or asphyxia in the fetal sheep. *Neurochem. Res.* 28, 215–223. doi: 10.1023/A:1022368915400

Marin, O. (2012). Interneuron dysfunction in psychiatric disorders. *Nat. Rev. Neurosci.* 13, 107–120. doi: 10.1038/nrn3155

Marin, O. (2013). Cellular and molecular mechanisms controlling the migration of neocortical interneurons. *Eur. J. Neurosci.* 38, 2019–2029. doi: 10.1111/ejn.12225

Marlow, N., Wolke, D., Bracewell, M. A., Samara, M., and E. P. S. Group (2005). Neurologic and developmental disability at six years of age after extremely preterm birth. *N. Engl. J. Med.* 352, 9–19. doi: 10.1056/NEJMoa041367

Marques-Smith, A., Lyngholm, D., Kaufmann, A. K., Stacey, J. A., Hoerder-Suabedissen, A., Becker, E. B., et al. (2016). A transient translaminar GABAergic interneuron circuit connects thalamocortical recipient layers in neonatal somatosensory cortex. *Neuron* 89, 536–549. doi: 10.1016/j.neuron.2016.01.015

McClendon, E., Chen, K., Gong, X., Sharifnia, E., Hagen, M., Cai, V., et al. (2014). Prenatal cerebral ischemia triggers dysmaturation of caudate projection neurons. *Ann. Neurol.* 75, 508–524. doi: 10.1002/ana.24100

McClendon, E., Shaver, D. C., Degener-O'Brien, K., Gong, X., Nguyen, T., Hoerder-Suabedissen, A., et al. (2017). Transient hypoxemia chronically disrupts maturation of preterm Fetal ovine subplate neuron arborization and activity. *J. Neurosci.* 37, 11912–11929. doi: 10.1523/JNEUROSCI.2396-17.2017

O'Shea, T. M., Allred, E. N., Dammann, O., Hirtz, D., Kuban, K. C., Paneth, N., et al. (2009). The ELGAN study of the brain and related disorders in extremely low gestational age newborns. *Early Hum. Dev.* 85, 719–725. doi: 10.1016/j.earlhumdev.2009.08.060

Panda, S., Dohare, P., Jain, S., Parikh, N., Singla, P., Mehdizadeh, R., et al. (2018). Estrogen treatment reverses prematurity-induced disruption in cortical interneuron population. *J. Neurosci.* 38, 7378–7391. doi: 10.1523/JNEUROSCI.0478-18.2018

Pardo, C. A., and Eberhart, C. G. (2007). The neurobiology of autism. *Brain Pathol.* 17, 434–447. doi: 10.1111/j.1750-3639.2007.00102.x

Paxinos, G., and Franklin, K. (2012). *Paxinos and Franklin's the mouse brain in stereotaxic coordinates* (Australia: Academic Press).

Phillips, M., and Pozzo-Miller, L. (2015). Dendritic spine dysgenesis in autism related disorders. *Neurosci. Lett.* 601, 30–40. doi: 10.1016/j.neulet.2015.01.011

Pinto, D., Pagnamenta, A. T., Klei, L., Anney, R., Merico, D., Regan, R., et al. (2010). Functional impact of global rare copy number variation in autism spectrum disorders. *Nature* 466, 368–372. doi: 10.1038/nature09146

Rangon, C. M., Schang, A. L., Van Steenwinckel, J., Schwendimann, L., Lebon, S., Fu, T., et al. (2018). Myelination induction by a histamine H3 receptor antagonist in a mouse model of preterm white matter injury. *Brain Behav. Immun.* 74, 265–276. doi: 10.1016/j.bbi.2018.09.017

Rees, S., Harding, R., and Walker, D. (2008). An adverse intrauterine environment: implications for injury and altered development of the brain. *Int. J. Dev. Neurosci.* 26, 3–11. doi: 10.1016/j.ijdevneu.2007.08.020

Schang, A. L., Van Steenwinckel, J., Chevenne, D., Alkmark, M., Hagberg, H., Gressens, P., et al. (2014). Failure of thyroid hormone treatment to prevent inflammation-induced white matter injury in the immature brain. *Brain Behav. Immun.* 37, 95–102. doi: 10.1016/j.bbi.2013.11.005

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., and Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. *Prog. Neurobiol.* 106-107, 1–16. doi: 10.1016/j.pneurobio.2013.04.001

Shen, H. H. (2018). Core concept: perineuronal nets gain prominence for their role in learning, memory, and plasticity. *Proc. Natl. Acad. Sci. USA* 115, 9813–9815. doi: 10.1073/pnas.1815273115

Smrt, R. D., and Zhao, X. (2010). Epigenetic regulation of neuronal dendrite and dendritic spine development. *Front. Biol.* 5, 304–323. doi: 10.1007/s11515-010-0650-0

Stolp, H. B., Dziegielewska, K. M., Ek, C. J., Potter, A. M., and Saunders, N. R. (2005). Long-term changes in blood-brain barrier permeability and white matter following prolonged systemic inflammation in early development in the rat. *Eur. J. Neurosci.* 22, 2805–2816. doi: 10.1111/j.1460-9568.2005.04483.x

Stolp, H. B., Turnquist, C., Dziegielewska, K. M., Saunders, N. R., Anthony, D. C., and Molnar, Z. (2011). Reduced ventricular proliferation in the foetal cortex following maternal inflammation in the mouse. *Brain* 134, 3236–3248. doi: 10.1093/brain/awr237

Supramaniam, V., Vontell, R., Srinivasan, L., Wyatt-Ashmead, J., Hagberg, H., and Rutherford, M. (2013). Microglia activation in the extremely preterm human brain. *Pediatr. Res.* 73, 301–309. doi: 10.1038/pr.2012.186

Tusor, N., Benders, M. J., Counsell, S. J., Nongena, P., Ederies, M. A., Falconer, S., et al. (2017). Punctate white matter lesions associated with altered brain development and adverse motor outcome in preterm infants. *Sci. Rep.* 7:13250. doi: 10.1038/s41598-017-13753-x

Ueno, H., Suemitsu, S., Okamoto, M., Matsumoto, Y., and Ishihara, T. (2017). Parvalbumin neurons and perineuronal nets in the mouse prefrontal cortex. *Neurosci.* 343, 115–127.

Van de Berg, W. D., Kwaijtaal, M., de Louw, A. J., Lissone, N. P., Schmitz, C., Faull, R. L., et al. (2003). Impact of perinatal asphyxia on the GABAergic and locomotor system. *Neuroscience* 117, 83–96. doi: 10.1016/S0306-4522(02)00787-X

Verney, C., Rees, S., Biran, V., Thompson, M., Inder, T., and Gressens, P. (2010). Neuronal damage in the preterm baboon: impact of the mode of ventilatory support. *J. Neuropathol. Exp. Neurol.* 69, 473–482. doi: 10.1097/NEN.0b013e3181dac07b

Vogt, D., Cho, K. K. A., Shelton, S. M., Paul, A., Huang, Z. J., Sohal, V. S., et al. (2018). Mouse Cntnap2 and human CNTNAP2 ASD alleles cell autonomously regulate PV+ cortical interneurons. *Cereb. Cortex* 28, 3868–3879. doi: 10.1093/cercor/bhx248

Vontell, R., Supramaniam, V., Thornton, C., Wyatt-Ashmead, J., Mallard, C., Gressens, P., et al. (2013). Toll-like receptor 3 expression in glia and neurons alters in response to white matter injury in preterm infants. *Dev. Neurosci.* 35, 130–139. doi: 10.1159/000346158

Vontell, R., Supramaniam, V., Wyatt-Ashmead, J., Gressens, P., Rutherford, M., Hagberg, H., et al. (2015). Cellular mechanisms of toll-like receptor-3 activation in the thalamus are associated with white matter injury in the developing brain. *J. Neuropathol. Exp. Neurol.* 74, 273–285. doi: 10.1097/NEN.0000000000000172

Wohr, M., Orduz, D., Gregory, P., Moreno, H., Khan, U., Vorckel, K. J., et al. (2015). Lack of parvalbumin in mice leads to behavioral deficits relevant to all human autism core symptoms and related neural morphofunctional abnormalities. *Transl. Psychiatry* 5:e525. doi: 10.1038/tp.2015.19

Wood, N. S., Costeloe, K., Gibson, A. T., Hennessy, E. M., Marlow, N., Wilkinson, A. R., et al. (2005). The EPICure study: associations and antecedents of neurological and developmental disability at 30 months of age following extremely preterm birth. *Arch. Dis. Child. Fetal Neonatal Ed.* 90, F134–F140. doi: 10.1136/adc.2004.052407

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Stolp, Fleiss, Arai, Supramaniam, Vontell, Birtles, Yates, Baburamani, Thornton, Rutherford, Edwards and Gressens. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 02 August 2019
doi: 10.3389/fphys.2019.00990

Impact of High-Dose Caffeine on the Preterm Ovine Cerebrum and Cerebellum

*Anzari Atik[1], Robert De Matteo[1], Meghan Boomgardt[2], Sandra Rees[3], Richard Harding[1], Jeanie Cheong[4], Shreya Rana[2], Kelly Crossley[2] and Mary Tolcos[2,5]**

[1] Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC, Australia, [2] The Ritchie Centre, Hudson Institute of Medical Research, Department of Obstetrics and Gynecology, Monash University, Clayton, VIC, Australia, [3] Department of Anatomy and Neuroscience, The University of Melbourne, Melbourne, VIC, Australia, [4] Department of Neonatal Services, Royal Women's Hospital, Victorian Infant Brain Studies, Murdoch Children's Research Institute, and Department of Obstetrics and Gynaecology, The University of Melbourne, Melbourne, VIC, Australia, [5] School of Health and Biomedical Sciences, RMIT University, Bundoora, VIC, Australia

Caffeine is one of the few treatments available for infants with apnea of prematurity. As the recommended dosing regimen is not always sufficient to prevent apnea, higher doses may be prescribed. However, little is currently known about the impact of high-dose caffeine on the developing brain; thus, our aim was to investigate the consequences of a high-dose regimen on the immature ovine brain. High-dose caffeine (25 mg/kg caffeine base loading dose; 20 mg/kg daily maintenance dose; $n = 9$) or saline ($n = 8$) was administered to pregnant sheep from 105 to 118 days of gestation (DG; term = 147 days); this is broadly equivalent to 28–33 weeks of human gestation. At 119DG, the cerebral cortex, striatum, and cerebellum were assessed histologically and by immunohistochemistry. Compared with controls, caffeine-exposed fetuses showed (i) an increase in the density of Ctip2-positive layers V–VI projection neurons ($p = 0.02$), Tbr1-positive layers V–VI projection neurons ($p < 0.0001$), astrocytes ($p = 0.03$), and oligodendrocytes ($p = 0.02$) in the cerebral cortex, (ii) a decrease in the density of Cux1-positive layers II–IV projection neurons ($p = 0.01$) in the cerebral cortex, and (iii) a reduction in the area of Purkinje cell bodies in the cerebellum ($p = 0.03$). Comparing high-dose caffeine-exposed fetuses with controls, there was no difference ($p > 0.05$) in: (i) the volume of the cerebral cortex or striatum, (ii) the density of neurons (total and output projection neurons) in the striatum, (iii) dendritic spine density of layer V pyramidal cells, (iv) the density of cortical GABAergic interneurons, microglia, mature oligodendrocytes or proliferating cells, (v) total cerebellar area or dimensions of cerebellar layers, or (vi) the density of cerebellar white matter microglia, astrocytes, oligodendrocytes, or myelin. Daily exposure of the developing brain to high-dose caffeine affects some aspects of neuronal and glial development in the cerebral cortex and cerebellum in the short-term; the long-term structural and functional consequences of these alterations need to be investigated.

Keywords: apnea of prematurity, cerebral cortex, sheep, striatum, neurodevelopment

OPEN ACCESS

Edited by:
Charles Evans Wood, University of Florida, United States

Reviewed by:
Barbara Stonestreet, Women & Infants Hospital of Rhode Island, United States
Jayanth Ramadoss, Texas A&M University, United States

***Correspondence:**
Mary Tolcos
mary.tolcos@rmit.edu.au

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *29 May 2019*
Accepted: *18 July 2019*
Published: *02 August 2019*

Citation:
Atik A, De Matteo R, Boomgardt M, Rees S, Harding R, Cheong J, Rana S, Crossley K and Tolcos M (2019) Impact of High-Dose Caffeine on the Preterm Ovine Cerebrum and Cerebellum. Front. Physiol. 10:990. doi: 10.3389/fphys.2019.00990

INTRODUCTION

Caffeine therapy has become an integral part of management of apnea of prematurity in very preterm infants. Infants who do not respond to the standard recommended caffeine treatment (20 mg/kg caffeine citrate (loading) followed by 5–10 mg/kg daily) may require higher doses (Scanlon et al., 1992; Steer et al., 2003; Mohammed et al., 2015). However there is a paucity of evidence to show that doses above the "standard dose" are safe for the developing brain. We have previously reported that daily high-dose caffeine had no detectable effects on the cerebral white matter (WM) of the immature ovine brain (Atik et al., 2014). Here we report the effects of high-dose caffeine on the developing cerebral cortex, striatum, and cerebellum in a clinically appropriate animal model.

Previous studies have shown both beneficial (Juarez-Mendez et al., 2006; Connolly and Kingsbury, 2010) and detrimental effects (Fuller et al., 1982; Kang et al., 2002; Desfrere et al., 2007; Black et al., 2008) of caffeine on the developing brain, using a range of animal models. Thus the impact of caffeine on the developing brain at the cellular level remains contentious. Reported beneficial effects of high-dose caffeine exposure include increased total dendritic length and arborization of layer III pyramidal neurons of the prefrontal cortex of rats (Juarez-Mendez et al., 2006) and reduced seizure susceptibility to some chemo-convulsants in both juvenile and adult rats (Guillet and Dunham, 1995). Caffeine administered to rat pups significantly reduced brain injury caused by hypoxia-ischemia, as indicated by a reduction in neuronal necrosis and infarction (Bona et al., 1995). Caffeine has also been associated with adverse effects on the developing brain including a reduction in cell proliferation in the subventricular zone (SVZ) and dentate gyrus, a reduction in astrocytogenesis in the cerebral cortex (Desfrere et al., 2007), and increased apoptosis throughout the cerebral hemispheres (Kang et al., 2002; Black et al., 2008). In preterm infants, high-dose caffeine treatment during the first 24 h of life increases the incidence of cerebellar hemorrhage compared with infants treated with standard doses of caffeine, with no apparent injury to the cerebellar white or gray matter (McPherson et al., 2015).

Previous studies on the neurodevelopmental consequences of caffeine exposure have largely been conducted in rodents, a species in which key aspects of brain development differ from humans in their timing relative to birth. Furthermore, these studies have used different regimens of caffeine administration, including a variety of doses, routes and durations, making it difficult not only to extrapolate findings to preterm infants, but also to draw conclusions on the cellular effects of high-dose caffeine. Here we have used sheep, a long-gestation species in which the gestational timing of major events in brain development is similar to that in humans. Importantly neurodevelopmental events occurring in the sheep fetus are similar to those occurring in the very preterm human infant.

It is likely that a degree of cerebral hypoxia is present in preterm infants with apnea of prematurity; this is often difficult to quantify precisely, and the direct association between acute intermittent hypoxia and specific brain injury is speculative. Here, and in a previous study (Atik et al., 2014), we have sought to examine the effects of 2 weeks of high-dose caffeine exposure on the developing brain, without the potentially confounding effects of cerebral hypoxia. We have already demonstrated that such exposure does not affect the microstructure of the cerebral WM (Atik et al., 2014). In the present study we assessed aspects of the microstructure of the cerebral cortex, striatum and cerebellum as these brain regions are critical to cognition, memory and learning, and neuronal loss and/or gliosis in these regions could lead to long-term deficits in neurocognitive function (Pierson et al., 2007). Importantly, deficits in these key aspects of brain function have been reported in very preterm infants (Saigal and Doyle, 2008).

Abbreviations: CC, corpus callosum; CN, caudate nucleus; Ctip2, COUP-TF interacting protein 2; Cux1, cut-like homeobox 1; Cx, cortex; DG, days of gestation; EGL, external granule layer; GFAP, glial fibrillary acidic protein; Hi, hippocampus; Iba-1, ionized binding adaptor molecule-1; IGL, internal granule layer; IR, immunoreactive; MB, midbrain; MBP, myelin basic protein; ML, molecular layer; NeuN, neuronal nuclei; OD, optical density; Olig2, oligodendrocyte transcription factor 2; Pu, putamen; St, striatum; SST, somatostatin; SVZ, subventricular zone; Tbr1, T-box brain 1; WM, white matter.

MATERIALS AND METHODS

Ethics Statement

All experimental procedures were approved by the Monash University Animal Ethics Committee.

Experimental Protocol

Date-mated pregnant ewes (Merino × Border Leicester, $n = 14$) underwent aseptic surgery at 99 days of gestation (DG; term is approximately 147DG) for implantation of catheters into a fetal femoral artery and vein, the amniotic sac and a maternal jugular vein as previously described (Atik et al., 2014). Five days after the surgery, a loading dose of caffeine base (25 mg/kg maternal weight, maternal i.v., Sigma-Aldrich, St. Louis, MO, United States) was administered to 9 of the ewes carrying either male or female fetuses, on 104DG (0.7 of term) followed by a daily maintenance dose (20 mg/kg maternal weight, maternal i.v.) from 105 to 118DG (0.7–0.8 of term; broadly equivalent to 28–33 weeks of human gestation) (Atik et al., 2014); control ewes received i.v. saline ($n = 8$, male and female fetuses). The caffeine dosing regimen was chosen in consultation with clinicians involved in the project and represents higher than normal doses which are similar to those currently being used in some neonatal intensive care units (Steer et al., 2003). It is necessary to administer caffeine to the ewe (based on maternal body weight) as our initial experiments revealed that caffeine administered directly to the fetus was rapidly lost to the ewe's circulation via placental transport; intravenous infusion of caffeine (25 mg/kg followed by 20 mg/kg caffeine for 2 days) directly to the fetus via its femoral vein increased fetal plasma caffeine concentrations by < 0.1 mg/L. We administered caffeine base rather than caffeine citrate to minimize the volume required. Caffeine citrate contains anhydrous citric acid and 50% anhydrous caffeine base; thus the dose of caffeine base is approximately half that of caffeine citrate. Caffeine concentrations, body and organ weights, and

physiological parameters were measured and have been reported previously (Atik et al., 2014). In brief, caffeine exposure did not alter fetal body weight, arterial pressure or oxygenation; the maximal fetal plasma caffeine concentration achieved (32 mg/L) was high relative to the range of concentrations measured in preterm infants treated with a standard dose. Both treatment groups received the same feed (lucerne chaff [export quality]; Southern Cross Feeds).

Tissue Collection

At 119DG (0.8 of term), ewes and fetuses were euthanized using an overdose of sodium pentobarbitone (130 mg/kg i.v.) and the fetuses delivered via Caesarean section. Fetal brains were transcardially perfused *in situ* with isotonic saline and 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The entire forebrain was cut coronally into blocks 5 mm thick (8–10 blocks per animal) and the cerebellum was bisected at the midline of the vermis. Blocks of the entire right cerebral and right cerebellar hemispheres were then post-fixed in 4% paraformaldehyde (4 days, 4°C) and embedded in paraffin. The analyses described below have been applied to fetal brains obtained in a previous study (Atik et al., 2014) but are entirely different from the analyses previously reported.

Histology and Immunohistochemistry

Serial sections (8 μm thick) were cut from each block of the cerebral and cerebellar hemispheres and 1 section per block stained with thionin or hematoxylin and eosin (cerebellum only) and qualitatively examined for hemorrhages and gross structural alterations. One section from equivalent sites from each of the four lobes of the right cerebral hemisphere (four sections per animal) and two sections of the cerebellum (separated by 80 μm) from each animal were immunostained to identify mature neurons (NeuN; cerebral hemispheres only), post-mitotic projection neurons in layers II–IV (cut-like homeobox gene, Cux1; cerebral hemispheres only), layers V–VI (Ctip2; cerebral hemispheres only), and layers V–VI [Tbr1; predominantly layer VI projection neurons (Hevner et al., 2001); cerebral hemispheres only], GABAergic interneurons (SST; cerebral hemispheres only), microglia (Iba-1), astrocytes (GFAP), oligodendrocytes within the entire lineage (Olig2), mature myelinating oligodendrocytes and myelinated axons (MBP), proliferating cells (Ki67; cerebral hemispheres only) and Purkinje cells (Calbindin; cerebellum only) (**Table 1**). Immunostaining was performed using the avidin-biotin complex elite kit (Vector Laboratories, Burlingame, CA, United States) as previously described (Atik et al., 2014). For each antibody, sections from control and caffeine-treated animals were stained simultaneously to reduce variability. There was no staining when the primary antibodies were omitted.

Golgi-Cox Staining

For each fetus, one block from the left cerebral hemisphere (approximately section 1160 according to the Michigan State University sheep brain atlas[1]) was processed for Golgi-cox impregnation (FD Rapid Golgi Stain Kit; FD Neurotechnologies, Inc., Columbia, MD, United States). Blocks were serially sectioned (100 μm thick) in the coronal plane with a cryostat (Leica CM3050, Leica Microsystems, Pty Ltd., Australia). Sections were mounted onto gelatin-coated slides, processed for Golgi visualization using materials supplied in the kit, dehydrated in graded alcohols and coverslipped.

Histological and Immunohistochemical Analysis

Analyses were performed on coded slides (observer blinded to group) from the right cerebral hemisphere and cerebellum (at the level of the vermis) using an image analysis system (ImageScope, Aperio Technologies, San Diego, CA, United States). Immunohistochemical analyses were performed on one section from each of the frontal, parietal, temporal, and occipital lobes (Ki67, parietal and temporal only) from each fetus; the same region within the gray matter and striatum was assessed in each animal (**Figure 1**). For the cerebellum, all analyses were performed on two sections per animal, and assessments made in early (lobule I or X) and late (lobule VII or VIII) developing lobules (Altman, 1969), and the deep WM, in randomly selected fields with the exception of total cross-sectional cerebellar areal measurements (see below). Data from early and late developing lobules and the deep WM were combined and averaged, except for the assessment of cerebellar layers. For each immunostain, the mean cell density was calculated for each fetus and for each region or bin, and a mean of means for control and caffeine-treated groups determined.

Volumetric Analysis of the Cerebral Cortex and Striatum

The cross-sectional areas of the cerebral cortex and striatum were separately measured in every 625th thionin-stained section (1 section per 5 mm block; n = 8 sections per animal) using a digitizer interfaced to the image analysis software (Tolcos et al., 2011). The total volume of the cerebral cortex and striatum were estimated according to the Cavalieri principle using the formula $V = \Sigma APt$, where V is the total volume, ΣA is the sum of the areas measured, P is the inverse of the sampling fraction and t is the section thickness (Gundersen et al., 1999).

Morphological Assessment of the Cerebellum

Cerebellar sections were scanned for signs of hemorrhage or overt damage. The cross-sectional area of the vermis was measured in two sections of the cerebellum (separated by 80 μm). Tracing of the total cerebellar area was performed at x4 objective on Aperio scanned slides. Automated area (mm^2) of the total cross-section of the cerebellum, the IGL and the WM was calculated by ImageScan Scope software. Area of the IGL and WM were expressed as a ratio of the total cerebellum area. The external granule layer (EGL) represents the transient progenitor site from which granule cells arise and thus differences in EGL width following high-dose caffeine administration would be indicative of developmental disturbances. In order to assess differences in the width of the EGL between control and caffeine cohorts, 40 sites per section were measured from the early developing (I or X) and late developing (VII or VIII) lobules (80 sites/cerebellum).

[1]https://www.msu.edu/∼brains/brains/sheep/index.html

TABLE 1 | Immunohistochemistry: primary and secondary antibodies.

1° antibody and dilution	Localizes	Supplier	2° antibody and dilution
*Mouse anti-NeuN 1:500	Mature neurons	MAB377; Millipore, Billerica, MA, United States	Biotinylated goat anti-mouse IgG; 1:200
*#Rat anti-Ctip2 1:500	Projection neurons in cortical layers V–VI	ab28448, Abcam, Cambridge, MA, United States	Biotinylated anti-rat IgG; 1:200
†#Rabbit anti-Cux1 1:200	Projection neurons in cortical layers II–IV	Orb156497, Biorbyt, Cambridge, United Kingdom	Biotinylated anti-rat IgG; 1:200
#Rabbit anti-Tbr1 1:100	Projection neurons in cortical layers V–VI	AB10554, Millipore, Billerica, MA, United States	Biotinylated goat anti-rabbit IgG; 1:200
ˆRabbit anti-SST 1:500	GABAergic interneurons	A0566, DAKO, Carpinteria, CA, United States	Biotinylated anti-rabbit IgG; 1:200
ˆMouse anti-Calbindin 1:500	Purkinje cells in cerebellum	SWANT	Biotinylated goat anti-mouse IgG; 1:200
*#Rabbit anti-Ki67 1:200	Proliferating cells in late G1, S, G2 and M phases	RM9106, Thermo Scientific, Waltham, MA, United States	Biotinylated anti-rabbit IgG; 1:200
*#Rabbit anti-Iba-1 1:1500 (cerebrum); 1:1000 (cerebellum)	Microglia	019-19741, WAKO Pure Chemical Industries, Osaka, Japan	Biotinylated anti-rabbit IgG; 1:200
*#Rabbit anti-GFAP 1:1000	Astrocytes	ZO2334, DAKO, Carpinteria, CA, United States	Biotinylated anti-rabbit IgG; 1:200
*Rabbit anti-Olig2 1:500	Oligodendrocytes	AB9610, Millipore, Billerica, MA, United States	Biotinylated anti-rabbit IgG; 1:200
*Rat anti-MBP 1:100	Mature myelinating oligodendrocytes	MAB395, Millipore, Billerica, MA, United States	Biotinylated anti-rat IgG; 1:200

*Pre-treated with *0.01 M citrate buffer (pH6) and microwaving; †Tris EDTA (pH9), heated in water bath; ˆ0.02% proteinase K (30 min, 37°C); #counterstained with 0.01% thionin.*

The width of the ML offers a surrogate measurement for assessing connectivity, as this layer is largely comprised of the Purkinje cell dendritic outgrowth as well as the parallel fibers of the granule cells. In order to assess the differences in the width of the ML following high-dose caffeine administration, 40 sites per cerebellar section were measured from the early developing (I or X) and late developing (VII or VIII) lobules (80 sites/cerebellum). Purkinje cell somal areas were measured in 40 calbindin-positive Purkinje cells (with a clearly defined cell body and nucleus) randomly selected from the early developing (I or X) and late developing (VII or VIII) lobules (80 cells/cerebellum). The mean Purkinje cell somal area was calculated for each fetus (late and early lobules combined), and a mean of means for control and caffeine-treated groups determined.

Analysis of Neurons, Glia, and Proliferating Cells in the Cerebral Cortex and Striatum

For each fetus, NeuN-immunoreactive (IR) neurons were counted in the striatum (3 fields in the caudate and 3 in putamen, total of 6 fields/section/fetus; field size, 0.40 mm^2) (**Figure 1**); assessment of NeuN-IR neuronal density in the cerebral cortex has previously been reported (Atik et al., 2014). Ctip2-IR neurons were counted in the cerebral cortex (1 field/gyrus, total of 3 fields/section/fetus, field size, 0.56 mm^2); each field was further divided into 3 bins (bin 1: cortical layer I; bin 2: layers II, III, and IV; bin 3: layers V and VI). Ctip2-IR neurons were also counted in the striatum (6 fields/section/fetus; field size, 0.40 mm^2). Cux1- and Tbr1-IR neurons were counted in layers II–IV (Cux1) or V–VI (Tbr1) (1 field/sulcus, total of 3 fields/section/fetus; field size, 0.75 mm^2). SST-IR interneurons were counted in 0.40 mm wide columns that spanned all 6 layers of the cerebral cortex (3 fields/gyrus; total of 12 fields/section/fetus; field size, 0.56 mm^2) and in the striatum (6 fields/section/fetus; field size, 0.40 mm^2).

Iba-1-IR microglia, GFAP-IR astrocytes, Olig2-IR oligodendrocytes, MBP-IR mature myelinating oligodendrocytes and Ki67-IR proliferating cells were counted in random fields of view throughout the cerebral cortex (12 fields/section/fetus; field size, 0.140 mm^2); Ki67-IR cells were also counted in the SVZ (9 fields/section/fetus; field size, 0.140 mm^2).

Analysis of the Dendritic Spine Density of Pyramidal Cells in the Cerebral Cortex

Dendritic spines were counted along the apical dendrite (50 μm segment and ∼5 μm from the soma) of layer V pyramidal cells (5 cells/one section/fetus) in region-matched, Golgi-stained sections from control (n = 6) and caffeine-treated (n = 7) fetuses (x100 magnification; oil immersion). The mean number of spines/10 μm segment of dendrite was calculated for each fetus and group means were obtained by averaging the mean values of each fetus. Pyramidal neurons were selected for analysis when they met the following criteria: (i) triangular-shaped soma with an apical dendrite perpendicular to the pial surface; (ii) complete impregnation of the cell with Golgi-stain that permitted visualization of the entire dendritic arbor; and (iii) processes were not obscured by other neurons, glia, or the vasculature. Basal dendrites were excluded from analysis due to the large degree of overlap with other dendrites.

Analysis of Glia and Myelination in the Cerebellum

In the cerebellum, the areal density of Iba-1-IR microglia and Olig2-IR oligodendrocytes were assessed in the WM [deep WM

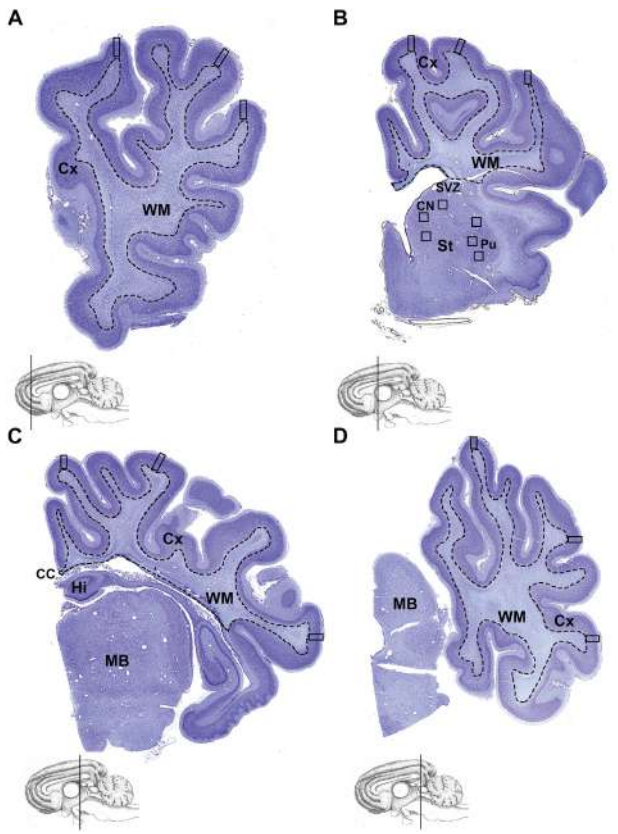


FIGURE 1 | Brain regions analyzed. Coronal, thionin-stained, hemi-sections of the cerebral hemisphere at the level of the **(A)** frontal, **(B)** parietal, **(C)** temporal, and **(D)** occipital lobe. Cerebral cortex (Cx; regions outside the dashed line) was examined for all histological and immunohistochemical analysis. Measurements were made in the cerebral cortex (squares indicate fields of view; 4 bins analyzed per square for NeuN; 3 bins analyzed per square for Ctip2; whole square without bins analyzed for SST). A total of 6 fields of view was analyzed from the striatum, with 3 fields of view selected from the caudate nucleus and 3 fields from the putamen **(B)**. Cx, cortex; CC, corpus callosum; Hi, hippocampus; St, striatum; SVZ, subventricular zone; CN, caudate nucleus; Pu, putamen; MB, midbrain.

and lobular WM combined; 8 fields/section/fetus; field size, 0.36 mm^2 (deep WM) and 0.12 mm^2 (lobular WM)]. The intensity of MBP-IR and GFAP-IR in the cerebellar WM was determined using a validated optical density (OD) analysis as described previously (Atik et al., 2014). From each section, OD was assessed in 3 fields from equivalent regions of the deep WM, 2 fields from equivalent regions of the WM from early developing (I or X) lobules, and 2 fields from equivalent regions of the WM from late developing (VII or VIII) lobules (total of 7 fields from each section). A correction was applied to each of these images by subtracting the OD measurement from a region of background staining. The mean OD was then calculated within each region, for each animal, and a mean of means determined for control and caffeine-treated animals. Imaging and analysis of each of the immunostains was performed in a single day using identical parameters to maintain consistency and eliminate error.

Qualitative Assessment of GFAP-IR in the Cerebellum

The morphology of GFAP-IR Bergmann glia was assessed qualitatively for evidence of fiber disorganization (tangled or irregularly orientated fibers), and disrupted fiber integrity (damaged/degenerating fibers and disconnected fibers: i.e., those that did not extend the full length from the IGL to the upper EGL) as previously described (McDougall et al., 2017; Tolcos et al., 2018). The density of GFAP-IR astrocytes in the IGL and ML was also qualitatively assessed.

Statistical Analysis

All data were analyzed using SPSS software (Version 20, SPSS, Inc., Chicago, IL, United States). Differences between treatment groups were analyzed by the Student's *t*-test for parametric data; if data failed a variance test (*F*-test), a Mann–Whitney *U*-test (non-parametric data) was used. Data are presented as mean of means ± SEM with $p < 0.05$ considered significant.

RESULTS

Cerebral Cortex and Striatum

Volumetric Analysis

Fetal exposure to high-dose caffeine did not affect the volumes of cortical gray matter (control: 6.74 ± 0.25 cm^3; caffeine: 7.16 ± 0.56 cm^3; $p > 0.05$) or striatum (control: 0.37 ± 0.05 cm^3; caffeine: 0.41 ± 0.04 cm^3; $p > 0.05$).

Neurons and Pyramidal Cell Dendritic Spine Density

The density of NeuN-IR neurons in the striatum was not different ($p > 0.05$) in control and caffeine-treated fetuses (**Figures 2A–C**). In the cerebral cortex, caffeine treatment led to a significantly greater density of Ctip2-IR neurons in bin 3 (layers V and VI) ($p = 0.02$; **Figures 2D–F**), with a tendency for an increase when all cortical bins were combined ($p = 0.07$; data not shown); in the striatum there was no difference ($p > 0.05$) between groups (**Figures 2G–I**). There was a significant decrease in the density of Cux1-IR neurons (control: 123.3 ± 8.5 cells/mm^2; caffeine: 86.5 ± 9.7 cells/mm^2; $p = 0.01$), and a significant increase in the density of Tbr1-IR neurons (control: 40.83 ± 1.1 cells/mm^2; caffeine: 65.5 ± 2.0 cells/mm^2; $p < 0.0001$) in the cerebral cortex of caffeine treated fetuses compared with controls.

There was no significant difference ($p > 0.05$) between groups in the linear density of dendritic spines on layer V pyramidal cells (**Figures 2J–L**). There was no qualitative difference between groups in the length or shape of the spines. The density of SST-IR interneurons was not different ($p > 0.05$) between groups in the cerebral cortex (**Figures 3A–C**) or striatum (**Figures 3D–F**).

Glia and Cell Proliferation

The density of Iba-1-IR microglia in the cerebral cortex did not differ ($p > 0.05$) between treatment groups (**Figures 4A–C**). Caffeine exposure led to an increase in the density of cortical GFAP-IR astrocytes ($p = 0.03$; **Figures 4D–F**) and an increase in the density of cortical Olig2-IR oligodendrocytes ($p = 0.02$; **Figures 4G–I**). There was no difference in the areal density of MBP-IR mature myelinating oligodendrocytes (**Figures 4J–L**) or

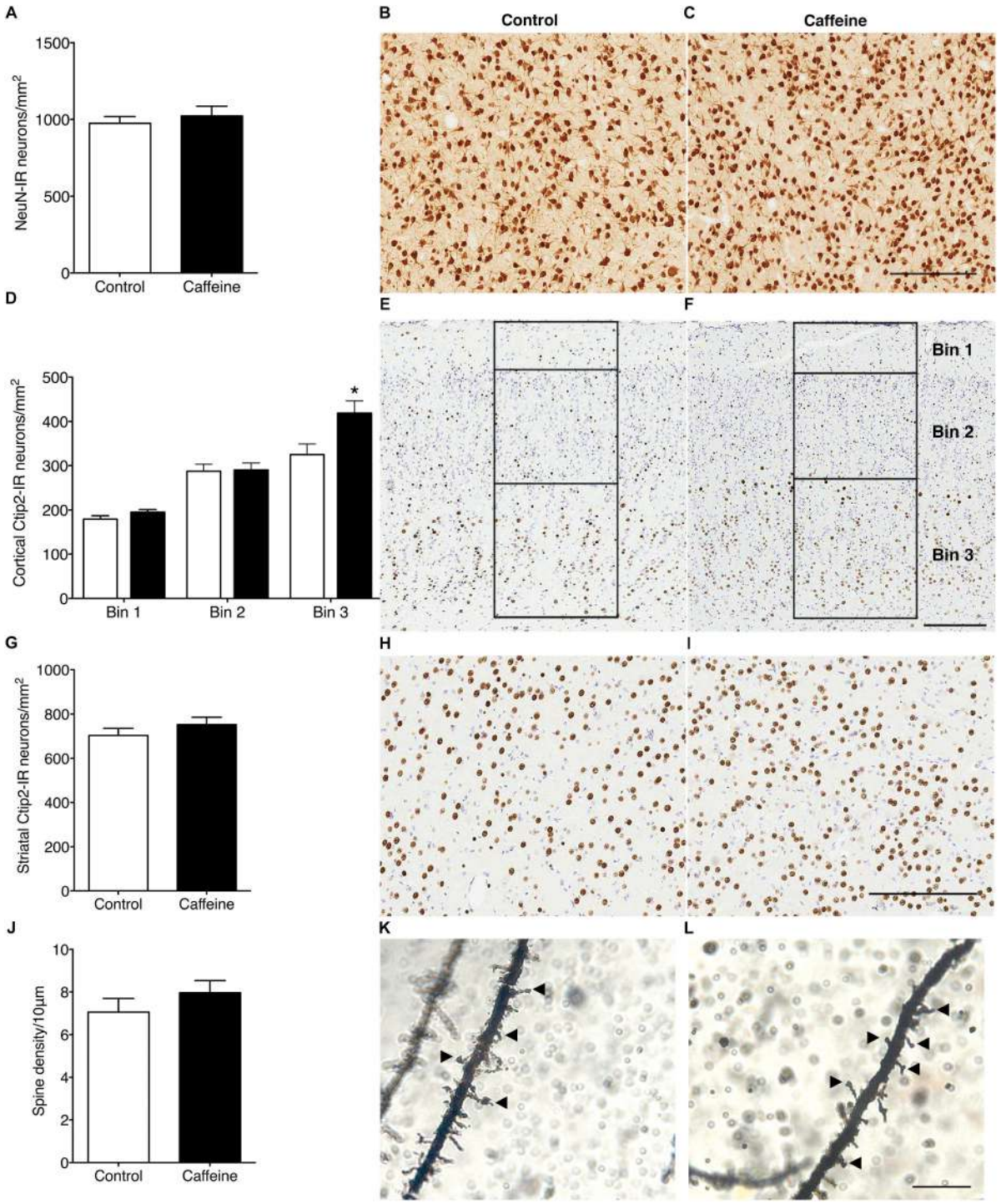


FIGURE 2 | NeuN-IR neurons, Ctip2-IR projection neurons and layer V pyramidal neuron dendritic spine density in control and caffeine-treated fetuses. NeuN-IR neuronal density in the striatum **(A)** was not different between control and caffeine-treated fetuses, illustrated by comparing images from the striatum in control **(B)** and caffeine-treated **(C)** fetuses at 119DG. There was a significant increase in Ctip2-IR projection neurons in bin 3 (layers V and VI) in the cerebral cortex **(D)**, in the caffeine group compared to controls, with no significant differences in all other bins. This is illustrated by comparing images of Ctip2-immunoreactivity in control **(E)** and caffeine-treated **(F)** fetuses. Bins were divided according to cortical layers (bin 1: cortical layer I; bin 2: layers II, III, and IV; bin 3: layers V and VI) as shown in **(E,F)**. There was no significant difference between control and caffeine-treated fetuses in Ctip2-IR neuronal density within the striatum **(G)**, illustrated by comparing images from the striatum of control **(H)** and caffeine-treated **(I)** fetuses. There was no significant difference between control and caffeine-treated fetuses in dendritic spine density per 10 μm of apical dendrite **(J)**. This is illustrated by comparing high power images of Golgi-cox stained sections from the cerebral cortex in control **(K)** and caffeine-treated **(L)** fetuses at 119DG. Arrowheads show dendritic spines on apical dendrites. Scale bar **(B,C,E,F,H,I)** = 200 μm; **(K,L)** = 10 μm. *$p < 0.05$.

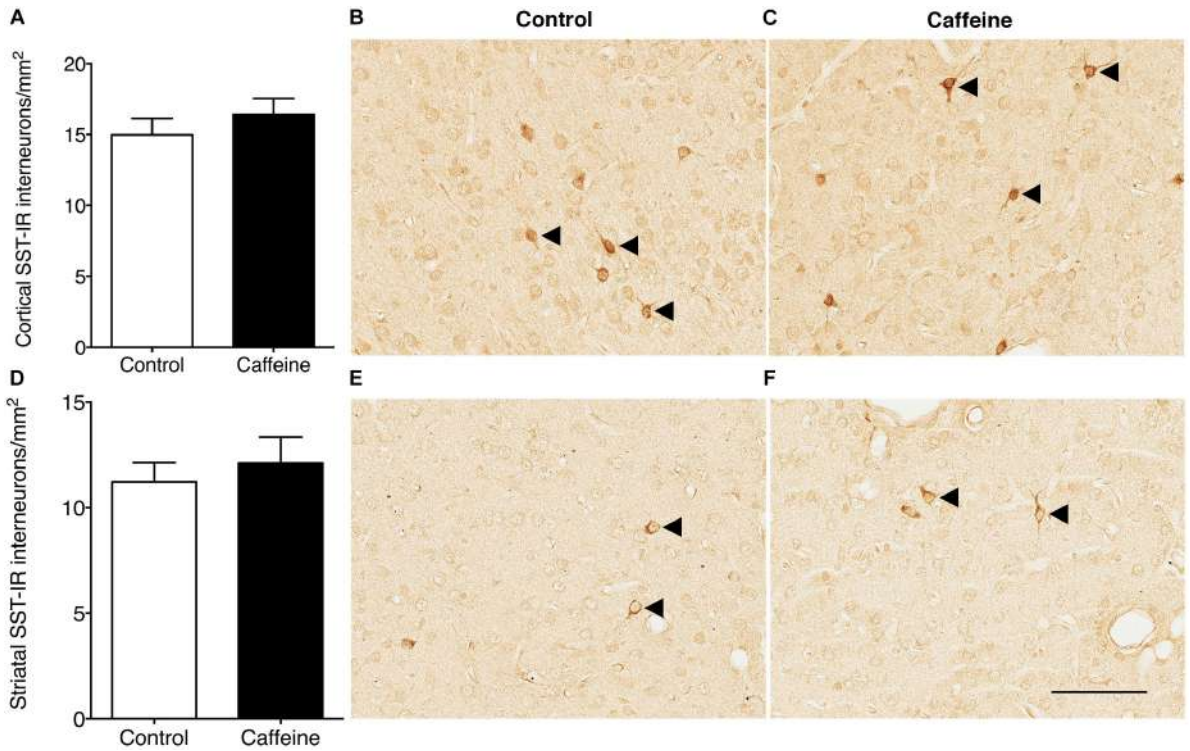


FIGURE 3 | SST-IR interneurons in the cerebral cortex and striatum of control and caffeine treated fetuses. SST-IR interneuron density was not affected by high-dose caffeine compared to controls in the cerebral cortex **(A)** or striatum **(D)**. This is illustrated by comparing images from the cerebral cortex **(B,C)** and striatum **(E,F)** in control **(B,E)** and caffeine-treated **(C,F)** fetuses. Arrowheads show SST-positive cells. Scale bar **(C–F)**: 100 μm.

Ki67-IR proliferating cells in cerebral cortex (**Figures 5A–C**), or in the density of Ki67-IR cells in the SVZ (**Figures 5D–F**) in control compared to caffeine-treated fetuses ($p > 0.05$).

Cerebellum

No hemorrhages or overt structural damage were observed. There was no significant difference in cerebellum weight (control: 3.0 ± 0.1 g; caffeine: 3.0 ± 0.1 g) or the ratio of cerebellum weight to brain weight (control: 0.08 ± 0.003; caffeine: 0.07 ± 0.002).

Morphological Analysis

In caffeine-treated fetuses compared to controls there was no difference ($p > 0.05$) in the total cross-sectional area of the cerebellum (control: 119.6 ± 5.1 mm^2; caffeine: 127.0 ± 6.9 mm^2; $p > 0.05$), the ML (control: 44.4 ± 1.8 mm^2; caffeine: 48.1 ± 3.4 mm^2; $p > 0.05$), the IGL (control: 47.6 ± 1.9 mm^2; caffeine: 49.4 ± 3.5 mm^2; $p > 0.05$), or WM (control: 27.7 ± 5.1 mm^2; caffeine: 29.5 ± 1.4 mm^2; $p > 0.05$), nor in the width of the EGL (control: 26.1 ± 1.2 μm; caffeine: 28.0 ± 0.4 μm; $p > 0.05$). There was however a reduction ($p < 0.05$) in the mean Purkinje cell somal area (control: 491.0 ± 11.1 μm^2; caffeine: 439.9 ± 17.4 μm^2; $p = 0.03$).

Glia and Myelination

In the WM, there was no difference ($p > 0.05$) in the areal density of Iba-1-IR microglia (control: 131.4 ± 22.6 cells/mm^2; caffeine: 126.1 ± 14.3 cells/mm^2; $p > 0.05$), Olig2-IR oligodendrocytes (control: 2449 ± 54.5 cells/mm^2; caffeine: 2464 ± 154.2 cells/mm^2; $p > 0.05$), nor in the OD of MBP-IR (control: 0.22 ± 0.006; caffeine: 0.22 ± 0.007; $p > 0.05$) or GFAP-IR (control: 0.14 ± 0.004; caffeine: 0.13 ± 0.004; $p > 0.05$) between groups. Qualitative analysis of GFAP-IR in the ML and IGL showed no difference between groups. There were also no observable differences in the morphology of the Bergmann glia fibers between groups: i.e., no evidence of fiber disorganization or disrupted fiber integrity.

DISCUSSION

This is the first study to assess the effects of high-dose caffeine on the developing cerebral cortex in a long-gestation, clinically relevant animal model. Key findings were that in the developing cortex, high-dose caffeine led to an increase in the density of Ctip2-IR projection neurons in layers V–VI, Tbr1-IR projection neurons in layers V–V1, and a decrease in Cux1-IR projection neurons in the upper cortical layers (II–IV). There was also an increase in the density of cortical GFAP-IR astrocytes and Olig2-IR oligodendrocytes but no change in dendritic spine density of layer V pyramidal neurons, nor in the density of interneurons, microglia, mature oligodendrocytes, or proliferating cells. Caffeine exposure had no effect on any of

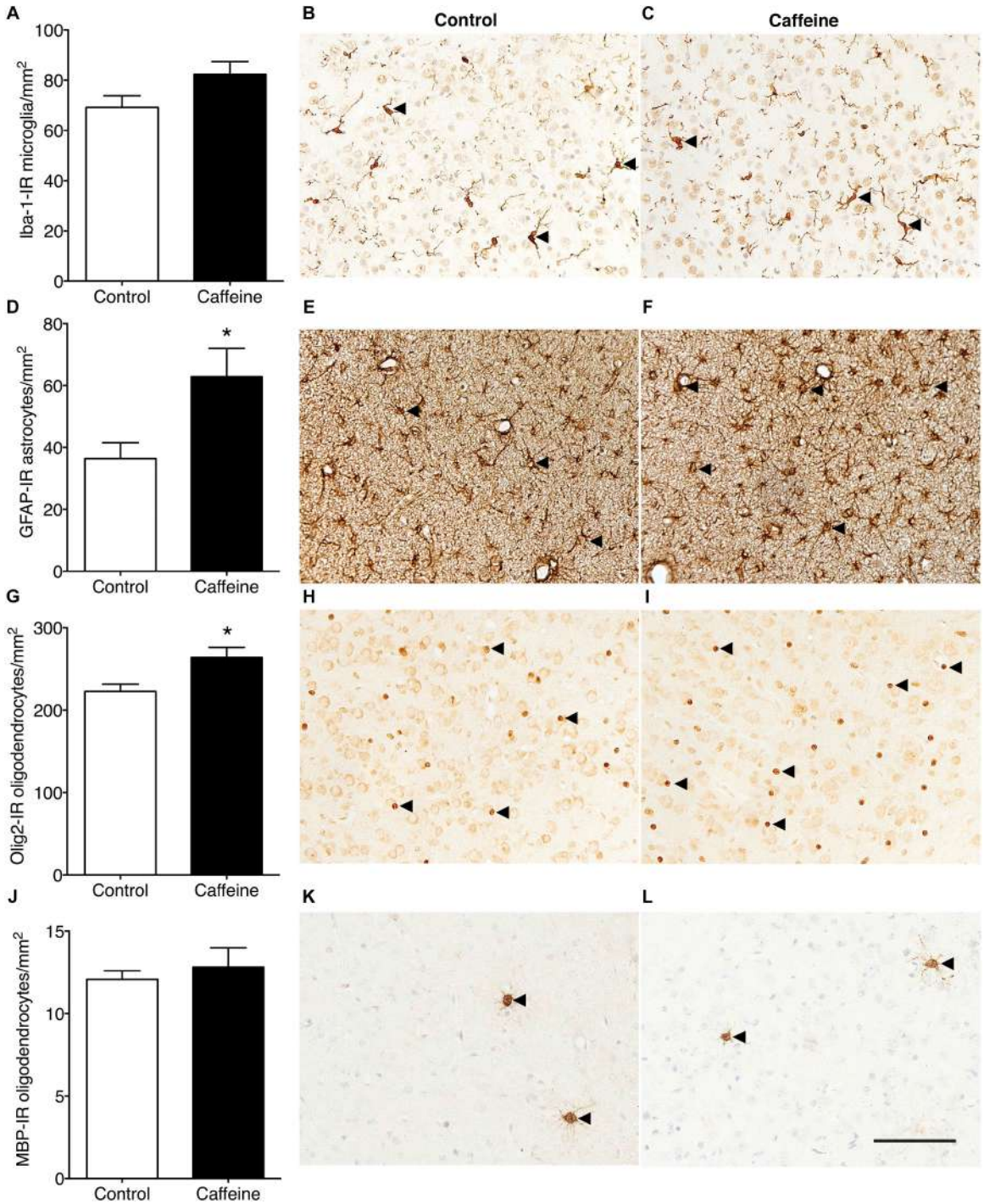


FIGURE 4 | Iba-1-IR microglia, GFAP-IR astrocytes and Olig2-IR and MBP-IR oligodendrocytes in the cerebral cortex of control and caffeine-treated fetuses. The density of Iba-1-IR microglia (resting and activated combined) was not different in caffeine-treated and control fetuses **(A)**, illustrated by comparing images from the cerebral cortex in control **(B)** and caffeine-treated **(C)** fetuses. There was a significant increase in the density of GFAP-IR astrocytes in caffeine-treated fetuses compared to controls **(D)**, evident by comparing images from the cerebral cortex in control **(E)** and caffeine-treated **(F)** fetuses. There was a significant increase in the density of Olig2-IR oligodendrocytes in the cerebral cortex in caffeine-treated fetuses compared to controls **(G)**. This is shown by comparing images from the cerebral cortex in control **(H)** and caffeine-treated **(I)** fetuses. The density of MBP-IR mature myelinating oligodendrocytes was not different in the cerebral cortex of caffeine-treated and control fetuses **(J)**, illustrated by comparing images from the cerebral cortex of control **(K)** and caffeine-treated **(L)** fetuses. In **(B,C)**, arrowheads show ramified microglia; in **(E,F)** arrowheads show astrocytes; in **(H,I)** arrowheads show Olig2-IR oligodendrocytes and in **(K,L)** arrowheads show MBP-IR mature oligodendrocytes. Scale bar = 100 μm. *$p < 0.05$.

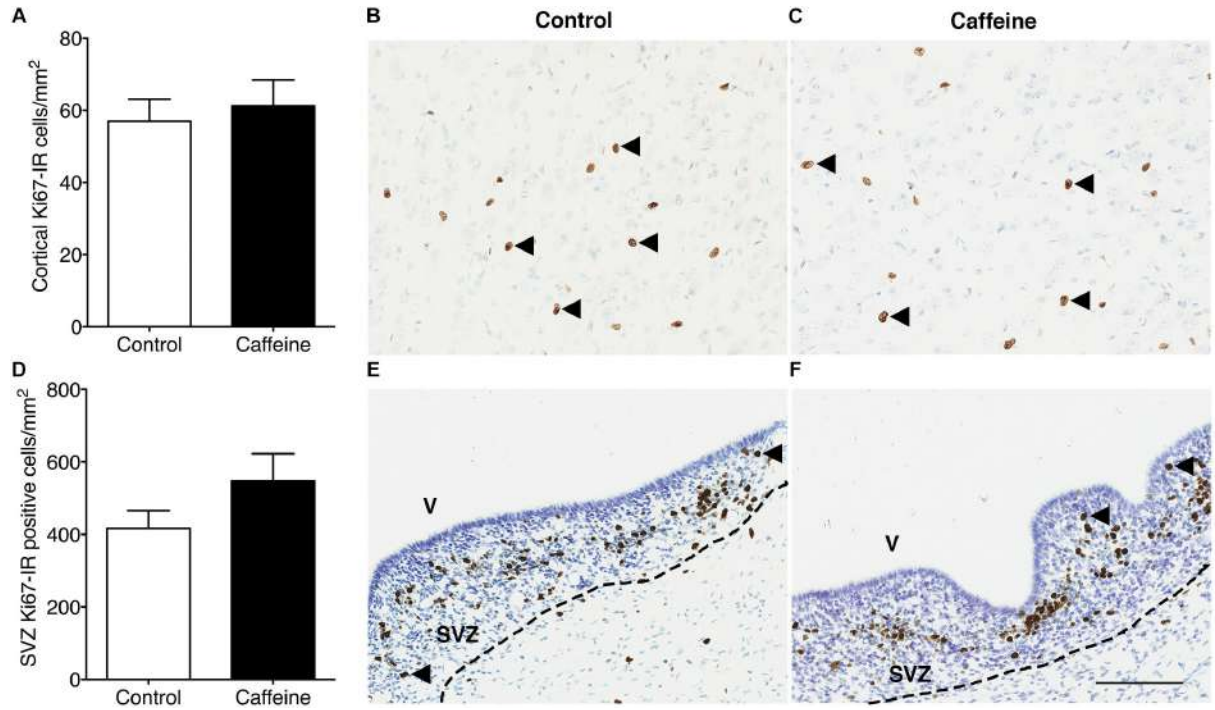


FIGURE 5 | Ki67-IR cells in the cerebral cortex and subventricular zone of control and caffeine-treated fetuses. The density of Ki67-IR cells in cerebral cortex **(A)** or subventricular zone **(D)** did not differ between control and caffeine-treated fetuses. This is illustrated by comparing images from the cerebral cortex **(B,C)** and subventricular zone **(E,F)** of control **(B,E)** and caffeine-treated **(C,F)** fetuses. Arrowheads show Ki67-IR cells. V, ventricle; SVZ, subventricular zone. Scale bar **(B,C,E,F)** = 100 μm.

the neuronal and glial parameters measured in the striatum and cerebellum apart from a small but significant reduction in mean Purkinje cell somal area.

Effects of High-Dose Caffeine on Pyramidal Neurons in the Cerebral Cortex

We previously reported that high-dose caffeine treatment does not affect neuronal density within the cerebral cortex (Atik et al., 2014); here we report that the volume of the cerebral cortex is also unaffected, making it unlikely that there are major changes in overall neuronal numbers. However to determine whether specific neuronal populations had been differentially affected, we identified excitatory pyramidal projection neurons of layers V–V1 using antibodies to Trb1 and Ctip2, a zinc-finger transcription factor; Ctip2 is more strongly expressed in sub-cortically projecting neurons than in inter- and intra-hemispherically projecting neurons (Arlotta et al., 2005). In addition we identified projection neurons in the upper layers (layers II–IV) with antibodies to Cux1, a transcription factor which regulates several genes involved in cellular proliferation and differentiation (Nepveu, 2001). We found that caffeine treatment resulted in an increase in the density of Ctip2-IR and Tbr1-IR neurons in the deeper cortical layers (early born projection neurons) and a decrease in Cux1-IR neurons in the upper layers (late born projection neurons). As this occurred in the absence of an overall increase in neuronal density, it suggests an upregulation in the expression of these proteins in previously low expressing cells.

As Ctip2 is a transcription factor critical for axonal outgrowth and extension as well as for axonal pathfinding by cortical projection neurons (Arlotta et al., 2005; Leyva-Diaz and Lopez-Bendito, 2013), an increase in Ctip2-immunoreactivity could lead to aberrant growth of subcortically projecting axons. This could occur at the expense of cortically projecting axonal growth as has been shown in rodents, when there is an imbalance of transcription factors (Alcamo et al., 2008; Britanova et al., 2008). Although we have not exhaustively examined developmental gene expression patterns it is clear that caffeine treatment results in alterations to neurochemical signatures which could affect appropriate axonal growth and target finding (Arlotta et al., 2005; Leyva-Diaz and Lopez-Bendito, 2013). The functional significance of these alterations is likely complex and calls for further investigation but underlines the need for examination at the cellular level when determining whether a specific regime has affected neuronal function. Although we found no change in the overall density of SST-IR (inhibitory) interneurons in the cerebral cortex of caffeine-treated fetuses, it should be noted that the SST-expressing interneurons are only one subpopulation of GABAergic interneurons, comprising approximately 23% of the entire GABAergic interneuron population (Gonchar et al., 2007); thus other populations may be differentially affected by caffeine.

High-Dose Caffeine and Synaptic Connectivity in the Cerebral Cortex

To determine the effects of high-dose caffeine on synaptic connectivity, we assessed the linear density of spines on the apical dendrites of layer V pyramidal neurons. We found no effect on spine density but recognize that other parameters of dendritic morphology such as the overall extent of the dendritic arbor need to be assessed. A study of the effects of caffeine exposure on dendritic morphology in newborn rats similarly showed no differences in spine density; however an increase in dendritic length was observed (Juarez-Mendez et al., 2006).

Effects of High-Dose Caffeine on Neuroglia in the Cerebral Cortex

We previously reported that high-dose caffeine does not result in an increase in the areal density of microglia, astroglia, or oligodendroglia in the developing ovine cerebral WM (Atik et al., 2014). Here we report that, in the cerebral cortex, the same regimen resulted in astrogliosis in the absence of microgliosis; however the mechanism underlying this cortical gliosis is uncertain. Astrogliosis is commonly associated with neural damage induced by an insult such as cerebral hypoxia (Brand and Bignami, 1969). Cerebral hypoxia may have occurred in our animals although we have previously shown that fetal arterial oxygen saturation and arterial pressure were unaffected throughout the treatment period; however, cerebral blood flow was not measured (Atik et al., 2014). Caffeine is known to decrease cerebral blood flow (Tracy et al., 2010) and acts as a neuronal stimulant (Nehlig et al., 1992), possibly increasing neuronal metabolic demand; both factors operating in concert could cause cerebral hypoxia, affecting neuronal survival. However, we saw no evidence of neuronal apoptosis and neuronal densities were not reduced in our model (Atik et al., 2014), although it is possible that cell death may have occurred at an earlier time point. In preterm baboons administered caffeine to stimulate breathing, cortical gliosis was also present in the absence of overt cell death (Loeliger et al., 2006). The authors (Loeliger et al., 2006) argued that caffeine might increase the vulnerability of cortical gray matter to intermittent hypoperfusion while preserving the cerebral WM. Further studies are required to determine whether cortical astrogliosis persists in the long-term in our model. Such persistence could have implications for neural function as astrocytes can either be beneficial or detrimental to neural tissue (Sofroniew and Vinters, 2010). Our findings are not in accord with those of Desfrere et al. (2007) who found transient and dose-dependent inhibition of astrocytogenesis in the developing mouse cortex following postnatal caffeine exposure; the different findings are possibly due to differences in species, dosing regimen, or timing of exposure.

In addition to astrogliosis, we have shown that high-dose caffeine increased the areal density of Olig2-IR oligodendrocytes in the cerebral cortex. There was no evidence of increased cell proliferation in the cerebral cortex or SVZ to account for the increased density; however, caffeine might have influenced the proliferation of oligodendrocytes at an earlier time point than the one examined in this study. The fate of this increased pool of Olig2-IR cells is uncertain, as it was not associated with an increase in the density of myelinating oligodendrocytes. A proportion of cells in the oligodendrocyte lineage in the cerebral cortex become perineuronal satellite cells (Szuchet et al., 2011) and are thought to provide support to neurons, possibly via regulation of the microenvironment (Dewar et al., 2003). Perineuronal oligodendrocytes also maintain a reservoir of untranslated transcripts encoding major myelin proteins (Szuchet et al., 2011). It is possible that the density of this population of oligodendrocytes is increased as a result of high-caffeine exposure but we were not able to investigate this.

Effects of High Dose Caffeine on the Cerebellum

The degree of caffeine exposure that we used had no effect on the cerebellar parameters measured here apart from a small (∼10%) but significant decrease in the area of the Purkinje cell body. Such a decrease could ultimately have an effect on dendritic growth but the lack of a reduction in the area of the ML (of which the Purkinje cell dendritic arbor is a major component) does not support the likelihood of such an outcome. We have previously shown that the most rapid expansion of the Purkinje cell dendritic tree in fetal sheep occurs between 100 and 120DG (Rees and Harding, 1988), coinciding with the time course of the present study. If caffeine exposure were to affect dendritic growth we would have expected to identify it during this period of rapid expansion.

Are There Long-Term Developmental Outcomes Following High-Dose Caffeine Administration?

Overall, caffeine exposure causes some relatively subtle but specific changes in the developing cerebral cortex while having a minimal effect in the cerebellum and no effect in the striatum; the reason for this differential effect is not clear and does not appear to be related to different developmental stages of the regions (Rees et al., 1997). Whether or not the subtle changes that we report in neuronal and glial cell development persist postnatally, or manifest into long-term functional deficits is unknown. In humans, clinical trials assessing the immediate and longer-term implications of high-dose caffeine therapy for apnea of prematurity are now emerging. Magnetic resonance imaging and neurobehavioral testing undertaken at term equivalent age, and at 2 years of age, showed that very preterm infants who received high-dose (80 mg/kg caffeine citrate; loading dose) compared with standard dose (20 mg/kg loading dose) caffeine, had a higher incidence of cerebellar hemorrhage with subsequent alterations in motor performance (McPherson et al., 2015).

CONCLUSION

Using an animal model in which the brain is at a stage of development resembling that of the very preterm infant, we have shown that daily administration of high-dose caffeine for 2 weeks affects aspects of neuronal and glial cell development in the cerebral cortex but has minimal effects on the cerebellum

and no effect on the striatum. Although the long-term structural and functional consequences of our findings are not yet known, our data, coupled with recent adverse findings in preterm infants (McPherson et al., 2015), highlight the need to consider both the benefits of high-dose caffeine [e.g., improved extubation from mechanical ventilation (Steer et al., 2004; Mohammed et al., 2015)], and potentially adverse effects. It is therefore important that investigators continue to study the neurodevelopmental impact of high-dose caffeine in clinically relevant, long-gestation animal models.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

RH, JC, SaR, RDM, and MT designed the experiments. AA, RDM, ShR, MB, KC, and MT collected the data. AA, ShR, and MB analyzed the data. AA, RH, JC, SaR, RDM, MB, KC, and MT interpreted the data. AA, RH, RDM, SaR, and MT wrote and revised the manuscript. All authors approved the final version of the manuscript.

FUNDING

Funding for this study was provided by the National Health and Medical Research Council of Australia (ID 628312) to MT, JC, RDM, SaR, and RH, Early Career Fellowship ID 1053787 to JC, and the Victorian Government's Operational Infrastructure Support Program (Government of Victoria).

ACKNOWLEDGMENTS

We thank Ms. Natasha Blasch for her technical assistance. Experiments were performed at Monash University and the Hudson Institute of Medical Research.

REFERENCES

Alcamo, E. A., Chirivella, L., Dautzenberg, M., Dobreva, G., Farinas, I., Grosschedl, R., et al. (2008). Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron* 57, 364–377. doi: 10.1016/j.neuron.2007.12.012

Altman, J. (1969). Autoradiographic and histological studies of postnatal neurogenesis. 3. Dating the time of production and onset of differentiation of cerebellar microneurons in rats. *J. Comp. Neurol.* 136, 269–293.

Arlotta, P., Molyneaux, B. J., Chen, J., Inoue, J., Kominami, R., and Macklis, J. D. (2005). Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron* 45, 207–221.

Atik, A., Cheong, J., Harding, R., Rees, S., De Matteo, R., and Tolcos, M. (2014). Impact of daily high-dose caffeine exposure on developing white matter of the immature ovine brain. *Pediatr. Res.* 76, 54–63. doi: 10.1038/pr.2014.55

Black, A. M., Pandya, S., Clark, D., Armstrong, E. A., and Yager, J. Y. (2008). Effect of caffeine and morphine on the developing pre-mature brain. *Brain Res.* 1219, 136–142. doi: 10.1016/j.brainres.2008.04.066

Bona, E., Aden, U., Fredholm, B. B., and Hagberg, H. (1995). The effect of long term caffeine treatment on hypoxic-ischemic brain damage in the neonate. *Pediatr. Res.* 38, 312–318.

Brand, M. M., and Bignami, A. (1969). The effects of chronic hypoxia on the neonatal and infantile brain. A neuropathological study of five premature infants with the respiratory distress syndrome treated by prolonged artificial ventilation. *Brain* 92, 233–254.

Britanova, O., De Juan Romero, C., Cheung, A., Kwan, K. Y., Schwark, M., Gyorgy, A., et al. (2008). Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. *Neuron* 57, 378–392. doi: 10.1016/j.neuron.2007.12.028

Connolly, S., and Kingsbury, T. J. (2010). Caffeine modulates CREB-dependent gene expression in developing cortical neurons. *Biochem. Biophys. Res. Commun.* 397, 152–156. doi: 10.1016/j.bbrc.2010.05.054

Desfrere, L., Olivier, P., Schwendimann, L., Verney, C., and Gressens, P. (2007). Transient inhibition of astrocytogenesis in developing mouse brain following postnatal caffeine exposure. *Pediatr. Res.* 62, 604–609.

Dewar, D., Underhill, S. M., and Goldberg, M. P. (2003). Oligodendrocytes and ischemic brain injury. *J. Cereb. Blood Flow Metab.* 23, 263–274.

Fuller, G. N., Divakaran, P., and Wiggins, R. C. (1982). The effect of postnatal caffeine administration on brain myelination. *Brain Res.* 249, 189–191.

Gonchar, Y., Wang, Q., and Burkhalter, A. (2007). Multiple distinct subtypes of GABAergic neurons in mouse visual cortex identified by triple immunostaining. *Front. Neuroanat.* 1:3. doi: 10.3389/neuro.05.003.2007

Guillet, R., and Dunham, L. (1995). Neonatal caffeine exposure and seizure susceptibility in adult rats. *Epilepsia* 36, 743–749.

Gundersen, H. J., Jensen, E. B., Kieu, K., and Nielsen, J. (1999). The efficiency of systematic sampling in stereology–reconsidered. *J. Microscopy* 193, 199–211.

Hevner, R. F., Shi, L., Justice, N., Hsueh, Y., Sheng, M., Smiga, S., et al. (2001). Tbr1 regulates differentiation of the preplate and layer 6. *Neuron* 29, 353–366.

Juarez-Mendez, S., Carretero, R., Martinez-Tellez, R., Silva-Gomez, A. B., and Flores, G. (2006). Neonatal caffeine administration causes a permanent increase in the dendritic length of prefrontal cortical neurons of rats. *Synapse* 60, 450–455.

Kang, S. H., Lee, Y. A., Won, S. J., Rhee, K. H., and Gwag, B. J. (2002). Caffeine-induced neuronal death in neonatal rat brain and cortical cell cultures. *Neuroreport* 13, 1945–1950.

Leyva-Diaz, E., and Lopez-Bendito, G. (2013). In and out from the cortex: development of major forebrain connections. *Neuroscience* 254, 26–44. doi: 10.1016/j.neuroscience.2013.08.070

Loeliger, M., Inder, T., Cain, S., Ramesh, R. C., Camm, E., Thomson, M. A., et al. (2006). Cerebral outcomes in a preterm baboon model of early versus delayed nasal continuous positive airway pressure. *Pediatrics* 118, 1640–1653.

McDougall, A. R. A., Wiradjaja, V., Azhan, A., Li, A., Hale, N., Wlodek, M. E., et al. (2017). Intrauterine growth restriction alters the postnatal development of the rat cerebellum. *Dev. Neurosci.* 39, 215–227. doi: 10.1159/000470902

McPherson, C., Neil, J. J., Tjoeng, T. H., Pineda, R., and Inder, T. E. (2015). A pilot randomized trial of high-dose caffeine therapy in preterm infants. *Pediatr. Res.* 78, 198–204. doi: 10.1038/pr.2015.72

Mohammed, S., Nour, I., Shabaan, A. E., Shouman, B., Abdel-Hady, H., and Nasef, N. (2015). High versus low-dose caffeine for apnea of prematurity: a randomized controlled trial. *Eur. J. Pediatr.* 174, 949–956. doi: 10.1007/s00431-015-2494-8

Nehlig, A., Daval, J. L., and Debry, G. (1992). Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. *Brain Res. Brain Res. Rev.* 17, 139–170.

Nepveu, A. (2001). Role of the multifunctional CDP/Cut/Cux homeodomain transcription factor in regulating differentiation, cell growth and development. *Gene* 270, 1–15.

Pierson, C. R., Folkerth, R. D., Billiards, S. S., Trachtenberg, F. L., Drinkwater, M. E., Volpe, J. J., et al. (2007). Gray matter injury associated with periventricular leukomalacia in the premature infant. *Acta Neuropathol.* 114, 619–631.

Rees, S., and Harding, R. (1988). The effects of intrauterine growth retardation on the development of the Purkinje cell dendritic tree in the cerebellar cortex of fetal sheep: a note on the ontogeny of the Purkinje cell. *Int. J. Dev. Neurosci.* 6, 461–469.

Rees, S., Stringer, M., Just, Y., Hooper, S. B., and Harding, R. (1997). The vulnerability of the fetal sheep brain to hypoxemia at mid-gestation. *Brain Res. Dev. Brain Res.* 103, 103–118.

Saigal, S., and Doyle, L. W. (2008). An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet* 371, 261–269. doi: 10.1016/S0140-6736(08)60136-1

Scanlon, J. E., Chin, K. C., Morgan, M. E., Durbin, G. M., Hale, K. A., and Brown, S. S. (1992). Caffeine or theophylline for neonatal apnoea? *Arch. Dis. Child.* 67, 425–428.

Sofroniew, M. V., and Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol.* 119, 7–35.

Steer, P., Flenady, V., Shearman, A., Charles, B., Gray, P. H., Henderson-Smart, D., et al. (2004). High dose caffeine citrate for extubation of preterm infants: a randomised controlled trial. *Arch. Dis. Child. Fetal Neonatal Ed.* 89, F499–F503.

Steer, P. A., Flenady, V. J., Shearman, A., Lee, T. C., Tudehope, D. I., and Charles, B. G. (2003). Periextubation caffeine in preterm neonates: a randomized dose response trial. *J. Paediatr. Child Health* 39, 511–515.

Szuchet, S., Nielsen, J. A., Lovas, G., Domowicz, M. S., De Velasco, J. M., Maric, D., et al. (2011). The genetic signature of perineuronal oligodendrocytes reveals their unique phenotype. *Eur. J. Neurosci.* 34, 1906–1922. doi: 10.1111/j.1460-9568.2011.07922.x

Tolcos, M., Bateman, E., O'dowd, R., Markwick, R., Vrijsen, K., Rehn, A., et al. (2011). Intrauterine growth restriction affects the maturation of myelin. *Exp. Neurol.* 232, 53–65. doi: 10.1016/j.expneurol.2011.08.002

Tolcos, M., Mcdougall, A., Shields, A., Chung, Y., O'dowd, R., Turnley, A., et al. (2018). Intrauterine growth restriction affects cerebellar granule cells in the developing guinea pig brain. *Dev. Neurosci.* 40, 162–174. doi: 10.1159/000487797

Tracy, M. B., Klimek, J., Hinder, M., Ponnampalam, G., and Tracy, S. K. (2010). Does caffeine impair cerebral oxygenation and blood flow velocity in preterm infants? *Acta Paediatr.* 99, 1319–1323. doi: 10.1111/j.1651-2227.2010.01828.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Atik, De Matteo, Boomgardt, Rees, Harding, Cheong, Rana, Crossley and Tolcos. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Physiology

ORIGINAL RESEARCH
published: 13 November 2019
doi: 10.3389/fphys.2019.01351

Curcumin: Novel Treatment in Neonatal Hypoxic-Ischemic Brain Injury

Eridan Rocha-Ferreira[1,2†], Claudia Sisa[1†], Sarah Bright[1], Tessa Fautz[1], Michael Harris[1], Ingrid Contreras Riquelme[1], Chinedu Agwu[1], Tugce Kurulday[1,3], Beenaben Mistry[1], Daniel Hill[1,4], Sigrun Lange[5] and Mariya Hristova[1]*

[1]Department of Maternal and Fetal Medicine, Perinatal Brain Repair Group, UCL Institute for Women's Health, London, United Kingdom, [2]Department of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [3]Department of Molecular Biology and Genetics, Izmir Institute of Technology, İzmir, Turkey, [4]Department of Visual Neuroscience, Glaucoma and Retinal Neurodegeneration Group, UCL Institute of Ophthalmology, London, United Kingdom, [5]School of Life Sciences, Tissue Architecture and Regeneration Research Group, University of Westminster, London, United Kingdom

OPEN ACCESS

Edited by:
Mary Tolcos,
RMIT University, Australia

Reviewed by:
Tracey Bjorkman,
University of Queensland, Australia
Deirdre M. Murray,
University College Cork, Ireland

***Correspondence:**
Mariya Hristova
m.hristova@ucl.ac.uk

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *26 January 2019*
Accepted: *10 October 2019*
Published: *13 November 2019*

Citation:
Rocha-Ferreira E, Sisa C, Bright S, Fautz T, Harris M, Contreras Riquelme I, Agwu C, Kurulday T, Mistry B, Hill D, Lange S and Hristova M (2019) Curcumin: Novel Treatment in Neonatal Hypoxic-Ischemic Brain Injury. Front. Physiol. 10:1351. doi: 10.3389/fphys.2019.01351

Hypoxic-ischemic encephalopathy (HIE) is a major cause of mortality and morbidity in neonates, with an estimated global incidence of 3/1,000 live births. HIE brain damage is associated with an inflammatory response and oxidative stress, resulting in the activation of cell death pathways. At present, therapeutic hypothermia is the only clinically approved treatment available for HIE. This approach, however, is only partially effective. Therefore, there is an unmet clinical need for the development of novel therapeutic interventions for the treatment of HIE. Curcumin is an antioxidant reactive oxygen species scavenger, with reported anti-tumor and anti-inflammatory activity. Curcumin has been shown to attenuate mitochondrial dysfunction, stabilize the cell membrane, stimulate proliferation, and reduce injury severity in adult models of spinal cord injury, cancer, and cardiovascular disease. The role of curcumin in neonatal HIE has not been widely studied due to its low bioavailability and limited aqueous solubility. The aim of this study was to investigate the effect of curcumin treatment in neonatal HIE, including time of administration and dose-dependent effects. Our results indicate that curcumin administration prior to HIE in neonatal mice elevated cell and tissue loss, as well as glial activation compared to HI alone. However, immediate post-treatment with curcumin was significantly neuroprotective, reducing grey and white matter tissue loss, TUNEL+ cell death, microglia activation, reactive astrogliosis, and iNOS oxidative stress when compared to vehicle-treated littermates. This effect was dose-dependent, with 200 μg/g body weight as the optimal dose-regimen, and was maintained when curcumin treatment was delayed by 60 or 120 min post-HI. Cell proliferation measurements showed no changes between curcumin and HI alone, suggesting that the protective effects of curcumin on the neonatal brain following HI are most likely due to curcumin's anti-inflammatory and antioxidant properties, as seen in the reduced glial and iNOS activity. In conclusion, this study suggests curcumin as a potent neuroprotective agent with potential for the treatment of HIE. The delayed application of curcumin further increases its clinical relevance.

Keywords: curcumin, hypoxia, ischemia, neuroprotection, neonate, oxidative stress

INTRODUCTION

Neonatal hypoxic-ischemic (HI) brain injury has an incidence of 1–3 per 1,000 live births (Rocha-Ferreira and Hristova, 2016), and results in almost 1 million neonatal deaths worldwide (Lawn et al., 2005; Rocha-Ferreira and Hristova, 2016). Approximately, 30% of HI cases will develop lifelong disabilities, including cerebral palsy, seizures, and cognitive impairments (Rocha-Ferreira and Hristova, 2016; Lundgren et al., 2018). The severity of such disabilities depends on the stage of gestation at which the HI event occurs and its duration (Sanders et al., 2010).

The pathology of HI brain damage is characterized by an initial primary energy loss phase, where oxygen and glucose deprivation in the cell causes a drop in the mitochondrial oxidative phosphorylation, resulting in reduced adenosine triphosphate (ATP) availability, triggering excitotoxicity (Rocha-Ferreira and Hristova, 2016), neurotoxicity (Sanders et al., 2010), and oxidative stress (Hope et al., 1984; Penrice et al., 1997). Early after the HI insult, damaged neuronal cells stimulate a pro-inflammatory immune response where activated microglia produce cytokines such as IL-1β and TNFα, proteases, and complement factors (Rocha-Ferreira and Hristova, 2016).

After successful resuscitation, a short latent/recovery period occurs. However, during reperfusion, the majority of the oxidative markers are produced, and in cases of an initial prolonged HI insult, the primary energy failure cannot be compensated, therefore, leading to a secondary energy drop (Rocha-Ferreira and Hristova, 2016). Inflammatory processes and continued excitotoxicity lead to an impaired equilibrium between pro- and anti-inflammatory cytokines, as well as significant damage of the mitochondria machinery (Peng and Greenamyre, 1998; Puka-Sundvall et al., 2000). This is associated with increased levels of hydrogen peroxide (H_2O_2) and nitrogen oxide (NO), overproduction of free radicals, and reactive oxygen species (ROS) which, together with the persistent inflammation, stimulate necrosis (Li et al., 1998), apoptosis (Johnston et al., 2002), and autophagy (Rocha-Ferreira and Hristova, 2016) cell death pathways.

Therapeutic hypothermia (TH) is the standard clinical treatment applied in moderate to severe injury; however, it does not guarantee total recovery of the treated neonates with effectiveness of only 55% of cases and the remaining infants still develop neurological deficits (Gluckman et al., 2005). Thus, further studies on improving TH success rate and finding therapeutic alternatives are urgently required.

Curcumin, the major phytochemical component of the plant *Curcuma longa*, is extracted from its rhizomes (turmeric), and regularly consumed in South Asian diets (Shishodia et al., 2005; Pescosolido et al., 2013). Except for turmeric usage as dietary spice and coloring agent, curcumin has been studied for its therapeutic role in several pathological conditions; its effects have been investigated in cancer (Naksuriya et al., 2014; Ahmad et al., 2016), inflammation (Kim et al., 2003; Sandur et al., 2007), infections, cardiovascular diseases (Nishiyama et al., 2005; Liu and Hong, 2006), fibrosis, and neurological disorders (Spagnuolo et al., 2016). Such pharmacological success relies on the phytochemical abilities of acting on many critical pathways, showing anti-inflammatory (Tham et al., 2010), anti-oxidant (Alexandrow et al., 2012), anti-microbial (Moghadamtousi et al., 2014), and anti-apoptotic (Yu et al., 2010; Jaisin et al., 2011) effects, as well as capability to promote stem cell differentiation (Gu et al., 2012; Mujoo et al., 2012; Chen et al., 2014). Effects of curcumin on mitochondrial function have also been reported (Bavarsad et al., 2018; He et al., 2018; Hsiao et al., 2018; Momekova et al., 2018; Naserzadeh et al., 2018) and furthermore, hormetic effects of curcumin are receiving increased attention (Moghaddam et al., 2018). Curcumin pleiotropic activity is due to its chemical structure, and specifically to the o-methoxy phenolic groups (Priyadarsini and Indira, 2014), which are central for chemical reactions. In fact, curcumin scavenging abilities on ROS and free radicals (Trujillo et al., 2014) seem to rely on this functional group, as well as its modulatory role on pro-inflammatory cytokines [tumor necrosis factor alpha (TNFα), interleukin (IL)1 (Alexandrow et al., 2012), and IL6 (Maheshwari et al., 2006)] and its inhibition of signal transducer and activator of transcription 3 (STAT3) phosphorylation (Maheshwari et al., 2006; Alexandrow et al., 2012). As STAT3 is crucial for astrocyte differentiation (Hong and Song, 2014), its inhibition can reduce reactive astrogliosis post-HI. Clinical trials have demonstrated remarkable safety profile and good tolerance for curcumin in humans at doses of 8 g/day (Dhillon et al., 2008). Moreover, the small molecular weight (368.385 g/mol), and dimensions allow it to cross the blood-barrier (BBB) (Priyadarsini and Indira, 2014), increase researchers interest for curcumin application in neurodegenerative disorders including traumatic brain injury (Wu et al., 2006), Alzheimer's (Ray et al., 2011; Mutsuga et al., 2012), and Parkinson's (Mythri et al., 2011) diseases.

The pathways on which curcumin acts, overlap with those activated after HI injury, including oxidative stress and inflammation. So far curcumin has been vaguely tested in neonatal HI injury in rats, either through oral administration (Cui et al., 2017) or through intraperitoneal single dose delivery of nanoparticle- or DMSO-dissolved curcumin (Joseph et al., 2018). Both studies report attenuation of damage; however, there is no systematic approach to dose response or long-term protection. In fact, Joseph et al. report no effect of the DMSO-dissolved curcumin suggesting that it is only the nanoparticle-loaded curcumin showing protection (Joseph et al., 2018). The aim of our study was to assess the short- and long term-effects of curcumin administered immediately after neonatal HI insult, to determine the lowest neuroprotective dose of the compound and whether delayed administration at 60 and 120 min post-HI would show neuroprotection. Our observations show that immediate or delayed administration of curcumin strongly protects the neonatal brain following HI insult. These protective effects are though not a consequence of changes in cell proliferation, but possibly related to alterations in STAT3 phosphorylation and prohibitin (PHB) protein levels. The STAT3 Y705 phosphorylation site is responsible for the transcription properties of STAT3 and previous results from our group showed bilateral upregulation and involvement of STAT3 Y705 in neonatal HI brain damage (Hristova et al., 2016). The STAT3 S727 phosphorylation site is downstream of extracellular signal-regulated kinase (ERK), respectively of RAS and is responsible for mitochondrial survival in cancer studies (Gough et al., 2009).

PHB is a pleiotropic multifaceted protein and an essential factor in mitochondrial homeostasis, biogenesis, degradation, and response to stress (Peng et al., 2015; Ande et al., 2017; Hernando-Rodríguez and Artal-Sanz, 2018). In mitochondria, PHB acts as a scaffold protein and is thus crucial for regulation of mitochondrial architecture, mitochondrial dynamics, morphology, and biogenesis, and furthermore stabilizes the mitochondrial genome (Merkwirth et al., 2012; Peng et al., 2015).

Our data demonstrate that the neuroprotective effects of curcumin are likely the consequence of changes in the levels of PHB and of STAT3 phosphorylation (Y705 and S727); thus affecting inflammation and mitochondrial dysfunction post-HI. Therefore, curcumin could be considered a potent candidate for neuroprotective treatment in neonatal HI brain damage.

MATERIALS AND METHODS

HI Insult

All animal experiments and care protocols were carried out according to the UK Animals (Scientific Procedures) Act 1986 and approved by the Home Office (PPL70/8784). The ARRIVE guidelines were followed. All experiments involved postnatal day 7 C57/Bl6 mice (P7) bred in house.

The surgical procedures were performed as previously described (Hristova et al., 2010; Kendall et al., 2012; Lange et al., 2014; Rocha-Ferreira et al., 2015). Briefly, male and female P7 mice were anesthetized with isoflurane (5% induction and 1.5% maintenance). The left common carotid artery was permanently occluded with 8/0 polypropylene suture and the wound closed with tissue glue. The mice recovered at 36°C and were returned to the dam for 2 h. The pups were then placed in a hypoxia chamber and exposed to humidified 8% oxygen/92% nitrogen (3 L/min) at 36°C for 60 min, resulting in moderate to severe brain damage (Lange et al., 2014; Hristova et al., 2016).

The P7 rodent HI model, though slightly preterm, presents phenotypical similarities to the grey and white matter injury observed in humans, i.e., tissue loss, cell-death, microglia-mediated immune response, and astrogliosis as well as alteration in neurobehavioral performance (Vannucci and Vannucci, 1997).

Pharmacological Treatment

Curcumin (LKT Laboratories, UK) was dissolved in 100% DMSO to concentrations of 20, 44, 100, 200, and 400 ug/ul. The animals were injected 20 min before, immediately after, or after a 60 or 120 min delay following a 60 min HI insult. The animals received a single intraperitoneal injection of 0.5 ul/g body weight (BW) resulting in doses of 10, 22, 50, 100, or 200 μg/g, respectively, based on previous studies (Shukla et al., 2008; Zhao et al., 2010).

Tissue Sample Preparation

The animals were sacrificed at 48 h or at 21 days post-HI by intraperitoneal injection of pentobarbitone and perfused with 30 ml (for 48 h) or 90 ml (for 21 days) 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed, post fixed in 4% paraformaldehyde/0.1 M PB for 1 h at 4°C, and cryoprotected in 30% sucrose/PB solution for 24 h as previously described (Rocha-Ferreira et al., 2015; Hristova et al., 2016). The brains were then frozen on dry ice, cut on a cryostat into sequential 40 μm sections, and stored at −80°C until used.

Immunohistochemistry and Histological Analysis

Five sections from each brain (400 μm apart) were rehydrated in distilled water and stained using immunohistochemistry as previously described (Möller et al., 1996). Briefly, the sections were incubated overnight with rat anti-CD11b αM integrin subunit (1:5,000, Serotec, UK), rabbit polyclonal anti-GFAP (1:6,000, DAKO, UK), rabbit anti-iNOS (1:500, Santa Cruz, USA), polyclonal rat anti-BrdU (1:400, Abcam, UK), or rabbit anti-myelin basic protein (MBP) (1:200, Abcam, Cambridge, UK) primary antibodies, for 1 h with biotinylated goat anti-rabbit or -rat (1:100, Vector, UK) secondary antibodies, followed by incubation with Avidin-Biotinylated horseradish peroxidase Complex (Vector, UK) and visualization with diaminobenzidine/H_2O_2 (Fisher Scientific, UK). The visualization for BrdU antibody required Co/Ni enhancement.

Five further sections from each brain with the same spacing were stained using Terminal transferase mediated d-UTP nick end labeling (TUNEL) (Roche, UK). The staining procedure followed the manufacturer protocol with Co/Ni enhancement.

Five more sections per brain with the same spacing were stained with Cresyl-Violet (Nissl).

To detect and identify the different types of proliferating cells following neonatal HI, we used double labeling for BrdU and: rabbit polyclonal anti-IBA1 (microglia, 1:2,000, Wako, Japan), rabbit polyclonal anti-GFAP (astroglia, 1:6,000, DAKO, UK), guinea pig polyclonal anti-NG2 (oligodendrocyte precursors, 1:400, Bill Stallcup, USA), or mouse monoclonal anti-NeuN (neurons, 1:25,000, Millipore, UK). The protocol was performed as previously described (Hristova et al., 2010). The sections were covered with VectaShield (Vector) and stored in the dark at 4°C before use. The number of BrdU positive and the double positive cells was counted in three fields at ×20 magnification. The percentage of the double positive cells as a fraction of the overall number was calculated and presented.

AlphaM Score

Immunohistochemistry for αM integrin as an early microglial activation marker (Raivich et al., 1999; Hristova et al., 2010; Kendall et al., 2012; Lange et al., 2014; Rocha-Ferreira et al., 2015), was performed as previously described (Rocha-Ferreira et al., 2015; Hristova et al., 2016). Semi-quantitative scores were allocated to each brain region (cortex, pyriform cortex, hippocampus, striatum, thalamus, and external capsule) by two independent observers blinded to the treatment of the groups.

TUNEL, BrdU, and iNOS

TUNEL positive cell death or iNOS and BrdU positive cells were assessed at 48 h following HI through bilateral counting of the number of positive cells in three different optical fields at ×20 magnification. Cortex, pyriform cortex, hippocampus,

striatum, thalamus, and external capsule were assessed for TUNEL and BrdU. Due to the exclusive expression of iNOS in hippocampus, this marker was assessed only in that region. The counts were averaged per animal and per group.

Optical Luminosity

The intensity of the GFAP or MBP staining in the tissue was assessed using optical luminosity values (Rocha-Ferreira et al., 2015; Hristova et al., 2016). Images for ipsilateral and contralateral sides were captured with a Sony AVT-Horn 3CCD color video camera (24 bit RGB, 760 × 570 pixel resolution) in three different optical fields in the regions of interest. We used Optimas 6.5 software to obtain the mean and standard deviation (SD) for optical luminosity values (OLV). SD was subtracted from the mean for each image and the resulting value was subtracted from the values acquired for the surrounding glass (Möller et al., 1996). OLVs for GFAP assessment were evaluated in cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule, while for MBP assessment OLVs were recorded in external capsule and striatum.

Infarct Volume Measurement

The Cresyl-Violet stained sections were scanned and imported into Optimas 6.5 image analysis software. The areas of intact staining in the cortex, pyriform cortex, hippocampus, striatum, thalamus, and external capsule were outlined and bilaterally measured using Fiji Image J (NIH, USA). In the long-term assessment experiments the intact staining of the different regions as well as the whole hemisphere were outlined and bilaterally measured. The percentage tissue loss was then calculated by converting the measured injured and uninjured areas into square millimeters and then transformed to a volume through multiplication by 400 μm. The sum of these volumes was then used to calculate the percentage of surviving brain tissue as ipsilateral/contralateral × 100 (Kendall et al., 2006).

Behavioral Assessment

The slipping test was performed at 21 days (P28) post-HI. It is a modification of the balance beam test (Luong et al., 2011) used for evaluation of motor balance and co-ordination. This test detects motor deficits resulting from central nervous system lesions, as well as aging and genetic and pharmacological interventions (Luong et al., 2011). The apparatus consists of a metal grid 50 cm in length, placed about 20 cm above a table top between the housing cage and a new clean cage. The starting point is the clean cage and the finish consisted of the housing nesting cage. The animals were allowed to walk freely on the grid for 1 min. The task was recorded, and the videos were reviewed by two team members blinded to the mouse groups. The number of missed steps was counted and presented as a percentage of the total number of steps for each animal. The results were then averaged per group.

At P7, the levels of testosterone in male and female mice do not differ (Clarkson and Herbison, 2016). However, at 21 days, testosterone levels between male and female mice are different and a lot of clinical and experimental evidence suggests important differences between males and females, with increased loss of male hippocampal volume after chronic postnatal hypoxia (Mayoral et al., 2009; Gobinath et al., 2017). Thus, the assessments for 21 days took gender into account.

Western Blot Analysis

The animals were sacrificed at 1 h post HI by intraperitoneal injection of pentobarbitone and hippocampus was extracted from treated and un-treated brains and snaps frozen before homogenization in RIPA+ buffer (Sigma, UK) containing 10% protease inhibitor complex (Sigma, UK). The time point was chosen as coinciding with time of maximum expression of phosphorylated STAT3 Y705 (Hristova et al., 2016). Total protein was thereafter extracted from the homogenized hippocampal tissue as follows: the homogenized tissue was incubated with the RIPA+ buffer on a shaking platform on ice for 2 h, pipetting gently with regular intervals. Thereafter, the homogenates were centrifuged at 16,000 g at 4°C for 20 min and the supernatant containing the extracted protein collected. The protein extracts were re-constituted in 2 × Laemmli sample buffer (BioRad, UK) containing 5% β-mercaptoethanol (Sigma, UK) and boiled for 5 min at 100°C before separation by SDS-PAGE, using 4–20% Mini-Protean TGX protein gels (BioRad, UK), followed by Semi-Dry Western blotting analysis. Approximately, 5 μg of protein was loaded per lane and even transfer to nitrocellulose membranes (0.45 μm, BioRad, UK) was assessed using Ponceau S staining (Sigma, UK). The membranes were blocked for 1 h at room temperature (RT) in 5% bovine serum albumin (BSA, Sigma, UK) in Tris-buffered saline (TBS) with 0.001% Tween20 (TBS-T), followed by overnight incubation at 4°C with the following primary antibodies: anti-prohibitin (Abcam, UK), Phospho-Stat3 (Ser727) (Cell Signaling, UK and Abcam, UK), or Phospho-Stat3 (Tyr705) (Cell Signaling, UK and Abcam, UK). Thereafter, membranes were washed three times for 10 min in TBS-T, incubated for 1 h at RT with an HRP-labeled anti-rabbit IgG secondary antibody (BioRad, UK), followed by five TBS-T washes and one final TBS wash, and thereafter they were visualized using ECL (Amersham, UK) and the UVP BioDoc-ITTM System. HRP-conjugated anti-β-actin antibody (1/5,000 in TBS-T, Abcam, UK) was used for internal loading control and densitometry analysis was carried out using ImageJ.

Statistics

Statistical significance was assessed through repeated testing using Mixed Linear Model with SPSS 23.0 and GraphPad Prism 7.0 software, treating region as the repeated measure. For each outcome six regions of the brain were examined. It is likely that with repeated measures such as the observations from a single subject are correlated, the first stage of the analysis included the observations from all the regions tested in a single mixed model with a random subject effect, to produce an estimate of the treatment effect and associated inference that accounts for the correlations in the data arising from the repeated measures. Further *post hoc* Student *t*-tests were carried out to assess evidence for subregional differences, $p < 0.05$. For comparison of more than two groups, we used two-way ANOVA

with *post hoc* Tukey or Bonferroni tests to assess evidence for subregional differences, $p < 0.05$. If parametric analysis was inappropriate, the non-parametric Kruskal-Wallis test was used, followed by Bonferroni-corrected pairwise-contrasts to investigate differences between treatment-conditions. For each outcome, the main effect from the mixed linear model or the Kruskal-Wallis test is reported, followed by the results from the individual regional *t*-tests. In our data, a main effect is the effect of an independent variable (treatment) on a dependent variable (damage marker) averaged across the levels of any other independent variables (brain regions). All data are presented as Mean + SEM.

RESULTS

Intraperitoneal Pre-treatment With Curcumin Increases the Glial Response Following HI-Insult in the Neonate

To determine the biological impact of pre-treatment with curcumin, the animals were injected intraperitoneally with 100 μg/g BW curcumin and the effect on brain volume loss, TUNEL+ cell death, and on reactive astrogliosis and microglial activation at 48 h were examined following the HI-insult. As shown in **Figure 1**, curcumin pre-treatment had no main effect

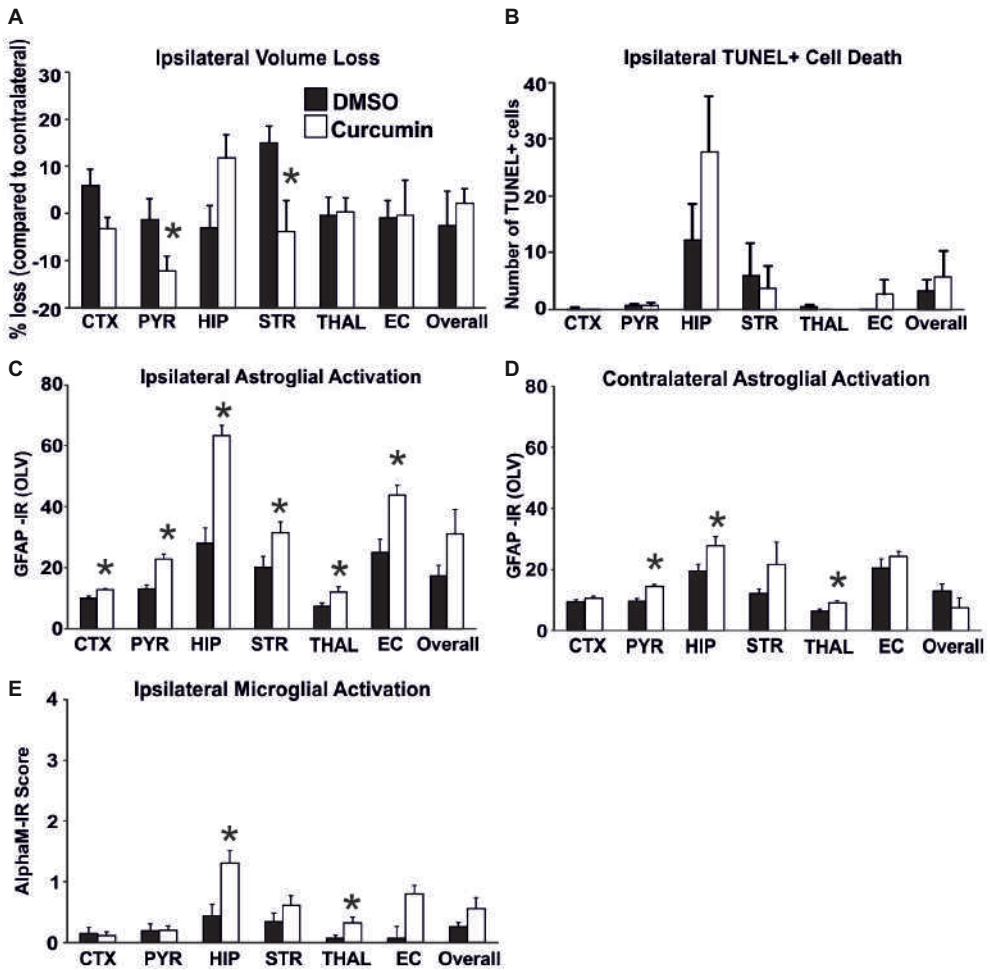


FIGURE 1 | Intraperitoneal injection of curcumin (100 μg/g BW) in P7 mice 20 min before HI does not affect tissue damage and cell death, but significantly increases glial response. **(A)** Ipsilateral forebrain Nissl staining (Cresyl-Violet, at rostral parietal level) – Quantification of ipsilateral brain tissue volume loss of DMSO and curcumin pre-treated animals at 48 h following HI-insult. Curcumin pre-treatment ($n = 12$) did not affect volume loss compared to DMSO-treated littermates ($n = 11$) (Mixed Linear Model treating region as a repeated measure $p = 0.156$). However significant, individual decrease (*t*-test) was registered in pyriform cortex ($p = 0.04$) and striatum ($p = 0.02$). **(B)** The number of TUNEL+ dying cells (per 20x eye-field) at 48 h following HI, was not affected in the curcumin pre-treated group compared to DMSO-treated littermates (Mixed Linear Model treating region as a repeated measure $p = 0.053$). **(C,D)** GFAP immunoreactivity at 48 h – quantification of the ipsilateral **(C)** and contralateral **(D)** side in optical luminosity values (OLV, Mean + SEM). Note the increased levels of GFAP immunoreactivity in the curcumin pre-treated animals, with significant, individual increase (*t*-test) in ipsilateral cortex ($p = 0.002$), pyriform cortex ($p = 0.0001$), hippocampus ($p = 3\times10^{-5}$), striatum ($p = 0.038$), thalamus ($p = 0.026$), and external capsule ($p = 0.003$), and contralateral pyriform cortex ($p = 0.0002$), hippocampus ($p = 0.04$), and thalamus ($p = 0.013$). Mixed Linear Model treating region as a repeated measure revealed $p = 0.0001$ for ipsilateral and $p = 0.007$ for contralateral side, respectively. **(E)** Activation of αM+ microglia – ipsilateral αM microglial activation score (Mean + SEM). Pre-treatment with 100 μg/g BW curcumin increased αM+ microglial activation with significant, individual increase (*t*-test) in hippocampus ($p = 0.008$) and thalamus ($p = 0.03$). Mixed Linear Model treating region as a repeated measure revealed $p = 0.019$. (*$p < 0.05$). Abbreviations: CTX, cerebral cortex; EC, external capsule; HIP, hippocampus; PYR, pyriform cortex; STR, striatum; THAL, thalamus.

on brain volume loss (**Figure 1A**) and TUNEL+ cell death (**Figure 1B**). However, individual significant attenuation of brain volume loss (*t*-test) was observed in pyriform cortex and striatum (**Figure 1A**). Interestingly, an increase of tissue loss, although not significant, was observed in the hippocampus of curcumin treated animals. Pre-treatment with 100 μg/g BW of curcumin significantly increased HI-induced and predominantly ipsilateral reactive astrogliosis and microglial activation (**Figures 1C–E**). Compared to DMSO-treated littermates, the curcumin pre-treated animals revealed more GFAP-immunoreactivity (**Figures 1C,D**). Assessment across the different forebrain regions through Mixed Linear Model treating region as a repeated measure revealed a clear increase on the ipsilateral, and milder increase on the contralateral side ($p = 0.0001$ and $p = 0.007$, respectively), with individual significant increase of 20–50% in all studied ipsilateral regions, and of 30–40% in contralateral pyriform cortex, hippocampus and striatum ($p < 0.05$ in *t*-test). Curcumin pre-treatment also increased microglial activation score (**Figure 1E**) based on the αM integrin immunoreactivity. Regional assessment revealed an increase in activation score in the curcumin pre-treated group (Mixed Linear Model treating region as a repeated measure $p = 0.019$) with significant increase of 30–50% in hippocampus and thalamus (**Figure 1E**).

Immediate Post-treatment With Curcumin Reduces Brain Damage Following Neonatal HI-Insult

Compared to DMSO-treated animals, intraperitoneal injection of 200 μg/g BW curcumin straight after the HI-insult significantly reduced brain damage markers (tissue loss, TUNEL+ cell death, reactive astrogliosis, and microglial activation) at 48 h post-HI. As shown in **Figures 2A–C**, curcumin post-treatment markedly decreased ipsilateral forebrain tissue loss. Regional assessment presented in **Figure 2A** revealed strong decrease across the different forebrain regions (Mixed Linear Model treating region as a repeated measure, $p = 0.001$). Treatment with curcumin significantly reduced tissue loss in relation to DMSO-treated littermates by 50–90% in cortex, pyriform cortex, hippocampus, striatum, external capsule, and overall forebrain area ($p < 0.05$ in *t*-test). **Figure 2B** shows large infarct in cortex and hippocampus of the DMSO-treated animal and its sparing in the curcumin-treated littermate (**Figure 2C**).

A similar effect of the curcumin post-treatment was also observed for TUNEL+ cell death (**Figures 2D–F**). **Figure 2D** shows that curcumin treatment straight after 60 min HI significantly reduced the number of TUNEL+ cells compared to DMSO-treated littermates (Mixed Linear Model treating region as a repeated measure $p = 0.036$), with individual significant decrease of 70–90% in cortex, pyriform cortex, hippocampus, and overall ($p < 0.05$ in *t*-test). The TUNEL+ cells displayed the typical pyknotic nuclear morphology (**Figure 2E**-insert, ipsilateral hippocampus DMSO).

In addition to cell death and brain tissue loss, curcumin post-treatment also decreased HI-induced and predominantly ipsilateral reactive astrogliosis and microglial activation. Compared to DMSO-treated animals, their curcumin-treated littermates revealed less GFAP immunoreactivity (**Figures 2G,M**) with substantially reduced amount of GFAP+ astroglial processes (**Figures 2H,I**). Assessment across the different forebrain regions through Mixed Linear Model treating region as a repeated measure revealed a clear decrease on the ipsilateral, and milder reduction on the contralateral side (main effect $p = 0.005$ and $p = 0.0001$, respectively) with individual significant decrease of 30–50% in all six ipsilateral regions and overall, and of 30% in contralateral cortex and pyriform cortex ($p < 0.05$, *t*-test).

Curcumin post-treatment had a similar effect on microglia activation score (**Figure 2J**) based on αM integrin immunoreactivity (**Figures 2K,L**). Regional assessment shown in **Figure 2J** revealed a reduction in activation score in the curcumin-treated group (Mixed Linear Model treating region as a repeated measure, main effect $p = 0.019$), with significant decrease of 70–90% in all six individual ipsilateral brain regions ($p < 0.05$ in *t*-test).

Compared to DMSO-treated animals, curcumin post-treated littermates had higher levels of myelination assessed through MBP immunoreactivity (**Figure 2N**), with individual significant increase of 20% in external capsule ($p < 0.05$, *t*-test).

Curcumin post-treatment also reduced the levels of oxidative stress assessed through iNOS immunoreactivity, compared to DMSO-treated littermates, with individual significant decrease of 50% in hippocampus (**Figure 2O**, $p < 0.05$, *t*-test).

Immediate Post-treatment With Curcumin Reduces Ipsilateral Volume Loss in Males, Females and Combined (Males + Females), Decreases Myelin Loss in Males, but Does Not Provide Functional Protection Assessed Through the Slipping Test

To evaluate the long-term effects of curcumin following immediate injection of 200 μg/g post HI, the levels of tissue loss were assessed through Nissl staining, the degree of myelination was evaluated through MBP immunoreactivity, and motor balance and co-ordination were assessed through the slipping test (Rocha-Ferreira et al., 2018) at day 21 post HI (P28).

Intraperitoneal injection of 200 μg/g curcumin straight after HI decreased tissue loss in males at 21 days post HI compared to DMSO-treated littermates and untreated HI controls (**Figure 3A**). Immediate curcumin treatment had no effect on tissue loss in females compared to DMSO treated or untreated HI controls (**Figure 3B**). The levels of tissue loss in females were lower compared to males (**Figure 3D**). Although the differences did not reach significant values, the lower level of tissue loss in females suggests increased susceptibility of males to the insult. The levels of tissue loss in the curcumin group were significantly lower compared to DMSO-treated littermates and untreated HI controls (**Figure 3C**). Subregional assessment of tissue loss at 21 days post HI in males (**Figure 3E**) and combined (males + females) (**Figure 3G**) suggested that immediate treatment with curcumin at 200 μg/g immediately post HI reduced tissue damage in all studied regions with significant decrease in thalamus. Curcumin treatment did not affect subregional differences in tissue loss in females (**Figure 3F**).

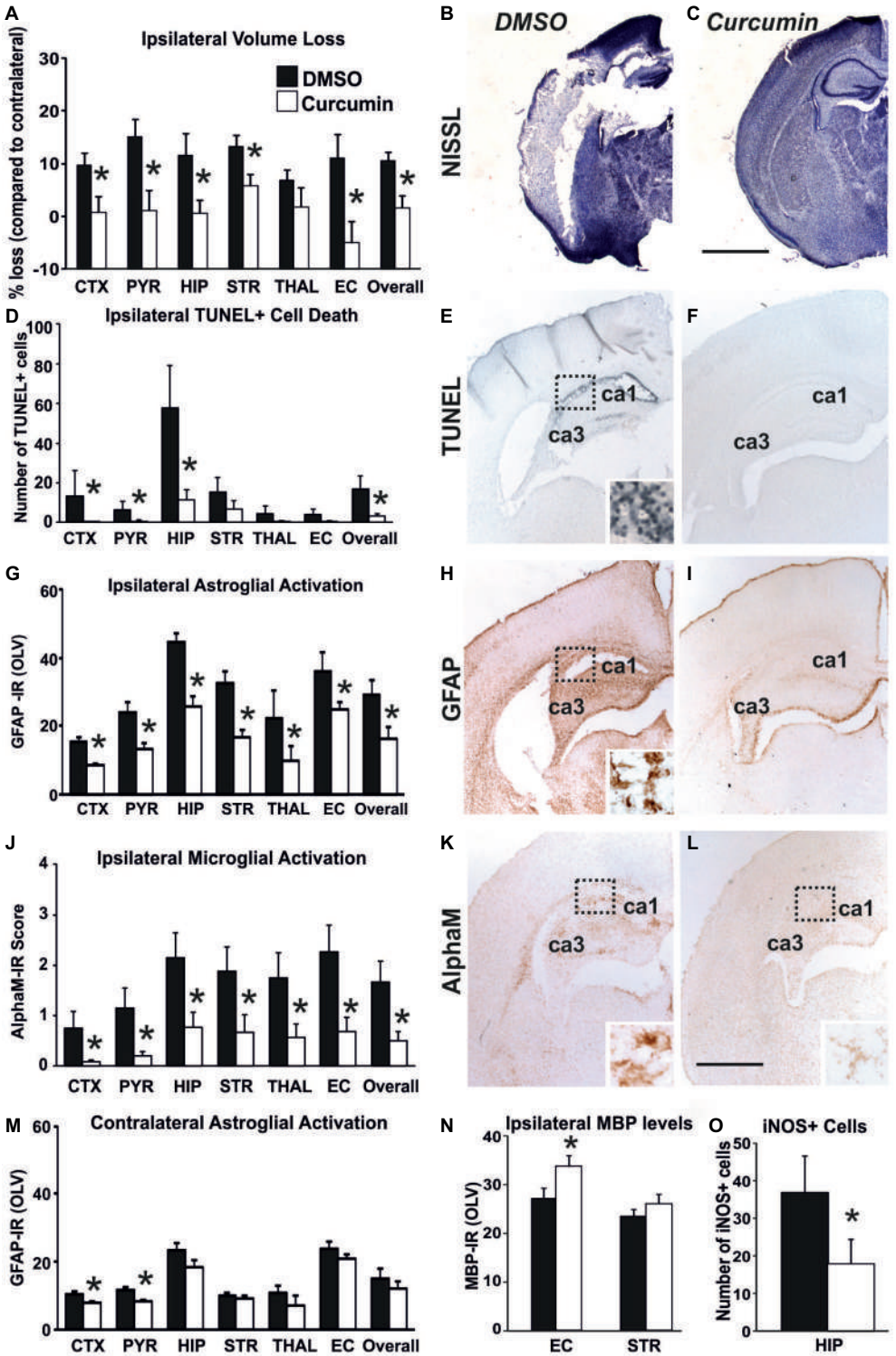


FIGURE 2 | Intraperitoneal injection of curcumin (200 μg/g BW) in P7 mice immediately post-HI significantly reduces tissue damage, cell death and glial response. **(A–C)** Ipsilateral forebrain Nissl staining (Cresyl-Violet, at rostral parietal level) - Quantification of ipsilateral brain tissue volume loss **(A)** at 48 h following HI-insult of DMSO **(B)** and curcumin **(C)** treated animals at 48 h following HI-insult. Curcumin treatment (n = 11) reduced volume loss compared to DMSO-treated littermates (n = 11) with significant, individual decrease (t-test) in cortex (p = 0.026), pyriform cortex (p = 0.011), hippocampus (p = 0.038), striatum (p = 0.020), external capsule (p = 0.015) and overall volume loss (p = 0.003). Mixed Linear Model treating region as a repeated measure revealed p = 0.001. **(D–F)** TUNEL+ staining of dying brain cells with fragmented DNA at 48 h following HI-insult – Quantification **(D)** (number of TUNEL+ cells per 20× eye-field, Mean + SEM) and histochemical overview of the ipsilateral forebrain in DMSO **(E)** and curcumin **(F)** treated animals. Note the typical pyknotic nuclear morphology of the TUNEL+ cells

(Continued)

FIGURE 2 | observed in the DMSO group (**E**- insert, hippocampus) and the lack of such cells in the curcumin group **(F)**. Curcumin treatment reduced TUNEL+ cell death across all 6 examined forebrain regions, with significant, individual decrease (*t*-test) in cortex ($p = 0.04$), pyriform cortex ($p = 0.049$), hippocampus ($p = 0.046$), and overall ($p = 0.048$). Mixed Linear Model treating region as a repeated measure revealed $p = 0.036$. **(G–I,M)** GFAP immunoreactivity at 48 h - Quantification of the ipsilateral **(D)** and contralateral, non-occluded side **(M)** in optical luminosity values (OLV, Mean + SEM), and low magnification ipsilateral overview in DMSO **(H)** and curcumin **(I)** treated animals. The insert in **H** shows higher magnification of the dotted region in rostro-parietal cortex. Note the reduced levels of GFAP immunoreactivity in the curcumin treated animals, with significant, individual decrease (*t*-test) in ipsilateral cortex ($p = 4 \times 10^{-5}$), pyriform cortex ($p = 0.004$), hippocampus ($p = 0.049$), striatum ($p = 0.001$), thalamus ($p = 0.001$), external capsule ($p = 0.009$) and overall ($p = 0.048$) in **G**, and in contralateral cortex ($p = 0.01$) and pyriform cortex ($p = 0.0004$) in **M**. Mixed Linear Model treating region as a repeated measure revealed $p = 0.005$ for the ipsilateral, and $p = 0.0001$ for the contralateral side. **(J–L)** Activation of αM+ microglia – Ipsilateral αM microglial activation score (**J**, Mean + SEM) and low magnification ipsilateral overview in DMSO **(K)** and curcumin **(L)** treated animals. Note the strong microglial activation in DMSO-treated animals with αM+ cells showing phagocytic morphology at high magnification (K-insert, hippocampus), compared to the curcumin treated brains exhibiting a ramified phenotype (L-insert). Curcumin treatment reduced αM+ microglial activation across all six examined forebrain regions, with significant, individual decrease (*t*-test) in cortex ($p = 0.03$), pyriform cortex ($p = 0.01$), hippocampus ($p = 0.03$), striatum ($p = 0.04$), thalamus ($p = 0.04$), external capsule ($p = 0.01$) and overall ($p = 0.02$). Mixed Linear Model treating region as a repeated measure $p = 0.019$. DMSO ($n = 11$) and curcumin ($n = 11$) in all assessments. **(N)** MBP immunoreactivity at 48 h - Quantification of the ipsilateral external capsule and striatum in optical luminosity values (OLV, Mean + SEM). Note the increased levels of MBP immunoreactivity in the curcumin treated animals, with significant, individual increase (*t*-test) in ipsilateral external capsule ($p = 0.045$). Mixed Linear Model treating region as a repeated measure $p = 0.056$. **(O)** iNOS immunoreactivity at 48 h – Quantification of the number of iNOS+ cells in ipsilateral hippocampus (number of iNOS+ cells per 20× eye-field, Mean + SEM). Note the reduced number of iNOS+ cells (*t*-test) in the curcumin treated group compared to DMSO-treated littermates ($p = 0.041$). (*$p < 0.05$). Abbreviations: CTX, cerebral cortex; EC, external capsule; HIP, hippocampus; PYR, pyriform cortex; STR, striatum; THAL, thalamus. Scale bars: **(B,C)** = 2,000 μm; **(E,F,H,I,K,L)** = 1,000 μm; inserts = 62 μm.

Compared to untreated male HI control animals, curcumin post-treated littermates had an increase in myelination assessed through MBP immunoreactivity (**Figure 3H**) of 23% in striatum ($p < 0.05$, two-way ANOVA). Immediate post-HI treatment with 200 μg/g curcumin did not affect myelin loss in females (**Figure 3I**) or combined (males + females) (**Figure 3J**).

Assessment of motor balance and co-ordination through slipping test at 21 days post HI of animals treated immediately after the insult with 200 μg/g curcumin, DMSO, or HI controls showed slightly decreased number of missed steps in curcumin-treated males (**Figure 3K**), females (**Figure 3L**) and combined (males + females) (**Figure 3M**); however, the differences did not reach significance (Kruskal-Wallis test).

Immediate Post-treatment With Curcumin Reduces Brain Damage Following Neonatal HI-Insult in a Dose-Dependent Manner

Curcumin dose of 200 μg/g BW resulted in significant neuroprotection (**Figure 2**). To determine whether a lower dose of curcumin post-treatment immediately after the HI-insult would provide neuroprotective effects, three groups of animals were injected intraperitoneally with doses of 50, 22, and 10 μg/g BW curcumin in 0.5 μl/g BW DMSO. The control littermates in each group received the same volume of DMSO only. Brain damage markers (tissue loss, TUNEL+ cell death, reactive astrogliosis, and microglial activation) were assessed at 48 h post-HI. As shown in **Figure 4**, the dose of 50 μg/g (**Figure 4A**) significantly reduced the levels of microglial activation (Mixed Linear Model treating region as a repeated measure $p = 0.035$), with significant individual decrease of 50–65% in cortex, hippocampus, striatum, and external capsule ($p < 0.05$, *t*-test). Similarly, the dose of 50 μg/g BW curcumin significantly reduced the levels of ipsilateral tissue loss (Mixed Linear Model treating region as a repeated measure $p = 0.0001$), with significant individual decrease of 75–90% in cortex, hippocampus, and overall (**Supplementary Figure S1A**, $p < 0.05$, *t*-test). The levels of TUNEL+ cell death were also reduced by the dose of 50 μg/g BW curcumin (Mixed Linear Model treating region as a repeated measure $p = 0.016$) with significant individual reduction of 75–90% in hippocampus and external capsule (**Supplementary Figure S1D**, $p < 0.05$, *t*-test). The dose of 50 μg/g markedly reduced also the levels of ipsilateral reactive astrogliosis (Mixed Linear Model treating region as a repeated measure $p = 0.014$), with significant individual reduction of 44–55% in pyriform cortex, hippocampus, thalamus, and external capsule (**Supplementary Figure S1G**, $p < 0.05$, *t*-test). In a similar pattern, the dose of 50 μg/g significantly reduced contralateral astroglial activation (Mixed Linear Model treating region as a repeated measure $p = 0.005$), with significant individual decrease in pyriform cortex ($p < 0.05$, **Supplementary Figure S1J**). The doses of 22 and 10 μg/g BW curcumin did not have an effect on microglial activation (**Figures 4B,C**), ipsilateral volume loss, TUNEL+ cell death or reactive astrogliosis (**Supplementary Figures S1B,C,E,F,H,I,K,L**).

Delayed Post-treatment With Curcumin at 60 and 120 min Post HI Reduces Brain Damage Following Neonatal HI-Insult

To investigate whether delayed application of curcumin would provide similar neuroprotection to the one achieved through immediate post-HI treatment, 200 μg/g BW curcumin was applied at 60 and 120 min following a neonatal HI insult and the brain damage markers were assessed at 48 h post HI. Treatment at 60 min reduced volume loss by 60–70% in cortex, hippocampus, striatum, thalamus, and overall compared to HI controls (**Figure 5A**, $p < 0.05$, Kruskal-Wallis test with Bonferroni correction), but had no effect compared to DMSO-treated littermates and no main effect of the treatment was observed. Application of curcumin at 60 min post HI significantly reduced TUNEL+ cell death compared to HI- and DMSO-treated littermates (**Figure 5C**, $p < 0.05$, Kruskal-Wallis test). Pairwise comparison between the curcumin treated and HI groups revealed significant 80–90% decrease of the number of TUNEL+ cells in cortex, pyriform cortex, hippocampus and overall (**Figure 5C**, $p < 0.05$, Kruskal-Wallis test with Bonferroni correction). Compared to DMSO-treated littermates curcumin

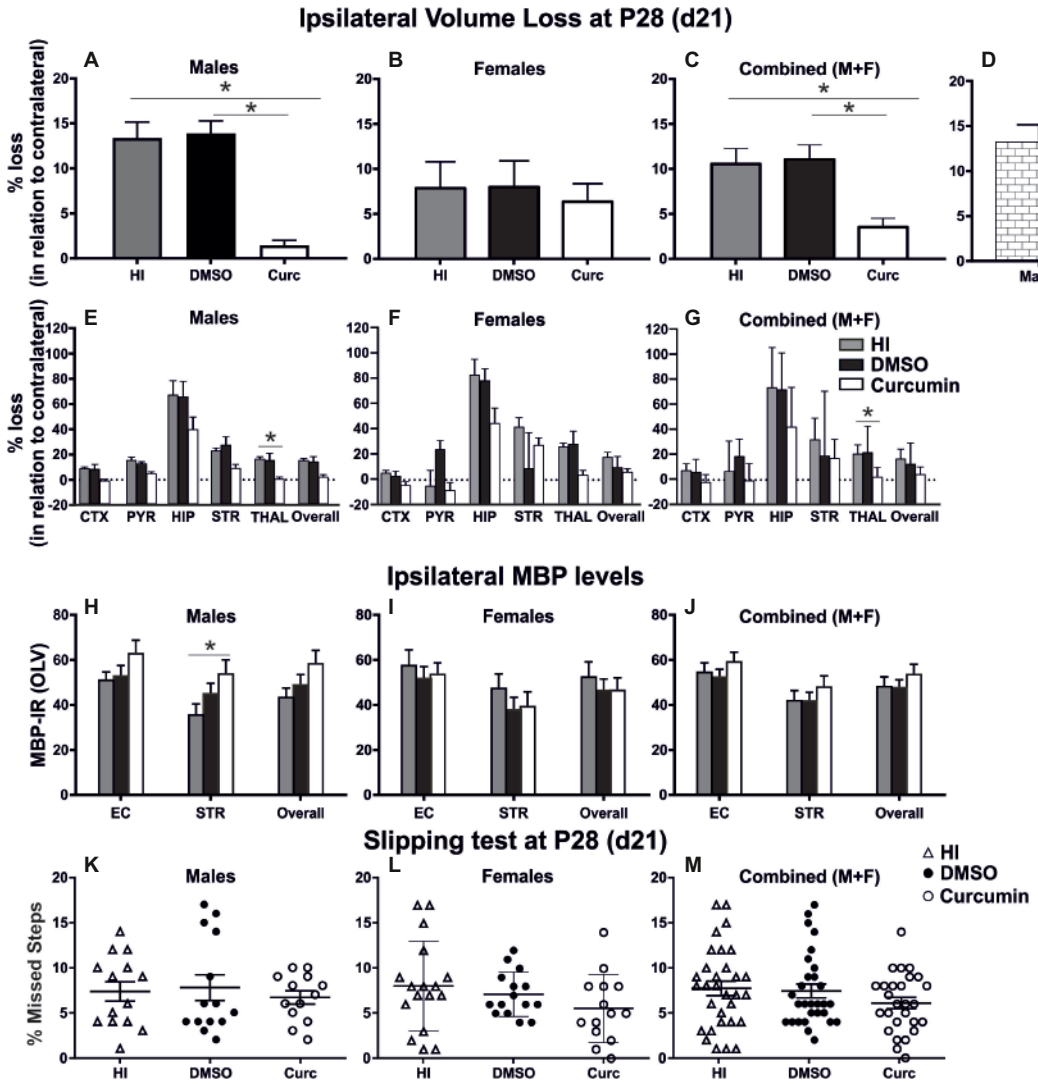


FIGURE 3 | Intraperitoneal injection of curcumin in P7 mice post-HI reduces ipsilateral volume loss in males, females and combined (males + females), decreases myelin loss in males, but does not provide functional protection assessed through slipping test. **(A)** Intraperitoneal injection of 200 μg/g curcumin straight after HI decreased tissue loss at 21 days post HI (P28) in males compared to DMSO-treated littermates and untreated HI controls (curcumin $n = 11$, DMSO $n = 8$, HI $n = 9$; curcumin vs. HI $p = 0.0017$, curcumin vs. DMSO $p = 0.0003$). **(B)** Curcumin treatment at 200 μg/g straight after HI did not affect tissue loss at 21 days post HI in females compared to DMSO-treated littermates or to untreated HI controls. (curcumin $n = 8$, DMSO $n = 7$, HI $n = 8$). **(C)** The levels of tissue loss in the curcumin treated animals decreased compared to DMSO-treated littermates and untreated HI controls (curcumin $n = 19$, DMSO $n = 15$, HI $n = 17$; curcumin vs. HI $p = 0.0024$, curcumin vs. DMSO $p = 0.0014$). **(D)** The level of tissue loss observed in females was lower compared to the males, however the differences did not reach significant values ($p = 0.085$). This suggests higher susceptibility of the males to HI insult. **(E–G)** Subregional assessment of tissue loss at 21 days post HI in males (**E**, curcumin $n = 11$, DMSO $n = 8$, HI $n = 9$), females (**F,** curcumin $n = 8$, DMSO $n = 7$, HI $n = 8$) and combined (males + females) (**G**, curcumin $n = 19$, DMSO $n = 15$, HI $n = 17$). Curcumin treatment with 200 μg/g immediately post HI resulted in a reduction of subregional tissue loss in all studied regions in males **(E)** and combined (males + females) **(G)** with significant differences observed in thalamus (males $p = 0.0292$, combined (males + females) $p = 0.0242$). **(F)** Curcumin treatment had no effect on subregional differences in tissue loss in females. **(H–J)** MBP immunoreactivity at 21 days post HI (P28). Quantification of the ipsilateral external capsule, striatum, and overall in optical luminosity values (OLV, Mean + SEM). **(H)** In males curcumin treatment with 200 μg/g immediately post HI resulted in increased levels of MBP immunoreactivity compared to untreated HI controls (curcumin $n = 11$, DMSO $n = 8$, HI $n = 9$), with significant differences in striatum (Two-way ANOVA with *post hoc* Tukey's test, $p = 0.042$). In females (**I**, curcumin $n = 8$, DMSO $n = 7$, HI $n = 8$) and combined (males + females) (**J**, curcumin $n = 19$, DMSO $n = 15$, HI $n = 17$), immediate treatment with 200 μg/g curcumin had no effect on MBP immunoreactivity. **(K–M)** Curcumin treatment with 200 μg/g immediately post HI did not affect the number of missed steps (slipping test) at 21 days (P28) post HI in males (**K**, curcumin $n = 13$, DMSO $n = 14$, HI $n = 14$), females (**L**, curcumin $n = 14$, DMSO $n = 15$, HI $n = 17$) and combined (males + females) (**M**, curcumin $n = 27$, DMSO $n = 29$, HI $n = 31$). *(*$p < 0.05$)*. Abbreviations: CTX, cerebral cortex; EC – external capsule; HIP – hippocampus; PYR, pyriform cortex; STR, striatum; THAL, thalamus.

treatment at 60 min post HI significantly reduced TUNEL+ cell death by 80–90% in cortex, pyriform cortex, hippocampus and overall (**Figure 5C**, $p < 0.05$, Kruskal-Wallis test with Bonferroni correction). Administration of curcumin at 60 min post HI reduced microglial activation compared to DMSO-treated littermates and HI controls, although the main effect

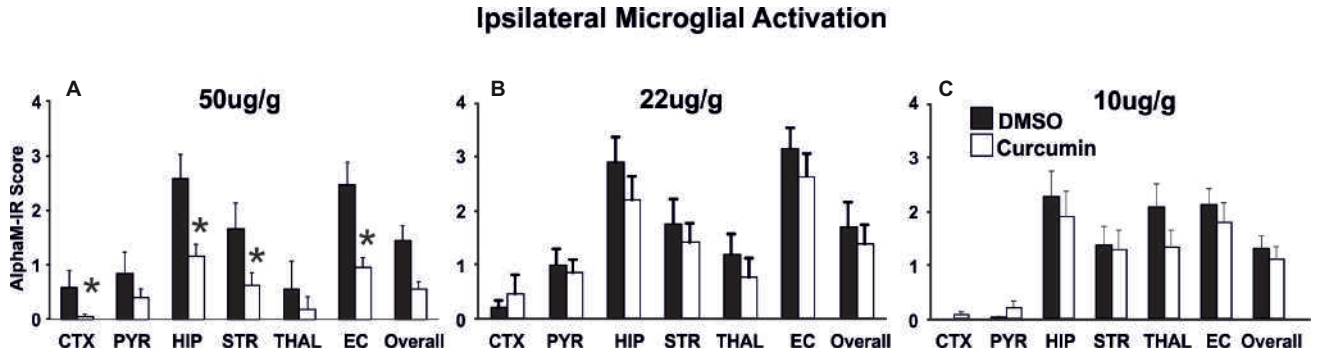


FIGURE 4 | Intraperitoneal injection of curcumin in P7 mice post-HI significantly reduces ipsilateral αM microglial activation score in a dose dependent manner. **(A)** Intraperitoneal injection of 50 μg/g BW curcumin decreased αM+ microglial activation compared to DMSO-treated littermates, with significant, individual decrease (*t*-test) in cortex ($p = 0.038$), hippocampus ($p = 0.035$), striatum ($p = 0.035$) and external capsule ($p = 0.046$). Mixed Linear Model treating region as a repeated measure revealed $p = 0.035$. **(B,C)** Intraperitoneal curcumin injection of 22 μg/g **(B)** or 10 μg/g **(C)** did not have an effect on αM+ microglial activation compared to DMSO-treated littermates. (**A**, curcumin 50 μg/g $n = 8$, DMSO $n = 6$, **B**, curcumin 22 μg/g $n = 12$, DMSO $n = 10$, **C**, curcumin 10 μg/g $n = 11$, DMSO $n = 11$). *(*p < 0.05)* Abbreviations: CTX, cerebral cortex; EC, external capsule; HIP, hippocampus; PYR, pyriform cortex; STR, striatum; THAL, thalamus.

of treatment did not reach significance (**Figure 5E**). Curcumin treatment at 60 min post HI reduced microglial activation compared to HI controls with significant, individual decrease of 30% in external capsule (**Figure 5E**, $p < 0.05$, Kruskal-Wallis test with Bonferroni correction). Compared to DMSO-treated littermates curcumin treatment at 60 min post-HI reduced microglial activation with significant, individual decrease of 90% in cortex (**Figure 5E**, $p < 0.05$, Kruskal-Wallis test with Bonferroni correction). Curcumin application at 60 min post HI did not affect ipsilateral astroglial activation (**Figure 5G**).

Application of curcumin at 120 min post HI surprisingly increased the level of tissue loss but there was no significant main effect of treatment ($p > 0.05$, one-way ANOVA). However pairwise comparison revealed significant increase of 50% in volume loss in the cortex of curcumin treated compared to HI and to DMSO-treated littermates (**Figure 5B,** $p < 0.05$, one-way ANOVA with Bonferroni correction). Delayed administration of curcumin at 120 min post HI significantly reduced the number of TUNEL+ cells compared to HI- and DMSO-treated littermates (**Figure 5D,** $p < 0.05$, one-way ANOVA). Pairwise comparison revealed significant 60–90% decrease of TUNEL+ cell death in curcumin treated compared to HI animals in hippocampus, striatum and overall (**Figure 5D,** $p < 0.05$, one-way ANOVA with Bonferroni correction). Pairwise comparison between curcumin and DMSO-treated animals revealed significant 50–85% decrease of TUNEL+ cell death in hippocampus, striatum and overall (**Figure 5D,** $p < 0.05$, one-way ANOVA with Bonferroni correction). Administration of curcumin at 120 min post HI reduced microglial activation compared to HI- and DMSO-treated littermates (**Figure 5F,** $p < 0.05$, Kruskal-Wallis test). Pairwise comparison between curcumin treated and HI animals revealed significant 40–60% decrease of microglial activation in pyriform cortex, hippocampus, striatum, thalamus, external capsule and overall (**Figure 5F,** $p < 0.05$, Kruskal-Wallis test with Bonferroni correction). Pairwise comparison revealed significant 50% decrease of microglial activation in curcumin compared to DMSO-treated littermates in pyriform cortex, external capsule and overall (**Figure 5F,** $p < 0.05$, Kruskal-Wallis test with Bonferroni correction). Administration of curcumin at 120 min post HI reduced ipsilateral reactive astrogliosis in cortex by 18% compared to HI and by 38% compared to DMSO-treated littermates (**Figure 5H,** $p < 0.05$, Kruskal-Wallis test with Bonferroni correction); however, no main effect of the treatment was observed.

Post-treatment With Curcumin Immediately Post HI Has No Effect on the Levels of Cellular Proliferation at 48 h

To determine whether the neuroprotective effects of immediate curcumin treatment post HI are due to a boost of cellular proliferation, we assessed the number of BrdU positive cells in P7 naïve animals, naïve animals injected with 200 μg/g curcumin, and HI animals injected immediately post insult with either 200 μg/g curcumin or DMSO. The number of BrdU+ cells in naïve untreated animals or naïve and HI animals treated with curcumin or DMSO did not differ between the groups in any of the tested brain regions (**Figure 6A**). Although curcumin treatment did not result in differences in cell proliferation levels in naïve or HI animals, we assessed its effect on the proliferation of different cell types, i.e., microglia, astroglia, oligodendrocyte precursors and neurons through double labeling for BrdU and IBA1 (microglia), GFAP (astroglia), NG2 (oligodendrocyte precursors), and NeuN (neurons) and calculation of the percentage of the double positive cells over the BrdU positive ones.

Curcumin treatment of naïve or HI animals immediately after insult did not affect the percentage of IBA1 and BrdU double positive (**Figure 6B**), GFAP and BrdU double positive (**Figure 6C**), NG2 and BrdU double positive (**Figure 6D**), or NeuN and BrdU double positive (**Figure 6E**) over BrdU positive cells per region and group. The percentage of proliferating microglia (**Figures 6F,N,J**), astroglia (**Figures 6G,O,K**), and oligodendrocytes (**Figures 6H,P,L**) was relatively low (10–20% of the total number of BrdU positive cells) compared to the neurons which comprised 80–90% of the BrdU positive cells (**Figures 6I,Q,M**).

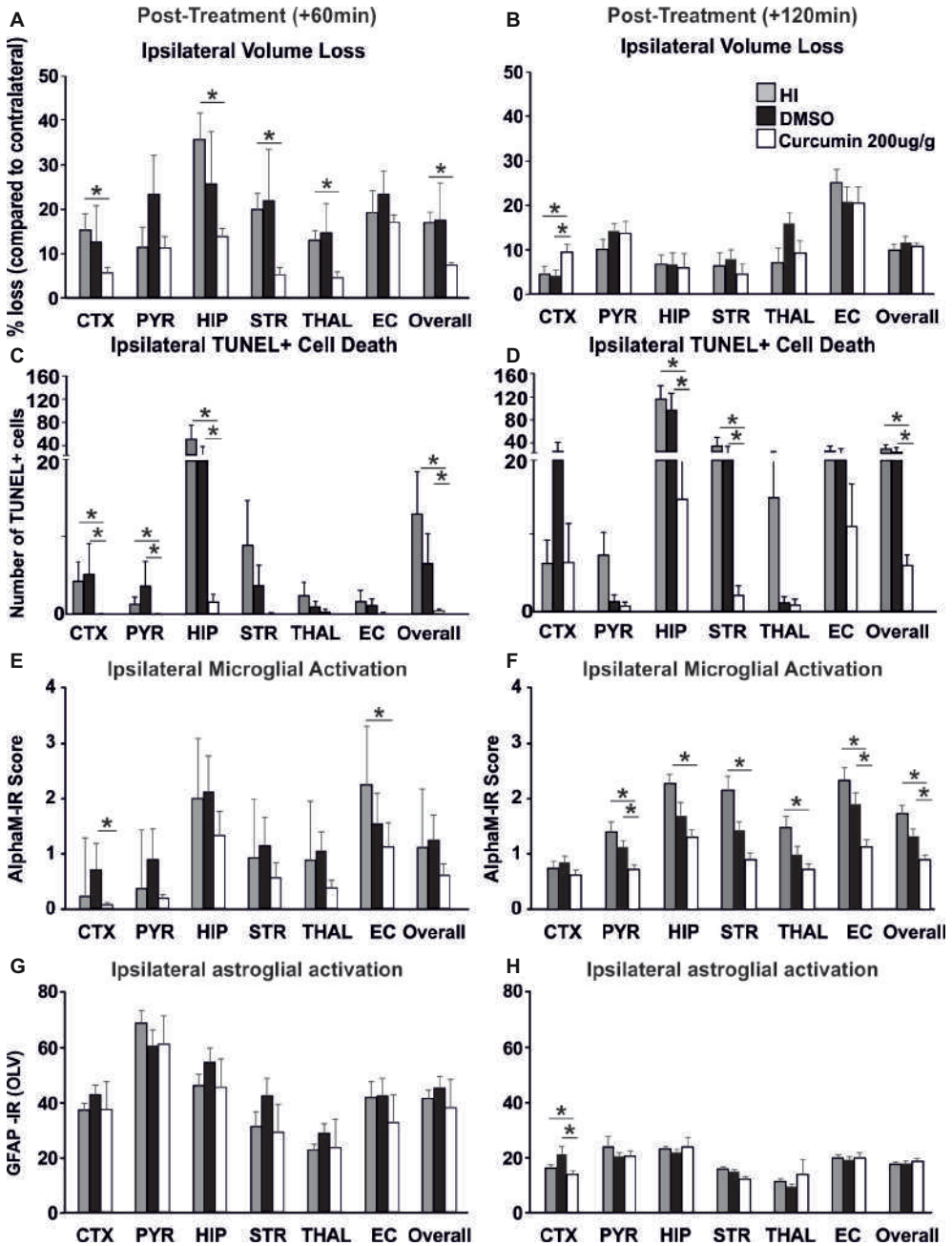


FIGURE 5 | Intraperitoneal injection of curcumin (200 μg/g BW) in P7 mice at 60 min or 120 min post-HI significantly reduces tissue damage, cell death and glial response. **(A)** Curcumin treatment at 60 min post HI (n = 8) reduced volume loss compared to HI controls (n = 10) with significant, individual decrease (Kruskal-Wallis test with Bonferroni correction) in cortex (p = 0.036), hippocampus (p = 0.007), striatum (p = 0.003), thalamus (p = 0.005), and overall volume loss (p = 0.002). However, no main effect of the treatment was observed. No differences were registered between curcumin and DMSO (n = 7) treated littermates. **(B)** Administration of curcumin at 120 min post HI (n = 7) surprisingly increased the level of tissue loss however no significant main effect of treatment was observed. Pairwise comparison (one-way ANOVA with Bonferroni correction) revealed significant increase of volume loss in cortex in curcumin treated compared to HI (n = 7, p = 0.05) and to DMSO-treated littermates (n = 7, p = 0.017). **(C)** Curcumin treatment at 60 min post HI reduced TUNEL+ cell death compared to HI- and DMSO-treated littermates (Kruskal-Wallis test). Pairwise comparison (Bonferroni correction) revealed significant decrease of TUNEL+ cell death in curcumin treated compared to HI animals in cortex (p = 0.064), pyriform cortex (p = 0.064), hippocampus (p = 0.003) and overall (p = 0.001). Pairwise comparison (Bonferroni correction) revealed significant decrease of TUNEL+ cell death in curcumin compared to DMSO-treated animals in cortex (p = 0.003), pyriform cortex (p = 0.013), hippocampus (p = 0.042), and overall (p = 0.012). **(D)** Administration of curcumin at 120 min post HI reduced TUNEL+ cell death compared to HI- and DMSO-treated littermates (tone-way ANOVA, main effect p = 0.001). Pairwise comparison (Bonferroni correction) revealed significant decrease of TUNEL+ cell death in curcumin treated compared to HI animals in hippocampus (p = 0.002), striatum (p = 0.02), and overall (p = 0.001). Pairwise comparison (Bonferroni correction) revealed significant decrease of TUNEL+ cell death in curcumin compared to DMSO-treated littermates in hippocampus (p = 0.012), striatum (p = 0.022) and overall (p = 0.002). **(E)** Administration of curcumin at 60 min post HI reduced microglial activation compared to DMSO-treated littermates and HI controls, *(Continued)*

FIGURE 5 | although the main effect of treatment did not reach significance. However, compared to HI controls, curcumin treatment reduced microglial activation with significant, individual decrease (Kruskal-Wallis test with Bonferroni correction) in external capsule ($p = 0.042$). Compared to DMSO-treated littermates curcumin treatment at 60 min post-HI reduced microglial activation with significant, individual decrease (Kruskal-Wallis test with Bonferroni correction) in cortex ($p = 0.028$). **(F)** Administration of curcumin at 120 min post HI reduced microglial activation compared to HI- and DMSO-treated littermates (Kruskal-Wallis test, main effect $p = 0.002$). Pairwise comparison (Bonferroni correction) revealed significant decrease of microglia activation in curcumin treated compared to HI animals in pyriform cortex ($p = 0.03$), hippocampus ($p = 0.002$), striatum ($p = 0.002$), thalamus ($p = 0.01$), external capsule ($p = 0.006$) and overall ($p = 0.004$). Pairwise comparison (Bonferroni correction) revealed significant decrease of microglia activation in curcumin compared to DMSO-treated littermates in pyriform cortex ($p = 0.018$), external capsule ($p = 0.022$), and overall ($p = 0.016$). **(G)** Curcumin treatment at 60 min post HI did not affect ipsilateral reactive astrogliosis. **(H)** Administration of curcumin at 120 min post HI reduced ipsilateral reactive astrogliosis (Kruskal-Wallis test with Bonferroni correction) in cortex compared to HI ($p = 0.049$) and DMSO-treated littermates ($p = 0.006$), however no main effect of the treatment was observed. (*$p < 0.05$). Abbreviations: CTX, cerebral cortex; EC – external capsule; HIP, hippocampus; PYR, pyriform cortex; STR, striatum; THAL, thalamus.

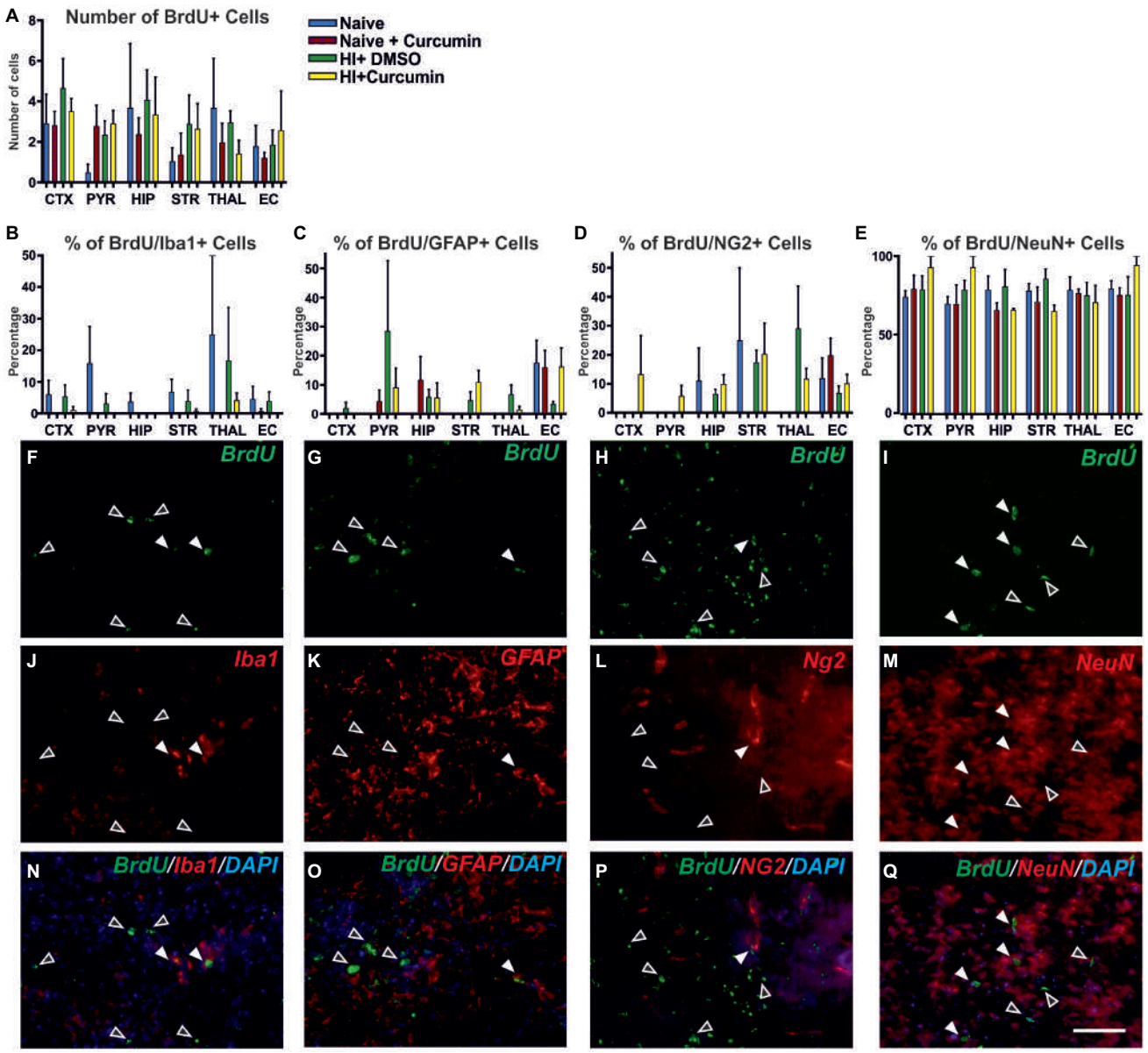


FIGURE 6 | Intraperitoneal injection of curcumin (200 μg/g BW) in P7 mice immediately post-HI has no effect on the levels of proliferation at 48 h. **(A)** Assessment of the number of BrdU+ cells in P7 naïve untreated animals or naïve and HI animals treated with curcumin or DMSO did not show differences between the groups in any of the regions. (naïve $n = 4$, naïve + curcumin $n = 5$, HI + DMSO $n = 7$, HI + curcumin $n = 8$). **(B)** Curcumin treatment in naïve or HI animals immediately after insult did not affect the percentage of IBA1 and BrdU double positive over BrdU+ cells per region and group. The percentage of proliferating IBA1+ cells was low (10–20% of the BrdU+ cells). **(C)** The percentage of GFAP and BrdU double positive over BrdU+ cells per region and *(Continued)*

FIGURE 6 | group was not affected by curcumin treatment in naïve or HI animals. The percentage of proliferating GFAP+ cells was low (10–20% of the BrdU+ cells). **(D)** The percentage of NG2 and BrdU double positive over BrdU+ cells per region and group was not affected by curcumin treatment in naïve or HI animals. The percentage of proliferating NG2+ cells was low (10–20% of the BrdU+ cells). **(E)** The percentage of NeuN and BrdU double positive over BrdU+ cells per region and group was not affected by curcumin treatment in naïve or HI animals. The percentage of proliferating NeuN+ cells comprised 80–90% of the BrdU+ cells and was the highest of all four studied cell types. **(F,J,N)** Immunofluorescence for rat polyclonal anti-BrdU **(F,N)** in green superimposed on the rabbit polyclonal anti-IBA1 **(J,N)** in red, and nuclear DAPI fluorescence in blue **(N)**. Note the co-localization of BrdU and IBA1 (white arrows) and the lack of such co- localization (empty arrows). **(G,K,O)** Immunofluorescence for rat polyclonal anti-BrdU **(G,O)** in green superimposed on the rabbit polyclonal anti-GFAP **(K,O)** in red, and nuclear DAPI fluorescence in blue **(O)**. Note the co-localization of BrdU and GFAP (full arrows) and the lack of such co-localization (empty arrows). **(H,L,P)** Immunofluorescence for rat polyclonal anti-BrdU **(H,P)** in green superimposed on the guinea pig polyclonal anti-NG2 **(L,P)** in red, and nuclear DAPI fluorescence in blue **(P)**. Note the co-localization of BrdU and NG2 (full arrows) and the lack of such co-localization (empty arrows). **(I,M,Q)** Immunofluorescence for rat polyclonal anti-BrdU **(I,Q)** in green superimposed on the mouse monoclonal anti-NeuN **(M,Q)** in red, and nuclear DAPI fluorescence in blue **(Q)**. Note the high level of co-localization of BrdU and NeuN (full arrows) and the lack of such co-localization (empty arrows). Abbreviations: CTX, cerebral cortex; EC – external capsule; HIP – hippocampus; PYR, pyriform cortex; STR, striatum; THAL, thalamus. Scale bar: 126 μm.

Post-treatment With Curcumin Immediately Post-HI Decreases Phosphorylated STAT3 in Hippocampus, While Increasing Ipsilateral PHB Protein Levels at 1 h

Western Blot analyses for phosphorylated STAT3 Y705 (pSTAT3 Y705) in ipsilateral and contralateral hippocampus from animals with no treatment (HI), DMSO- or 200 μg/g curcumin treatment and 1 h recovery demonstrated a slight bilateral increase in pSTAT3 Y705 in the DMSO-treated group compared to the HI controls; however, no significant differences were achieved (**Figure 7A**). Curcumin treatment significantly decreased pSTAT3 Y705 on the contralateral side compared to DMSO-treated animals but did not show significant reduction compared to the HI group. Curcumin treatment did not result in significant changes in the ipsilateral side (**Figure 7A**). Western Blot analyses for phosphorylated STAT3 S727 (pSTAT3 S727) under the same conditions showed a slight ipsilateral increase of pSTAT3 S727 in the DMSO-treated compared to HI control animals; however, significant differences were not reached (**Figure 7B**). Curcumin treatment significantly reduced the levels of pSTAT3 S727 compared to the DMSO-treated animals, but had no effect in comparison to the HI littermate controls. No differences between the three groups were registered on the contralateral side (**Figure 7B**).

Western Blots analyses for PHB at 1 h post-HI of animals with HI, DMSO, or 200 μg/g curcumin treatment showed ipsilateral increase of PHB protein levels in the curcumin compared to DMSO-treated animals, but not to HI littermate controls (**Figure 7C**). No significant differences were observed between the DMSO-treated animals and the HI littermate controls. No differences between the three groups were registered on the contralateral side (**Figure 7C**).

The values represent relative densitometry compared to β-actin, which was used as the internal loading control.

DISCUSSION

As shown in the current study, in a Rice-Vannucci model of severe HI insult in P7 mice, immediate, as well as delayed (60 or 120 min) application of curcumin after the HI insult clearly reduced forebrain cell death and tissue loss, as well as microglial and astroglial activation, in a dose dependent manner at 48 h post HI (**Figures 2,4,5; Supplementary Figure S1**). Our results show higher levels of MBP in the external capsule of the curcumin treated group at 48 h post HI and in the striatum of males at 21 days post HI, suggesting protected myelination compared to the DMSO-treated littermates and untreated HI control littermates (**Figures 2N,3H**). These data were in line with other studies demonstrating that decrease in MBP loss is associated with neuroprotection of white matter following neonatal HI injury (Carlsson et al., 2012; Cui et al., 2017). Our data were also in line with the results from other groups registering maintenance of myelin structure and upregulation of MBP expression in the cerebellar white matter in a model of sodium arsenite induced neurotoxicity in developing rat cerebellum (Kaushal et al., 2014). Surprisingly, we did not register similar effect in females (**Figure 3I**), which was also reflected in the combined (males + females) assessment (**Figure 3J**). This is in line with the fact that male sex is a well-established risk factor for poor neurodevelopmental outcome after birth asphyxia and that the male hippocampus, normally larger than the female, undergoes a greater volume loss compared to females (Mayoral et al., 2009). We also observed decrease in the number of iNOS positive cells in hippocampus following curcumin treatment post-HI (**Figure 2O**) suggesting reduction in oxidative stress. Our data is in line with previous studies in a rat model of neonatal HI where curcumin treatment prevented myelin loss, reduced iNOS expression and decreased caspase-3 dependent apoptosis (Cui et al., 2017). Additionally, our results also show reduction in TUNEL+ cell death and tissue loss (**Figure 2, Supplementary Figure S1**) thus broadening the spectrum of assessed cell death.

Our experiments showed attenuation of tissue loss at 21 days post HI in males, females and combined (males + females), (**Figures 3A–C**), however the effects were more pronounced in the male group with subregional significant differences observed in thalamus of males and combined (males + females) (**Figures 3E,G**). The levels of damage in the female group were lower compared to the males (**Figure 3D**). Sex-related susceptibility to brain damage is probably the reason for no visible effect of the curcumin treatment in females (**Figures 3B,F**). Immediate post-HI treatment with curcumin slightly improved motor balance and co-ordination, assessed through the slipping test, in each gender separately and combined; however, the differences did not prove significance. This result is in line with previous data from our group suggesting that the sensitivity of the slipping test might not be suitable for assessment of motor balance and co-ordination differences following neonatal HI (Rocha-Ferreira et al., 2018). At P28 the neonatal hippocampus is not fully developed (Semple

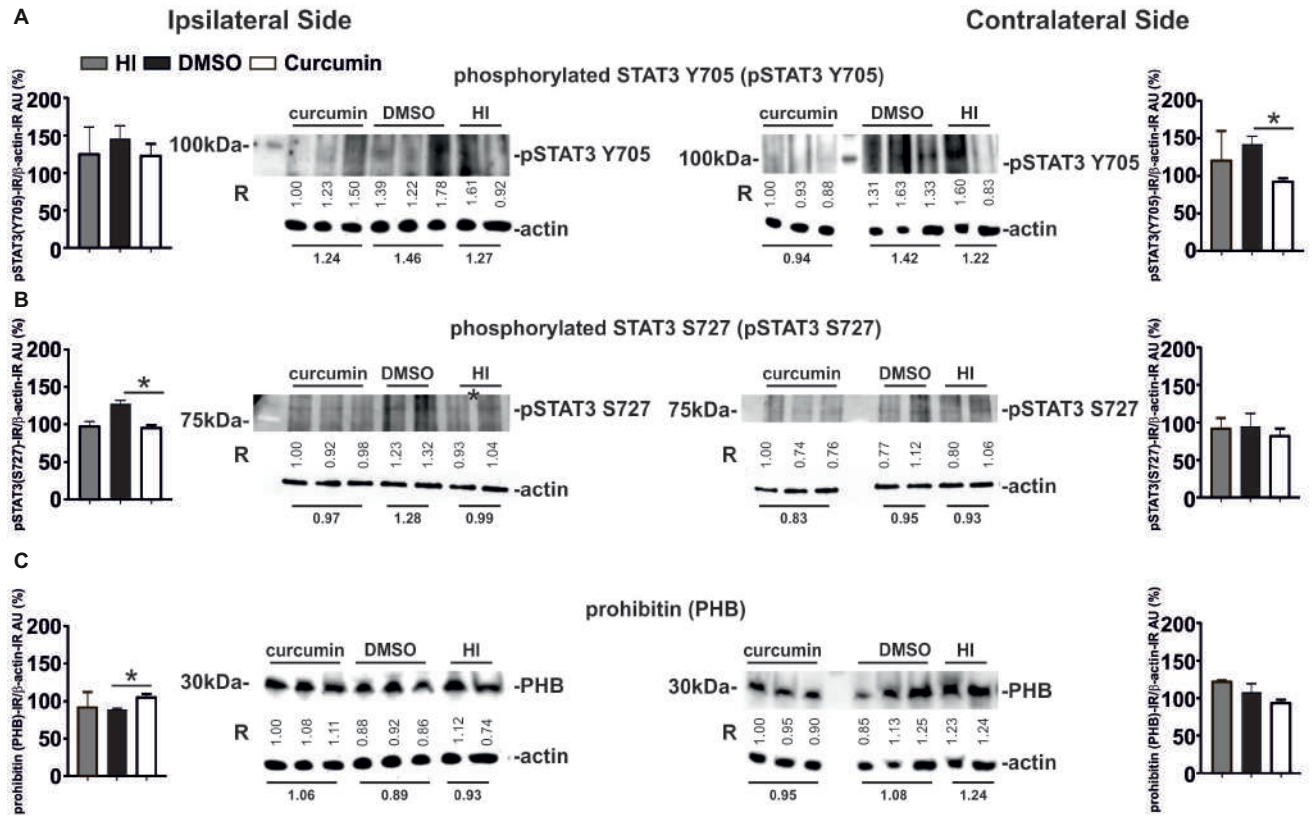


FIGURE 7 | Intraperitoneal injection of curcumin (200 μg/g BW) in P7 mice immediately post-HI decreases phosphorylated STAT3 Y705 and S727 in hippocampus, while increasing ipsilateral PHB protein levels. **(A)** Western Blots for pSTAT3 (Y705) in ipsilateral and contralateral hippocampus from animals with HI, DMSO or 200 μg/g curcumin treatment and 1 h recovery. β-actin protein levels served as control. Note the increase of STAT3 Y705 ipsi- and contralaterally in the DMSO-treated group (19 and 16%, respectively) when compared to HI. Curcumin treatment decreases STAT3 Y705 protein levels ipsi- and contralaterally by 3 and 22%, respectively when compared to HI, and by 15 and 33% ($p = 0.04$) when compared to DMSO treatment. **(B)** Western Blots for pSTAT3 (S727) in ipsilateral and contralateral hippocampus from animals with HI, DMSO or 200 μg/g curcumin treatment and 1 h recovery. β-actin protein levels served as control. Note the increase of STAT3 S727 ipsi- and contralaterally in the DMSO-treated group (29 and 2%, respectively) when compared to HI. Curcumin treatment decreases STAT3 Y705 protein levels ipsi- and contralaterally by 3 and 10%, respectively when compared to HI, and by 25% ($p = 0.009$) and 12% when compared to DMSO treatment. **(C)** Western Blots for PHB in ipsilateral and contralateral hippocampus from animals with HI, DMSO or 200 μg/g curcumin treatment and 1 h recovery. β-actin protein levels served as control. Note the ipsilateral 13 and 19% ($p = 0.0026$) increase of PHB protein levels in the curcumin treated animals compared to HI and DMSO, respectively. Interestingly curcumin treatment results in contralateral 23 and 12% decrease of PHB protein levels compared to HI and DMSO littermates, respectively. PHB levels in DMSO-treated littermates were reduced by 4% in the ipsilateral and by 13% in the contralateral side compared to littermate HI animals. The ratio between pSTAT3 or PHB and β-actin immunoreactivity is shown in arbitrary units (AU) set as 100% for the first line of curcumin treated animals on each of the blots. "R" represents relative densitometry compared to β-actin, which was used as the internal loading control.

et al., 2013) and it is possible that the damage is not reflected through motor and co-ordination challenges, thus assessments at later time points would be more indicative. Additionally, some data suggest that unilateral hippocampal damage is compensated by the contralateral healthy hippocampus, thus, obscuring behavioral outcome (Warburton et al., 2001; Jenkins et al., 2006). Therefore, more robust cognitive behavioral tests assessing short- and long-term memory post HI might prove more effective.

Curcumin is also a hormetin, thus demonstrating stimulatory properties at low doses and inhibitory ones at high doses. Therefore curcumin acts in a dose-dependent manner with lower doses being possibly more effective than higher ones, thus indicative of a hormetic response (Moghaddam et al., 2018). Dose response studies *in vivo* are essential for establishing whether a dietary factor affects organisms and cells *via* a hormetic mechanism. We tested four different doses of curcumin, i.e., 200, 50, 22, and 10 μg/g, and determined 50 μg/g as the minimal neuroprotective dose in neonatal HI brain damage (**Figure 4, Supplementary Figure S1**). As our data show no effect of the low doses (22 and 10 μg/g), and a protective effect at 50 and 200 μg/g, at the tested doses curcumin does not seem to exert hormetic properties when applied post injury in neonatal HI.

Curcumin pre-treatment with 100 μg/g BW significantly attenuates hypoxia-induced cerebral transvascular leakage, with concomitant downregulation in the expression of brain NFκB levels (Himadri et al., 2010). However, our results using the same pre-treatment curcumin dose but in the model of neonatal HI, increased the levels of brain damage markers (**Figure 1**) suggesting detrimental effect of the compound if present at the time of the insult. A possible explanation for the negative effect of curcumin in pre-treatment for HI can be attributed to the well-established anti-oxidant properties of the compound

(Marchiani et al., 2014; Akter et al., 2019). Thus, when present during the HI insult in the neonate, curcumin possibly additionally reduces blood oxygen levels compared to the DMSO-treated littermates, and actually proves damaging. Interestingly, all damage markers are increased in hippocampus of the curcumin treated group, which might be due to the high metabolic rate and oxygen demand of that region (Rocha-Ferreira and Hristova, 2016). Another possibility for the contradiction in the pre-treatment results is the different developmental stage: in adults with hypoxia-induced cerebral transvascular leakage all compensatory and antioxidant pathways are well established, compared to the underdeveloped neonatal brain subjected to HI injury.

Investigating the mechanisms behind the short-term neuroprotective effects of curcumin post-treatment following neonatal HI, we explored whether the treatment had an effect on total and cell-specific proliferation. HI brain damage naturally boosts cell proliferation in the neonatal brain (Rocha-Ferreira and Hristova, 2016), however due to the magnitude of the damage, that proliferation is insufficient to provide the necessary repair. Curcumin is reported to stimulate cell differentiation (Gu et al., 2012; Mujoo et al., 2012; Chen et al., 2014). Therefore, we investigated whether the neuroprotective effects of curcumin in neonatal HI could be attributed to an increase of the levels of cell proliferation thus stimulating repair. In line with previous studies (Rocha-Ferreira and Hristova, 2016), we observed some but not significant HI-induced increase in cell proliferation compared to naïve animals, and no significant effect of curcumin treatment. Thus, we conclude that at 48 h following neonatal HI brain damage the neuroprotection provided by curcumin post-treatment is not a result of effects on cell proliferation.

The inhibitory effect of curcumin on the transcription properties of STAT3, a downstream target of IL6 as a main participant in HI brain damage (Rocha-Ferreira and Hristova, 2016), is well documented (Alexandrow et al., 2012; Devi et al., 2015; Patel et al., 2019). Previous work from our group demonstrates that phosphorylated STAT3 Y705 is bilaterally upregulated in cortex and hippocampus following neonatal HI brain damage, and its inhibition results in neuroprotection (Hristova et al., 2016). Interestingly, at 1h post-HI, we observed only contralateral significant reduction in the protein levels of phosphorylated STAT3 Y705 in hippocampus of curcumin treated animals (**Figure 7A**) compared to DMSO-treated littermates but not to HI controls. Similar trend is also observed in the ipsilateral hippocampus; however, the differences were not significant. Detrimental effects of DMSO treatment have previously been reported (Galvao et al., 2014) and our results suggest that DMSO treatment has a harmful effect which is counteracted by curcumin application (**Figure 7A**). STAT3 is an important regulator in astrocyte differentiation (Hong and Song, 2014). Although, at 1h post-HI, we observed only contralateral reduction of pSTAT3 Y705 compared to DMSO-treated animals, we registered bilateral reduction of astroglial activation following post-treatment with 200 μg/g curcumin after neonatal HI (**Figures 2G,M**).

Curcumin treatment in a mouse model of stroke, reduced microglial activation and the infraction volume of the injured area compared to untreated controls (Liu et al., 2017). In the same study, curcumin treatment reduced microglial gene expression of pro-inflammatory and oxidative stress markers such as TNFα, IL12 and iNOS, and *in vitro* incubation of LPS- or IFN γ activated microglia with curcumin reduced the release of pro-inflammatory cytokines (Liu et al., 2017). Our data is in line with these findings suggesting that the observed reduction in tissue loss, cell death and glial response could rely on the ability of curcumin to resolve inflammation *via* downregulation of pro-inflammatory cytokines.

A small pool of STAT3 has recently been discovered in mitochondria (mito-STAT3), regulating mitochondrial electron transport chain, affecting mitochondrial metabolism and cellular function (Yang and Rincon, 2016). Mito-STAT3 activation is mediated by Ser727 phosphorylation and has been shown to be crucial in immunological effector function and in cancer progression. Mito-Stat3 suppresses ROS formation during cancer genesis, suggesting that targeting Ser727 phosphorylation and mito-STAT3 has strong potential in treating cancer. This effect of mito-STAT3 could be critical in neonatal HI as the formation of ROS is a major factor causing cell death during the secondary energy failure (Rocha-Ferreira and Hristova, 2016), although the mechanisms of action might not be necessarily the same as in cancer genesis. Our data demonstrates ipsilateral reduction of phosphorylated STAT3 S727 in hippocampus following curcumin treatment post-HI compared to DMSO-treated animals but not to HI controls (**Figure 7B**) at 1h post-HI. This suggests a detrimental role of DMSO-treatment in neonatal HI brain damage, which is counteracted by curcumin. STAT3 regulates a metabolic function in mitochondria through STAT3 S727 phosphorylation, supporting Ras-dependent malignant transformation (Gough et al., 2009). Previous data from our lab have confirmed that global pharmacological inhibition of ERK phosphorylation is strongly neuroprotective in neonatal HI brain damage (Thei et al., 2018). Thus, as mito-STAT3 is a downstream target of Ras/ERK (Yang and Rincon, 2016) our data were in line with these previous studies and reduced phosphorylation of STAT3 S727 could be neuroprotective.

Our data demonstrates bilateral increase of phosphorylated STAT3 Y705 and S727 levels in the DMSO-treated animals compared to HI controls at 1h post-HI, which is in line with data from other groups reporting that low doses of DMSO induce caspase-3 independent neuronal death that involves apoptosis-inducing factor (AIF) translocation from mitochondria to the nucleus and poly-(ADP-ribose)-polymerase (PARP) activation (Galvao et al., 2014). Thus our results showing decrease of only 3% ipsi- and 22% contralaterally for STAT3 Y705, and of 3% ipsi- and 10% contralaterally for STAT3 S727 in curcumin treated compared to HI control animals is likely a result of necessity for curcumin treatment to compensate for the detrimental effects of DMSO and provide further neuroprotection.

PHB is a mitochondrial protein which has emerged as an important modulator of neuronal survival in different injury models (Zhou et al., 2012; Hernando-Rodríguez and Artal-Sanz, 2018). PHB localizes to the inner membrane of mitochondria acting as a chaperone protein, but is also found in the nucleus, where it negatively regulates transcription. PHB is significantly increased in the whole cell and markedly decreased in the nuclear

matrix after curcumin treatment of HaCaT cells (Yang et al., 2014). Overexpression of PHB has been proven neuroprotective in a mouse model of middle cerebral artery occlusion (Kahl et al., 2018). Knocking down PHB by siRNA partly increased the apoptosis level of the neuronal cell line PC12 stimulated by H_2O_2 (Xu et al., 2014). Our results at 1h post-HI show ipsilateral increase of the protein levels of PHB in hippocampus following curcumin treatment post-HI (**Figure 7C**). This is in line with the effects observed by other groups, generally associating increased PHB expression with decrease of cell death, amelioration of mitochondrial dysfunction and neuroprotection. However, it is unclear whether, similarly to STAT3 Y705 (Hristova et al., 2016) PHB plays a different role in different cell types involved in neonatal HI, and whether its expression is time-dependent.

In conclusion, our data support a dose-dependent neuroprotection provided by immediate and delayed treatment with curcumin following neonatal HI injury. The precise mechanism of this protection is unclear; however, our results show effects of curcumin on oxidative stress and myelination, inflammation and transcription (STAT3 Y705) and mitochondrial dysfunction (STAT3 S727 and PHB). This makes curcumin an attractive therapeutic candidate in neonatal HI induced brain damage.

Although phase I clinical trials have shown that curcumin is safe even at high doses (12 g/day), its future use and clinical application is limited as a result of its poor bioavailability due to poor absorption, rapid metabolism, and rapid systemic elimination (Anand et al., 2007; Gupta et al., 2013). Therefore, future experiments should focus on increasing curcumin bioavailability and solubility with the development of aqueous solutions for clinical application.

ETHICS STATEMENT

All animal experiments and care protocols were carried out according to the UK Animals (Scientific Procedures) Act 1986 and approved by the Home Office (PPL70/8784). The ARRIVE guidelines were followed. All experiments involved postnatal day 7 C57/Bl6 mice (P7) bred in house.

AUTHOR CONTRIBUTIONS

ER-F, CS, and SL contributed to the collection and processing of data, writing and editing the manuscript. SB, TF, MHa, ICR, CA, TK, and BM assisted with the collection and processing of data. DH contributed to the collection and processing of data, editing the manuscript. MHr contributed to the design of the study, collection and processing of data, writing and editing the manuscript.

FUNDING

This work was supported by the BBSRC LIDo programme BB/M009513/1.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01351/full#supplementary-material

SUPPLEMENTARY FIGURE S1 | Intraperitoneal injection of curcumin in P7 mice post-HI significantly reduces in a dose dependent manner ipsilateral brain tissue volume loss, TUNEL+ cell death and ipsilateral and contralateral reactive astrogliosis. **(A)** Intraperitoneal injection of 50 µg/g BW curcumin decreased ipsilateral volume loss compared to DMSO treated littermates, with significant individual decrease (*t*-test) in cortex ($p = 0.018$), hippocampus ($p = 0.025$) and overall ($p = 0.0004$). Mixed Linear Model treating region as a repeated measure revealed $p = 0.0001$. **(B,C)** Intraperitoneal injection of 22 µg/g **(B)** and 10 µg/g **(C)** curcumin did not have an effect on volume tissue loss compared to DMSO treated littermates. **(D)** Intraperitoneal injection of 50 µg/g curcumin decreased ipsilateral TUNEL+ cell death compared to DMSO treated littermates, with significant individual decrease (*t*-test) in hippocampus ($p = 0.007$) and external capsule ($p = 0.0001$). Mixed Linear Model treating region as a repeated measure revealed $p = 0.016$. **(E,F)** Intraperitoneal injection of 22 µg/g **(E)** and 10 µg/g **(F)** curcumin did not have an effect on ipsilateral TUNEL+ cell death compared to DMSO treated littermates. **(G)** Intraperitoneal injection of 50 µg/g curcumin decreased ipsilateral reactive astrogliosis assessed though OLV of GFAP immunoreactivity compared to DMSO treated littermates, with significant individual decrease (*t*-test) in pyriform cortex ($p = 0.025$), hippocampus ($p = 0.033$), thalamus ($p = 0.030$) and external capsule ($p = 0.031$). Mixed Linear Model treating region as a repeated measure revealed $p = 0.014$. **(H,I)** Intraperitoneal injection of 22 µg/g **(H)** and 10 µg/g **(I)** curcumin did not have an effect on ipsilateral TUNEL+ cell death compared to DMSO treated littermates. **(J)** Intraperitoneal injection of 50 µg/g curcumin reduced contralateral reactive astrogliosis compared to DMSO treated littermates, with significant individual decrease (*t*-test) in pyriform cortex ($p = 0.009$). Mixed Linear Model treating region as a repeated measure revealed $p = 0.005$. **(K,L)** Intraperitoneal injection of 22 µg/g **(H)** and 10 µg/g **(I)** curcumin did not have an effect on contralateral astrogliosis compared to DMSO treated littermates. (**A**, curcumin 50 µg/g $n = 8$, DMSO $n = 6$, **B**, curcumin 22 µg/g $n = 12$, DMSO $n = 10$, **C**, curcumin 10 µg/g $n = 11$, DMSO $n = 11$) ($^*p < 0.05$). Abbreviations: CTX, cerebral cortex; EC, external capsule; HIP, hippocampus; PYR, pyriform cortex; STR, striatum; THAL, thalamus.

REFERENCES

Ahmad, M. Z., Alkahtani, S. A., Akhter, S., Ahmad, F. J., Ahmad, J., Akhtar, M. S., et al. (2016). Progress in nanotechnology-based drug carrier in designing of curcumin nanomedicines for cancer therapy: current state-of-the-art. *J. Drug Target.* 24, 273–293. doi: 10.3109/1061186X.2015.1055570

Akter, J., Hossain, M. A., Takara, K., Islam, M. Z., and Hou, D.-X. (2019). Antioxidant activity of different species and varieties of turmeric (Curcuma spp): isolation of active compounds. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 215, 9–17. doi: 10.1016/j.cbpc.2018.09.002

Alexandrow, M. G., Song, L. J., Altiok, S., Gray, J., Haura, E. B., and Kumar, N. B. (2012). Curcumin. *Eur. J. Cancer Prev.* 21, 407–412. doi: 10.1097/CEJ.0b013e32834ef194

Anand, P., Kunnumakkara, A. B., Newman, R. A., and Aggarwal, B. B. (2007). Bioavailability of curcumin: problems and promises. *Mol. Pharm.* 4, 807–818. doi: 10.1021/mp700113r

Ande, S. R., Xu, Y. X. Z., and Mishra, S. (2017). Prohibitin: a potential therapeutic target in tyrosine kinase signaling. *Signal Transduct. Target. Ther.* 2:17059. doi: 10.1038/sigtrans.2017.59

Bavarsad, K., Riahi, M. M., Saadat, S., Barreto, G., Atkin, S. L., and Sahebkar, A. (2018). Protective effects of curcumin against ischemia-reperfusion injury in the liver. *Pharmacol. Res.* 141, 53–62. doi: 10.1016/j.phrs.2018.12.014

Carlsson, Y., Wang, X., Schwendimann, L., Rousset, C. I., Jacotot, E., Gressens, P., et al. (2012). Combined effect of hypothermia and caspase-2 gene deficiency on neonatal hypoxic-ischemic brain injury. *Pediatr. Res.* 71, 566–572. doi: 10.1038/pr.2012.15 (Accessed March 13, 2015).

Chen, F., Wang, H., Xiang, X., Yuan, J., Chu, W., Xue, X., et al. (2014). Curcumin increased the differentiation rate of neurons in neural stem cells via wnt signaling in vitro study. *J. Surg. Res.* 192, 298–304. doi: 10.1016/j.jss.2014.06.026

Clarkson, J., and Herbison, A. E. (2016). Hypothalamic control of the male neonatal testosterone surge. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 371:20150115. Available at: http://rstb.royalsocietypublishing.org/content/371/1688/20150115 (Accessed September 5, 2017).

Cui, X., Song, H., and Su, J. (2017). Curcumin attenuates hypoxic-ischemic brain injury in neonatal rats through induction of nuclear factor erythroid-2-related factor 2 and heme oxygenase-1. *Exp. Ther. Med.* 14, 1512–1518. doi: 10.3892/etm.2017.4683

Devi, Y. S., DeVine, M., DeKuiper, J., Ferguson, S., and Fazleabas, A. T. (2015). Inhibition of IL-6 signaling pathway by curcumin in uterine decidual cells. *PLoS One* 10:e0125627. doi: 10.1371/journal.pone.0125627

Dhillon, N., Aggarwal, B. B., Newman, R. A., Wolff, R. A., Kunnumakkara, A. B., Abbruzzese, J. L., et al. (2008). Phase II trial of curcumin in patients with advanced pancreatic cancer. *Clin. Cancer Res.* 14, 4491–4499. doi: 10.1158/1078-0432.CCR-08-0024

Galvao, J., Davis, B., Tilley, M., Normando, E., Duchen, M. R., and Cordeiro, M. F. (2014). Unexpected low-dose toxicity of the universal solvent DMSO. *FASEB J.* 28, 1317–1330. doi: 10.1096/fj.13-235440

Gluckman, P. D., Wyatt, J. S., Azzopardi, D., Ballard, R., Edwards, A. D., Ferriero, D. M., et al. (2005). Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet* 365, 663–670. doi: 10.1016/S0140-6736(05)17946-X

Gobinath, A. R., Choleris, E., and Galea, L. A. M. (2017). Sex, hormones, and genotype interact to influence psychiatric disease, treatment, and behavioral research. *J. Neurosci. Res.* 95, 50–64. doi: 10.1002/jnr.23872

Gough, D. J., Corlett, A., Schlessinger, K., Wegrzyn, J., Larner, A. C., and Levy, D. E. (2009). Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science* 324, 1713–1716. doi: 10.1126/science.1171721

Gu, Q., Cai, Y., Huang, C., Shi, Q., and Yang, H. (2012). Curcumin increases rat mesenchymal stem cell osteoblast differentiation but inhibits adipocyte differentiation. *Pharmacogn. Mag.* 8, 202–208. doi: 10.4103/0973-1296.99285

Gupta, S. C., Patchva, S., and Aggarwal, B. B. (2013). Therapeutic roles of curcumin: lessons learned from clinical trials. *AAPS J.* 15, 195–218. doi: 10.1208/s12248-012-9432-8

He, H., Luo, Y., Qiao, Y., Zhang, Z., Yin, D., Yao, J., et al. (2018). Curcumin attenuates doxorubicin-induced cardiotoxicity *via* suppressing oxidative stress and preventing mitochondrial dysfunction mediated by 14-3-3γ. *Food Funct.* 9, 4404–4418. doi: 10.1039/C8FO00466H

Hernando-Rodríguez, B., and Artal-Sanz, M. (2018). Mitochondrial quality control mechanisms and the PHB (Prohibitin) complex. *Cell* 7:238. doi: 10.3390/cells7120238

Himadri, P., Kumari, S. S., Chitharanjan, M., and Dhananjay, S. (2010). Role of oxidative stress and inflammation in hypoxia-induced cerebral edema: a molecular approach. *High Alt. Med. Biol.* 11, 231–244. doi: 10.1089/ham.2009.1057

Hong, S., and Song, M.-R. (2014). STAT3 but not STAT1 is required for astrocyte differentiation. *PLoS One* 9:e86851. doi: 10.1371/journal.pone.0086851

Hope, P. L., Costello, A. M., Cady, E. B., Delpy, D. T., Tofts, P. S., Chu, A., et al. (1984). Cerebral energy metabolism studied with phosphorus NMR spectroscopy in normal and birth-asphyxiated infants. *Lancet* 2, 366–370. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6147452 (Accessed January 12, 2016).

Hristova, M., Cuthill, D., Zbarsky, V., Acosta-Saltos, A., Wallace, A., Blight, K., et al. (2010). Activation and deactivation of periventricular white matter phagocytes during postnatal mouse development. *Glia* 58, 11–28. doi: 10.1002/glia.20896

Hristova, M., Rocha-Ferreira, E., Fontana, X., Thei, L., Buckle, R., Christou, M., et al. (2016). Inhibition of signal transducer and activator of transcription 3 (STAT3) reduces neonatal hypoxic-ischaemic brain damage. *J. Neurochem.* 136, 981–994. doi: 10.1111/jnc.13490

Hsiao, Y.-T., Kuo, C.-L., Chueh, F.-S., Liu, K.-C., Bau, D.-T., and Chung, J.-G. (2018). Curcuminoids induce reactive oxygen species and autophagy to enhance apoptosis in human oral cancer cells. *Am. J. Chin. Med.* 46, 1145–1168. doi: 10.1142/S0192415X1850060X

Jaisin, Y., Thampithak, A., Meesarapee, B., Ratanachamnong, P., Suksamrarn, A., Phivthong-ngam, L., et al. (2011). Curcumin I protects the dopaminergic cell line SH-SY5Y from 6-hydroxydopamine-induced neurotoxicity through attenuation of p53-mediated apoptosis. *Neurosci. Lett.* 489, 192–196. doi: 10.1016/j.neulet.2010.12.014

Jenkins, T. A., Amin, E., Brown, M. W., and Aggleton, J. P. (2006). Changes in immediate early gene expression in the rat brain after unilateral lesions of the hippocampus. *Neuroscience* 137, 747–759. doi: 10.1016/J.NEUROSCIENCE.2005.09.034

Johnston, M. V., Nakajima, W., and Hagberg, H. (2002). Mechanisms of hypoxic neurodegeneration in the developing brain. *Neuroscientist* 8, 212–220. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12061501 (Accessed October 23, 2015).

Joseph, A., Wood, T., Chen, C.-C., Corry, K., Snyder, J. M., Juul, S. E., et al. (2018). Curcumin-loaded polymeric nanoparticles for neuroprotection in neonatal rats with hypoxic-ischemic encephalopathy. *Nano Res.* 11, 5670–5688. doi: 10.1007/s12274-018-2104-y

Kahl, A., Anderson, C. J., Qian, L., Voss, H., Manfredi, G., Iadecola, C., et al. (2018). Neuronal expression of the mitochondrial protein prohibitin confers profound neuroprotection in a mouse model of focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 38, 1010–1020. doi: 10.1177/0271678X17720371

Kaushal, P., Mehra, R. D., and Dhar, P. (2014). Curcumin induced up-regulation of myelin basic protein (MBP) ameliorates sodium arsenite induced neurotoxicity in developing rat cerebellum. *J. Anat. Soc. India* 63, 3–11. doi: 10.1016/J.JASI.2014.04.001

Kendall, G. S., Hristova, M., Zbarsky, V., Clements, A., Peebles, D. M., Robertson, N. J., et al. (2012). Distribution of pH changes in mouse neonatal hypoxic-ischaemic insult. *Dev. Neurosci.* 33, 505–518. doi: 10.1159/000333850

Kendall, G. S., Robertson, N. J., Iwata, O., Peebles, D., and Raivich, G. (2006). N-methyl-isobutyl-amiloride ameliorates brain injury when commenced before hypoxia ischemia in neonatal mice. *Pediatr. Res.* 59, 227–231. doi: 10.1203/01.pdr.0000196805.68082.22

Kim, J. E., Kim, A. R., Chung, H. Y., Han, S. Y., Kim, B. S., and Choi, J. S. (2003). In vitro peroxynitrite scavenging activity of diarylheptanoids from *Curcuma longa*. *Phyther. Res.* 17, 481–484. doi: 10.1002/ptr.1179

Lange, S., Rocha-Ferreira, E., Thei, L., Mawjee, P., Bennett, K., Thompson, P. R., et al. (2014). Peptidylarginine deiminases: novel drug targets for prevention of neuronal damage following hypoxic ischemic insult (HI) in neonates. *J. Neurochem.* 130, 555–562. doi: 10.1111/jnc.12744

Lawn, J. E., Cousens, S., and Zupan, J. (2005). 4 million neonatal deaths: when? Where? Why? *Lancet* 365, 891–900. doi: 10.1016/S0140-6736(05)71048-5

Li, Y., Powers, C., Jiang, N., and Chopp, M. (1998). Intact, injured, necrotic and apoptotic cells after focal cerebral ischemia in the rat. *J. Neurol. Sci.* 156, 119–132. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9588846 (Accessed October 31, 2015).

Liu, Y., and Hong, X.-Q. (2006). Effect of three different curcumin pigmens on the prdiferation of vascular smooth muscle cells by ox-LDL and the expression of LDL-R. *Zhongguo Zhong Yao Za Zhi* 31, 500–503.

Liu, Z., Ran, Y., Huang, S., Wen, S., Zhang, W., Liu, X., et al. (2017). Curcumin protects against ischemic stroke by titrating microglia/macrophage polarization. *Front. Aging Neurosci.* 9:233. doi: 10.3389/fnagi.2017.00233

Lundgren, C., Brudin, L., Wanby, A.-S., and Blomberg, M. (2018). Ante- and intrapartum risk factors for neonatal hypoxic ischemic encephalopathy. *J. Matern. Neonatal Med.* 31, 1595–1601. doi: 10.1080/14767058.2017.1321628

Luong, T. N., Carlisle, H. J., Southwell, A., and Patterson, P. H. (2011). Assessment of motor balance and coordination in mice using the balance beam. *J. Vis. Exp.* 49. doi: 10.3791/2376

Maheshwari, R. K., Singh, A. K., Gaddipati, J., and Srimal, R. C. (2006). Multiple biological activities of curcumin: a short review. *Life Sci.* 78, 2081–2087. doi: 10.1016/j.lfs.2005.12.007

Marchiani, A., Rozzo, C., Fadda, A., Delogu, G., and Ruzza, P. (2014). Curcumin and curcumin-like molecules: from spice to drugs. *Curr. Med. Chem.* 21, 204–222. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23590716 (Accessed January 24, 2019).

Mayoral, S. R., Omar, G., and Penn, A. A. (2009). Sex differences in a hypoxia model of preterm brain damage. *Pediatr. Res.* 66, 248–253. doi: 10.1203/PDR.0b013e3181b1bc34

Merkwirth, C., Martinelli, P., Korwitz, A., Morbin, M., Brönneke, H. S., Jordan, S. D., et al. (2012). Loss of prohibitin membrane scaffolds impairs mitochondrial architecture and leads to tau hyperphosphorylation and neurodegeneration. *PLoS Genet.* 8:e1003021. doi: 10.1371/journal.pgen.1003021

Moghadamtousi, S. Z., Kadir, H. A., Hassandarvish, P., Tajik, H., Abubakar, S., Zandi, K., et al. (2014). A review on antibacterial, antiviral, and antifungal activity of curcumin. *Biomed. Res. Int.* 2014:186864. doi: 10.1155/2014/186864

Moghaddam, N. S. A., Oskouie, M. N., Butler, A. E., Petit, P. X., Barreto, G. E., and Sahebkar, A. (2018). Hormetic effects of curcumin: what is the evidence? *J. Cell. Physiol.* 2014, 10060–10071. doi: 10.1002/jcp.27880

Möller, J. C., Klein, M. A., Haas, S., Jones, L. L., Kreutzberg, G. W., and Raivich, G. (1996). Regulation of thrombospondin in the regenerating mouse facial motor nucleus. *Glia* 17, –121, 32. doi: 10.1002/(SICI)1098-1136(199606)17:2<121::AID-GLIA4>3.0.CO;2-5

Momekova, D., Ugrinova, I., Slavkova, M., Momekov, G., Grancharov, G., Gancheva, V., et al. (2018). Superior proapoptotic activity of curcumin-loaded mixed block copolymer micelles with mitochondrial targeting properties. *Biomater. Sci.* 6, 3309–3317. doi: 10.1039/C8BM00644J

Mujoo, K., Nikonoff, L. E., Sharin, V. G., Bryan, N. S., Kots, A. Y., and Murad, F. (2012). Curcumin induces differentiation of embryonic stem cells through possible modulation of nitric oxide-cyclic GMP pathway. *Protein Cell* 3, 535–544. doi: 10.1007/s13238-012-2053-2

Mutsuga, M., Chambers, J. K., Uchida, K., Tei, M., Makibuchi, T., Mizorogi, T., et al. (2012). Binding of curcumin to senile plaques and cerebral amyloid angiopathy in the aged brain of various animals and to neurofibrillary tangles in Alzheimer's brain. *J. Vet. Med. Sci.* 74, 51–57. doi: 10.1292/jvms.11-0307

Mythri, R. B., Harish, G., Dubey, S. K., Misra, K., and Srinivas Bharath, M. M. (2011). Glutamoyl diester of the dietary polyphenol curcumin offers improved protection against peroxynitrite-mediated nitrosative stress and damage of brain mitochondria in vitro: implications for Parkinson's disease. *Mol. Cell. Biochem.* 347, 135–143. doi: 10.1007/s11010-010-0621-4

Naksuriya, O., Okonogi, S., Schiffelers, R. M., and Hennink, W. E. (2014). Curcumin nanoformulations: a review of pharmaceutical properties and preclinical studies and clinical data related to cancer treatment. *Biomaterials* 35, 3365–3383. doi: 10.1016/j.biomaterials.2013.12.090

Naserzadeh, P., Hafez, A. A., Abdorahim, M., Abdollahifar, M. A., Shabani, R., Peirovi, H., et al. (2018). Curcumin loading potentiates the neuroprotective efficacy of Fe_3O_4 magnetic nanoparticles in cerebellum cells of schizophrenic rats. *Biomed. Pharmacother.* 108, 1244–1252. doi: 10.1016/j.biopha.2018.09.106

Nishiyama, T., Mae, T., Kishida, H., Tsukagawa, M., Mimaki, Y., Kuroda, M., et al. (2005). Curcuminoids and Sesquiterpenoids in turmeric (*Curcuma longa* L.) suppress an increase in blood glucose level in type 2 diabetic KK-A^y mice. *J. Agric. Food Chem.* 53, 959–963. doi: 10.1021/jf0483873

Patel, S. S., Acharya, A., Ray, R. S., Agrawal, R., Raghuwanshi, R., and Jain, P. (2019). Cellular and molecular mechanisms of curcumin in prevention and treatment of disease. *Crit. Rev. Food Sci. Nutr.* 1–53. doi: 10.1080/10408398.2018.1552244

Peng, Y.-T., Chen, P., Ouyang, R.-Y., and Song, L. (2015). Multifaceted role of prohibitin in cell survival and apoptosis. *Apoptosis* 20, 1135–1149. doi: 10.1007/s10495-015-1143-z

Peng, T. I., and Greenamyre, J. T. (1998). Privileged access to mitochondria of calcium influx through N-methyl-D-aspartate receptors. *Mol. Pharmacol.* 53, 974–980.

Penrice, J., Lorek, A., Cady, E. B., Amess, P. N., Wylezinska, M., Cooper, C. E., et al. (1997). Proton magnetic resonance spectroscopy of the brain during acute hypoxia-ischemia and delayed cerebral energy failure in the newborn piglet. *Pediatr. Res.* 41, 795–802. doi: 10.1203/00006450-199706000-00001

Pescosolido, N., Giannotti, R., Plateroti, A., Pascarella, A., and Nebbioso, M. (2013). Curcumin: therapeutical potential in ophthalmology. *Planta Med.* 80, 249–254. doi: 10.1055/s-0033-1351074

Priyadarsini, K., and Indira, K. (2014). The chemistry of curcumin: from extraction to therapeutic agent. *Molecules* 19, 20091–20112. doi: 10.3390/molecules191220091

Puka-Sundvall, M., Hallin, U., Zhu, C., and Wang, X. (2000). NMDA blockade attenuates caspase-3 activation and DNA fragmentation after neonatal hypoxia–ischemia. *Dev. Neurosci.* 11, 2833–2836.

Raivich, G., Bohatschek, M., Kloss, C. U. A., Werner, A., Jones, L. L., and Kreutzberg, G. W. (1999). Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res. Rev.* 30, 77–105. doi: 10.1016/S0165-0173(99)00007-7

Ray, B., Bisht, S., Maitra, A., Maitra, A., and Lahiri, D. K. (2011). Neuroprotective and neurorescue effects of a novel polymeric nanoparticle formulation of curcumin (NanoCurc™) in the neuronal cell culture and animal model: implications for Alzheimer's disease. *J. Alzheimers Dis.* 23, 61–77. doi: 10.3233/JAD-2010-101374

Rocha-Ferreira, E., and Hristova, M. (2016). Plasticity in the neonatal brain following hypoxic-ischaemic injury. *Neural Plast.* 2016, 1–16. doi: 10.1155/2016/4901014

Rocha-Ferreira, E., Phillips, E., Francesch-Domenech, E., Thei, L., Peebles, D. M., Raivich, G., et al. (2015). The role of different strain backgrounds in bacterial endotoxin-mediated sensitization to neonatal hypoxic-ischemic brain damage. *Neuroscience* 311, 292–307. doi: 10.1016/j.neuroscience.2015.10.035

Rocha-Ferreira, E., Vincent, A., Bright, S., Peebles, D. M., and Hristova, M. (2018). The duration of hypothermia affects short-term neuroprotection in a mouse model of neonatal hypoxic ischaemic injury. *PLoS One* 13:e0199890. doi: 10.1371/journal.pone.0199890

Sanders, R. D., Manning, H. J., Robertson, N. J., Ma, D., Edwards, A. D., Hagberg, H., et al. (2010). Preconditioning and postinsult therapies for perinatal hypoxic–ischemic injury at term. *Anesthesiology* 113, 233–249. doi: 10.1097/ALN.0b013e3181dc1b84

Sandur, S. K., Pandey, M. K., Sung, B., Ahn, K. S., Murakami, A., Sethi, G., et al. (2007). Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism. *Carcinogenesis* 28, 1765–1773. doi: 10.1093/carcin/bgm123

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., and Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. *Prog. Neurobiol.* 106–107, 1–16. doi: 10.1016/j.pneurobio.2013.04.001

Shishodia, S., Sethi, G., and Aggarwal, B. B. (2005). Curcumin: getting back to the roots. *Ann. N. Y. Acad. Sci.* 1056, 206–217. doi: 10.1196/annals.1352.010

Shukla, P. K., Khanna, V. K., Ali, M. M., Khan, M. Y., and Srimal, R. C. (2008). Anti-ischemic effect of curcumin in rat brain. *Neurochem. Res.* 33, 1036–1043. doi: 10.1007/s11064-007-9547-y

Spagnuolo, C., Napolitano, M., Tedesco, I., Moccia, S., Milito, A., and Russo, G. L. (2016). Neuroprotective role of natural polyphenols. *Curr. Top. Med. Chem.* 16, 1943–1950. doi: 10.2174/1568026616666160204122449

Tham, C. L., Liew, C. Y., Lam, K. W., Mohamad, A.-S., Kim, M. K., Cheah, Y. K., et al. (2010). A synthetic curcuminoid derivative inhibits nitric oxide and proinflammatory cytokine synthesis. *Eur. J. Pharmacol.* 628, 247–254. doi: 10.1016/j.ejphar.2009.11.053

Thei, L., Rocha-Ferreira, E., Peebles, D., Raivich, G., and Hristova, M. (2018). Extracellular signal-regulated kinase 2 has duality in function between neuronal and astrocyte expression following neonatal hypoxic-ischaemic cerebral injury. *J. Physiol.* 596, 6043–6062. doi: 10.1113/JP275649

Trujillo, J., Granados-Castro, L. F., Zazueta, C., Andérica-Romero, A. C., Chirino, Y. I., and Pedraza-Chaverrí, J. (2014). Mitochondria as a target in the therapeutic properties of curcumin. *Arch. Pharm.* 347, 873–884. doi: 10.1002/ardp.201400266

Vannucci, R. C., and Vannucci, S. J. (1997). A model of perinatal hypoxic-ischemic brain damage. *Ann. N. Y. Acad. Sci.* 234–249. doi: 10.1111/j.1749-6632.1997.tb48634.x

Warburton, E. C., Baird, A., Morgan, A., Muir, J. L., and Aggleton, J. P. (2001). The conjoint importance of the hippocampus and anterior thalamic nuclei for allocentric spatial learning: evidence from a disconnection study in the rat. *J. Neurosci.* 21, 7323–7330. doi: 10.1523/JNEUROSCI.21-18-07323.2001

Wu, A., Ying, Z., and Gomez-Pinilla, F. (2006). Dietary curcumin counteracts the outcome of traumatic brain injury on oxidative stress, synaptic plasticity, and cognition. *Exp. Neurol.* 197, 309–317. doi: 10.1016/j.expneurol.2005.09.004

Xu, T., Fan, X., Tan, Y., Yue, Y., Chen, W., Gu, X., et al. (2014). Expression of PHB2 in rat brain cortex following traumatic brain injury. *Int. J. Mol. Sci.* 15, 3299–3318. doi: 10.3390/ijms15023299

Yang, R., and Rincon, M. (2016). Mitochondrial Stat3, the need for design thinking. *Int. J. Biol. Sci.* 12, 532–544. doi: 10.7150/ijbs.15153

Yang, H.-B., Song, W., Chen, L.-Y., Li, Q.-F., Shi, S.-L., Kong, H.-Y., et al. (2014). Differential expression and regulation of prohibitin during curcumin-induced apoptosis of immortalized human epidermal HaCaT cells. *Int. J. Mol. Med.* 33, 507–514. doi: 10.3892/ijmm.2014.1621

Yu, S., Zheng, W., Xin, N., Chi, Z.-H., Wang, N.-Q., Nie, Y.-X., et al. (2010). Curcumin prevents dopaminergic neuronal death through inhibition of the c-Jun N-terminal kinase pathway. *Rejuvenation Res.* 13, 55–64. doi: 10.1089/rej.2009.0908

Zhao, J., Yu, S., Zheng, W., Feng, G., Luo, G., Wang, L., et al. (2010). Curcumin improves outcomes and attenuates focal cerebral ischemic injury via antiapoptotic mechanisms in rats. *Neurochem. Res.* 35, 374–379. doi: 10.1007/s11064-009-0065-y

Zhou, P., Qian, L., D'Aurelio, M., Cho, S., Wang, G., Manfredi, G., et al. (2012). Prohibitin reduces mitochondrial free radical production and protects brain cells from different injury modalities. *J. Neurosci.* 32, 583–592. doi: 10.1523/JNEUROSCI.2849-11.2012

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Rocha-Ferreira, Sisa, Bright, Fautz, Harris, Contreras Riquelme, Agwu, Kurulday, Mistry, Hill, Lange and Hristova. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership