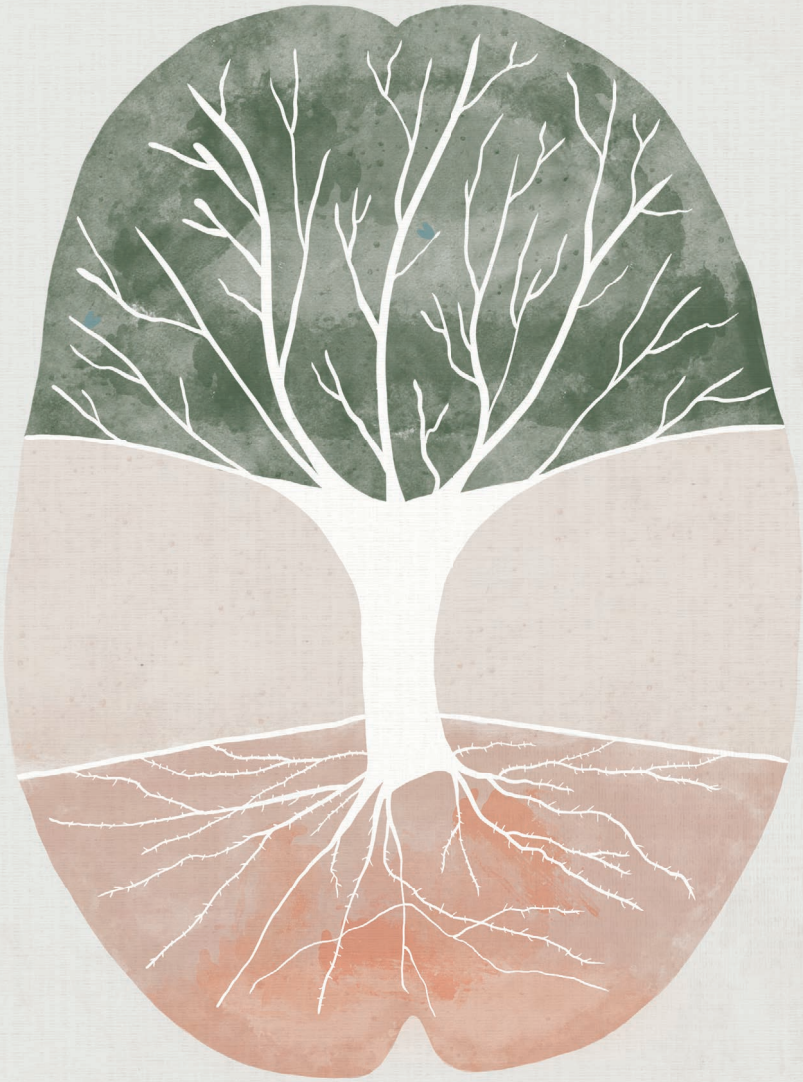
Novel strategies to improve brain development after Encephalopathy of Prematurity

from bench to incubator



UMC Utrecht Brain Center

Josine Vaes

Novel strategies to improve brain development after Encephalopathy of Prematurity: from bench to incubator

Josine Vaes

Novel strategies to improve brain development after Encephalopathy of Prematurity: from bench to incubator

PhD Thesis, Utrecht University, with a summary in Dutch.
Proefschrift, Universiteit Utrecht, met een samenvatting in het Nederlands.

Provided by thesis specialist Ridderprint, ridderprint.nl
Printing: Ridderprint
Layout and design: Jesse Haaksman, persoonlijkproefschrift.nl
Cover design: Dion Tavenier

ISBN: 978-94-6458-431-8

Publication of this thesis was financially supported by UMC Utrecht Brain Center, U-CyTech biosciences, ABN AMRO, ChipSoft, PeproTech, Oak Hill Bio and Noldus Information Technology.

Novel strategies to improve brain development after Encephalopathy of Prematurity: from bench to incubator

Nieuwe strategieën voor het verbeteren van hersenontwikkeling na extreme vroeggeboorte: van het laboratorium naar de couveuse
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

maandag 17 oktober 2022 des middags te 4.15 uur

door

Josine Elisabeth Gertruda Vaes

geboren op 30 september 1989
te Nederweert

Promotor:
Prof. dr. M.J.N.L. Benders

Copromotoren:
Dr. C.H.A. Nijboer
Dr. F. Groenendaal

Beoordelingscommissie:
Prof. dr. E.M. Hol
Prof. dr. K.P.J. Braun
Prof. dr. K. van der Ent
Prof. dr. C. Kemner
Prof. dr. M.M. van Weissenbruch

Maar je vindt soms een weg als je durft te verdwalen

Stef Bos

Table of contents

1

General introduction

INTRODUCTION

Worldwide numbers show that approximately 1 in 10 infants is born preterm, defined by the World Health Organization (WHO) as birth before 37 weeks of gestation. In the Netherlands the incidence of preterm birth is approximately 7-8% (Perined, 2019). Of all preterm children ~5% are born *extremely* preterm, i.e. before 28 weeks of pregnancy (Blencowe et al., 2012). Encephalopathy of Prematurity (EoP) is a major problem in infants born (extremely) prematurely and can lead to life-long neurodevelopmental impairments. In order to reduce EoP-related morbidity, in addition to improved clinical routine care, clinically-approved treatments are urgently needed. This thesis aims to explore promising treatment strategies for EoP, including mesenchymal stem cells (MSCs) and insulin-like growth factor I (IGF1), by investigating their therapeutic potential and underlying working mechanisms in experimental models of EoP. The ultimate goal of this thesis is to contribute to the translation of these treatments from bench-to-incubator in the near-future. To aid clinical translation, the use of IGF1 as a biomarker for EoP after extreme preterm birth is investigated in a clinical study. In this concise general introduction, the current knowledge regarding the pathophysiology, consequences and management of EoP will discussed briefly. In addition, two potential therapeutic options, MSCs and IGF1 will be described. This introduction will be concluded by the outline of this thesis.

Encephalopathy of Prematurity

In recent years, the umbrella term 'Encephalopathy of Prematurity' has been introduced to describe the distinct pattern of white and (subtle) gray matter abnormalities observed in preterm neonates (Fleiss et al., 2020; Volpe, 2009). These abnormalities are believed to result from impaired brain development, with peri- and postnatal insults, such as oxygen imbalances, inflammation and interrupted maternal supply of growth factors, interfering with the myriad of developmental processes that take place in the third trimester of pregnancy (Back & Miller, 2014; van Tilborg et al., 2016; Volpe, 2019). White matter injury (WMI), an evident finding in neonatal neuroimaging studies, is a key hallmark of EoP and has been the main focus of EoP-related research in recent years (Back, 2017; de Vries et al., 2013; Khwaja & Volpe, 2008; Ment et al., 2009; Volpe et al., 2011). The term 'preterm WMI' can be used to refer to a spectrum of pathological white matter changes in the developing brain. These changes can be divided into two subcategories based on neuropathological hallmarks, with cystic periventricular leukomalacia (cPVL) being characterized by tissue necrosis and

subsequent cyst formation (figure 1A/B), while diffuse white matter injury (dWMI) is characterized by diffuse, subtle dysmaturation of the white matter (hypomyelination) in absence of focal necrosis (figure 1C/D). Punctate white matter lesions (PWML), sometimes seen as a separate entity, are thought to result from small necrotic (ischemic or gliotic) lesions and can therefore be categorized in the PVL spectrum (Back, 2017; Lee, 2017; Volpe, 2017). At present, preterm dWMI is the most prevalent form of preterm WMI, with ~80% of affected preterm infants suffering from this type of WMI, associated with global hypomyelination of the brain (Back, 2017; Back & Miller, 2014). More details on the pathophysiology of preterm WMI, especially on the role of oligodendrocyte maturational arrest, can be found in **chapter 3** and **4** of this thesis. In the past few years, novel insights in the neurodevelopmental (cellular) programs that take place in the third trimester of human gestation have led to increased recognition of (subtle) gray matter deficits in EoP (Fleiss et al., 2020; Malik et al., 2013; Volpe, 2009). On a cellular level, GABAergic interneurons are believed to be a vulnerable neuronal subpopulation in EoP. Interestingly, disturbances in the development and subsequent functioning of interneurons have been proposed to play an important role in the pathophysiology of neurodevelopmental disorders, like autism-spectrum disorders, highly prevalent after preterm birth (Arshad et al., 2016; Fleiss et al., 2020; Marín, 2012). Additional information on the role of interneuron maldevelopment in EoP can be found in **chapter 8**.

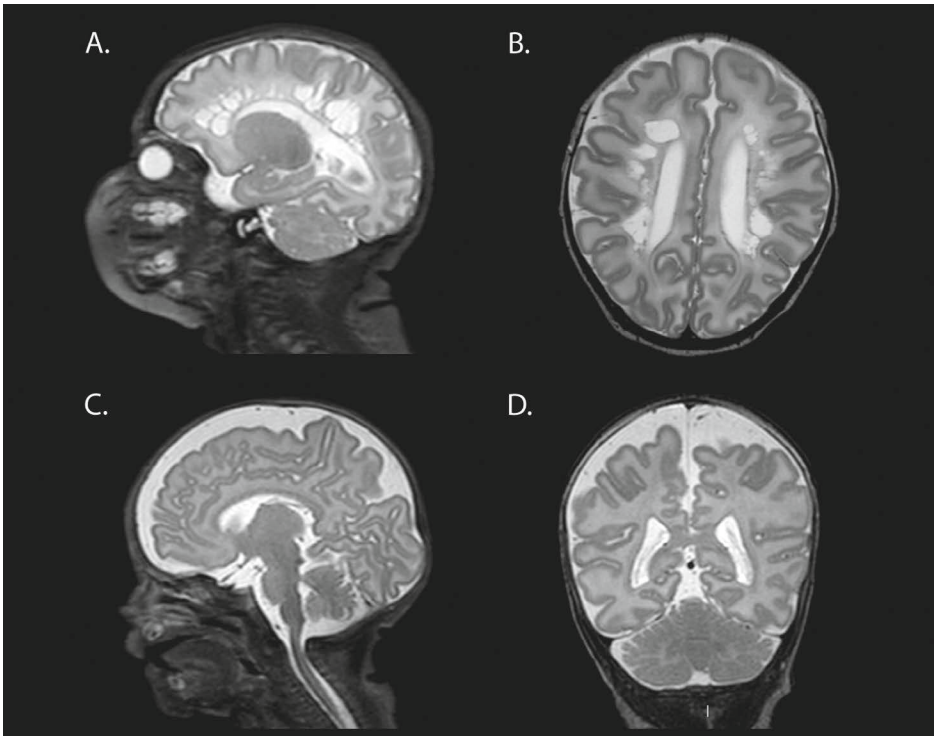


Figure 1. Two types of white matter injury in the preterm neonate as demonstrated on T2-weighted MRI scans at term-equivalent age (TEA). **A/B**. Cystic periventriculair leukomalacia, with formation of large cysts in the white matter, viewed in the sagittal (**A**) and axial (**B**) plane. The incidence and severity of cPVL has decreased considerably in the last decades. **C/D**. The most prevalent type of white matter injury in the preterm brain: diffuse white matter injury (dWMI), with diffuse white matter atrophy, mild ventriculomegaly, thinning of the corpus callosum and global volume reduction, viewed in a sagittal (**C**) and coronal (**D**) plane.

Long-term consequences of Encephalopathy of Prematurity

Over the last three decades, advances in clinical care, including the use of antenatal corticosteroids and early surfactant administration, have led to a gradual increase in neonates surviving (extreme) preterm birth. As a consequence of increased survival, more prematurely born children are impacted by EoP-induced adverse long-term neurodevelopmental consequences (Larroque et al., 2008; Moster et al., 2008). The risk of neurodevelopmental morbidity after preterm birth is inversely correlated with gestational age at birth, meaning that extremely preterm infants are most vulnerable (Deng, 2010). Multiple large (longitudinal) cohort studies have identified a wide range of (long-term) neurodevelopmental impairments encountered by very and extreme-

ly prematurely born infants, ranging from motor problems, cognitive impairments and learning disabilities to behavioral and social problems. These studies show that among extremely preterm infants ~10% suffer from cerebral palsy, while 20-40% is affected by cognitive impairment, though numbers may vary per country (Hirschberger et al., 2018; Johnson et al., 2009; Larroque et al., 2008; Marlow et al., 2005; Moster et al., 2008; Pierrat et al., 2017). Follow-up to adulthood showed no evidence for recovery of impaired cognitive functioning over time (Linsell et al., 2018). In addition, a higher prevalence of neurodevelopmental disorders has been reported in the preterm population compared to term-born peers. At school-age, ~8% of preterm children was diagnosed with autism-spectrum disorder, ~11% with attention-deficit-hyperactivity-disorder (ADHD) and ~10% with emotional disorders, including depression and anxiety (Hirschberger et al., 2018; Johnson & Marlow, 2011).

Novel treatment options

Although EoP can lead to life-long neurological impairments, at present there are no clinical treatment options available, aside from supportive NICU care. In later stages of life, symptomatic treatment can be offered, including physical, occupational, social and speech therapy and special education. Cell- and growth factor-based therapies have received an increasing amount of attention in the field of neonatal brain injury due to their potential to stimulate endogenous repair processes in experimental models of adult brain pathologies (Gronbach et al., 2018; Ophelders et al., 2020; van Velthoven et al., 2012; Wagenaar et al., 2017). One of these promising therapeutic options is (intranasally administered) mesenchymal stem cell (MSC) therapy (Archambault et al., 2017; van Velthoven et al., 2012; Wagenaar et al., 2017). Treatment with MSCs has been shown to repair gray and white matter deficits, dampen neuroinflammation and improve functional outcome in models of adult and neonatal (term) brain injury (Donega et al., 2014; Mueller et al., 2017; Nijboer et al., 2018; Paton et al., 2019; van Velthoven et al., 2010). MSCs are believed to exert their regenerative potential through secretion of trophic and immunomodulatory factors, contributing to a cerebral environment permissive of regeneration and development (Liang et al., 2014; Paul & Anisimov, 2013). The therapeutic potential of MSCs in EoP is extensively reviewed in **chapter 2** and **3**.

Insulin-like growth factor 1 (IGF1) is an endogenous growth factor of which blood levels are often low after preterm birth compared to fetal blood levels at corresponding post-menstrual ages (Hansen-Pupp et al., 2011; Hellstrom et al., 2016).

IGF1 has been shown to play an important role in normal development of gray- and white matter structures in the brain and regeneration in experimental models of cerebral injury (Beck et al., 1995; Cai et al., 2011; Hansen-Pupp et al., 2013; Hellström et al., 2016; Lin et al., 2005; Nieto-Estévez et al., 2016). A wide range of cerebral cell types, including oligodendrocytes and neurons are known to express the IGF receptor (IGFR), with IGF1 directly influencing survival and differentiation of these cell lineages (D'Ercole & Ye, 2008; Zeger et al., 2007). The potential of IGF1 supplementation therapy to boost brain development after preterm birth is thoroughly discussed in **chapter 4**.

At present, the gold standard for the identification of (subtle) brain abnormalities associated with EoP is MRI at term-equivalent age, when developmental processes, including myelination, are advancing (de Vries et al., 2013). Validated biomarkers to identify patients at risk for EoP at an earlier time point after (extreme) preterm birth are currently lacking (Douglas-Escobar & Weiss, 2012; Jin et al., 2015; Lu et al., 2018). Reliable, diagnostic biomarkers are urgently needed to enable clinicians to select patients that might benefit from regenerative therapies and allow timely administration at an early stage of the disease. The potential role of (the increase in) postnatal IGF1 blood levels as a predictive biomarker for EoP is addressed in **chapter 9**.

AIM AND OUTLINE OF THIS THESIS

The key aims of this thesis were to further elucidate the cellular and molecular mechanisms underlying cerebral dysmaturation in EoP and to investigate novel, clinically-applicable therapeutic strategies to support proper brain development after EoP.

Chapters 2, **3** and **4** are review articles that focus on the pathophysiology of EoP and discuss potential therapeutic options to prevent of repair EoP-related brain injury. In **chapter 2** the current standard of care and latest developments with respect to prevention and repair of periventricular-intraventricular hemorrhage (PIVH) and WMI, both prevalent patterns of preterm brain injury, are discussed. **Chapter 3** focuses on the lessons learned in the field of MSC therapy in previous studies of adult- and neonatal neurological diseases and subsequent translation of this knowledge to application of MSC therapy in preterm WMI. In addition, important considerations for clinical translation are discussed, including cellular source, timing of treatment and the route of administration. Furthermore, the potential of optimization strategies for cell-based treatments, including preconditioning and genetic engineering of MSCs, are reviewed. In **chapter 4** the current *in vitro, in vivo* and clinical literature on several promising trophic and immunomodulatory factors that contribute to healthy brain development and repair of oligodendrocytes, microglia, astrocytes and interneurons, key cellular players in EoP pathophysiology, is extensively reviewed. Moreover, the therapeutic potential and possible future clinical translation of these factors is evaluated.

In **chapter 5** and **6** the therapeutic potential of intranasal MSC therapy to repair WMI in an experimental model of EoP is investigated. More specifically, in **chapter 5** a novel multiple-hit model of EoP in newborn mice, incorporating two clinically relevant postnatal hits, is used to study the therapeutic efficacy of intranasal MSC therapy on both anatomical and functional level. Moreover, the working mechanisms of MSC therapy for EoP are investigated using *in vitro* primary glia cultures. Our follow-up study, described in **chapter 6**, investigates the underlying mechanisms of a limited treatment window for effective intranasal MSC treatment in the mouse model of EoP and studies the potential of MSC secretome modification to prolong the treatment window for intranasal application in EoP.

In **chapter 7** the regulation of endogenous IGF1 production is studied in our validated multiple-hit mouse model of EoP. Moreover, the potential of intranasal IGF1 supple-

tion therapy to restore myelination deficits in EoP is explored *in vivo*. In addition, the cell-specific mode of action of IGF1 on important key cell types in EoP is studied *in vitro*.

Chapter 8 focuses on maldevelopment of a previously overlooked cell type in EoP pathophysiology: the GABA-ergic interneuron. More specifically, two distinct, validated double-hit rodent models of EoP are used to evaluate changes in the number and distribution of cortical and hippocampal interneuron subtypes. Moreover, the potential of intranasal MSC and IGF1 treatment to restore interneuron deficits and social behavior is assessed in our EoP mouse model.

Chapter 9 explores the relationship between longitudinally measured postnatal IGF1 blood levels and brain development in extremely preterm infants. Neurodevelopmental outcome includes white matter maturation parameters, volumetric measurements and brain injury scores obtained using neuroimaging. Better understanding of the relationship between IGF1 levels and outcome could determine whether IGF1 levels are a reliable biomarker for EoP and will corroborate future clinical application of IGF1 therapy to support brain development in EoP.

To conclude, **chapter 10** summarizes the highlights of this thesis followed by a general discussion, critically evaluating the findings described in this thesis.

REFERENCES

Archambault, J., Moreira, A., McDaniel, D., Winter, L., Sun, L., & Hornsby, P. (2017). Therapeutic potential of mesenchymal stromal cells for hypoxic ischemic encephalopathy: A systematic review and meta-analysis of preclinical studies. *PLoS One, 12*(12), e0189895. doi:10.1371/journal.pone.0189895

Arshad, A., Vose, L. R., Vinukonda, G., Hu, F., Yoshikawa, K., Csiszar, A., . . . Ballabh, P. (2016). Extended Production of Cortical Interneurons into the Third Trimester of Human Gestation. *Cereb Cortex, 26*(5), 2242-2256. doi:10.1093/cercor/bhv074

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathologica, 134*(3), 331-349. doi:10.1007/s00401-017-1718-6

Back, S. A., & Miller, S. P. (2014). Brain injury in premature neonates: A primary cerebral dysmaturation disorder? *Annals of Neurology, 75*(4), 469-486. doi:10.1002/ana.24132

Beck, K. D., Powell-Braxton, L., Widmer, H. R., Valverde, J., & Hefti, F. (1995). Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. *Neuron, 14*(4), 717-730. doi:10.1016/0896-6273(95)90216-3

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A.-B., Narwal, R., . . . Lawn, J. E. (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. *The Lancet, 379*(9832), 2162-2172. doi:10.1016/S0140-6736(12)60820-4

Cai, Z., Fan, L.-W., Lin, S., Pang, Y., & Rhodes, P. G. (2011). Intranasal Administration of IGF-1 Protects against Lipopolysaccharide-induced Injury in the Developing Rat Brain. *Neuroscience, 194*, 195-207. doi:10.1016/j.neuroscience.2011.08.003

D'Ercole, a. J., & Ye, P. (2008). Minireview: Expanding the mind: Insulin-like growth factor I and brain development. *Endocrinology, 149*(12), 5958-5962. doi:10.1210/en.2008-0920

de Vries, L. S., Benders, M. J. N. L., & Groenendaal, F. (2013). Imaging the premature brain: ultrasound or MRI? *Neuroradiology, 55*(2), 13-22. doi:10.1007/s00234-013-1233-y

Deng, W. (2010). Neurobiology of injury to the developing brain. *Nature Reviews Neurology, 6*(1759-4766 (Electronic)), 328-336. doi:10.1038/nrneurol.2010.53

Donega, V., Nijboer, C. H., van Tilborg, G., Dijkhuizen, R. M., Kavelaars, A., & Heijnen, C. J. (2014). Intranasally administered mesenchymal stem cells promote a regenerative niche for repair of neonatal ischemic brain injury. *Experimental Neurology, 261*, 53-64. doi:10.1016/j.expneurol.2014.06.009

Douglas-Escobar, M., & Weiss, M. D. (2012). Biomarkers of brain injury in the premature infant. *Front Neurol, 3*, 185. doi:10.3389/fneur.2012.00185

Fleiss, B., Gressens, P., & Stolp, H. B. (2020). Cortical Gray Matter Injury in Encephalopathy of Prematurity: Link to Neurodevelopmental Disorders. *Front Neurol, 11*, 575-575. doi:10.3389/fneur.2020.00575

Gronbach, J., Shahzad, T., Radajewski, S., Chao, C. M., Bellusci, S., Morty, R. E., . . . Ehrhardt, H. (2018). The Potentials and Caveats of Mesenchymal Stromal Cell-Based Therapies in the Preterm Infant. *Stem Cells Int, 2018*, 9652897. doi:10.1155/2018/9652897

Hansen-Pupp, I., Hovel, H., Hellstrom, A., Hellstrom-Westas, L., Lofqvist, C., Larsson, E. M., . . . Ley, D. (2011). Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J Clin Endocrinol Metab, 96*(4), 1129-1135. doi:10.1210/jc.2010-2440

Hansen-Pupp, I., Hovel, H., Lofqvist, C., Hellstrom-Westas, L., Fellman, V., Huppi, P. S., . . . Ley, D. (2013). Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatr Res, 74*(5), 564-569. doi:10.1038/pr.2013.135

Hellstrom, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Lofqvist, C., van Marter, L., . . . Smith, L. E. (2016). Insulin-like growth factor 1 has multisystem effects on foetal and preterm infant development. *Acta Paediatr, 105*(6), 576-586. doi:10.1111/apa.13350

Hellström, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Ramenghi, L. A., Löfqvist, C., . . . Hård, A. L. (2016). Role of Insulinlike Growth Factor 1 in Fetal Development and in the Early Postnatal Life of Premature Infants. *Am J Perinatol, 33*(11), 1067-1071. doi:10.1055/s-0036-1586109

Hirschberger, R. G., Kuban, K. C. K., O'Shea, T. M., Joseph, R. M., Heeren, T., Douglass, L. M., . . . Investigators, E. S. (2018). Co-occurrence and Severity of Neurodevelopmental Burden (Cognitive Impairment, Cerebral Palsy, Autism Spectrum Disorder, and Epilepsy) at Age Ten Years in Children Born Extremely Preterm. *Pediatr Neurol, 79*, 45-52. doi:10.1016/j.pediatrneurol.2017.11.002

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

Johnson, S., Fawke, J., Hennessy, E., Rowell, V., Thomas, S., Wolke, D., & Marlow, N. (2009). Neurodevelopmental disability through 11 years of age in children born before 26 weeks of gestation. *Pediatrics, 124*(2), e249-257. doi:10.1542/peds.2008-3743

Johnson, S., & Marlow, N. (2011). Preterm Birth and Childhood Psychiatric Disorders. *Pediatric Research, 69*(8), 11-18. doi:10.1203/PDR.0b013e318212faa0

Khwaja, O., & Volpe, J. J. (2008). Pathogenesis of cerebral white matter injury of prematurity. *Archives of Disease in Childhood: Fetal & Neonatal Edition, 93*(2), F153-161. doi:10.1136/adc.2006.108837

Larroque, B., Ancel, P. Y., Marret, S., Marchand, L., Andre, M., Arnaud, C., . . . Kaminski, M. (2008). Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study. *The Lancet, 371*(9615), 813-820. doi:10.1016/s0140-6736(08)60380-3

Lee, Y. A. (2017). White Matter Injury of Prematurity: Its Mechanisms and Clinical Features. *J Pathol Transl Med, 51*(5), 449-455. doi:10.4132/jptm.2017.07.25

Liang, X., Ding, Y., Zhang, Y., Tse, H. F., & Lian, Q. (2014). Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant, 23*(9), 1045-1059. doi:10.3727/096368913×667709

Lin, S., Fan, L. W., Pang, Y., Rhodes, P. G., Mitchell, H. J., & Cai, Z. (2005). IGF-1 protects oligodendrocyte progenitor cells and improves neurological functions following cerebral hypoxia-ischemia in the neonatal rat. *Brain Res, 1063*(1), 15-26. doi:10.1016/j.brainres.2005.09.042

Linsell, L., Johnson, S., Wolke, D., O'Reilly, H., Morris, J. K., Kurinczuk, J. J., & Marlow, N. (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*(4), 363-370. doi:10.1136/archdischild-2017-313414

Lu, H., Huang, W., Chen, X., Wang, Q., Zhang, Q., & Chang, M. (2018). Relationship between premature brain injury and multiple biomarkers in cord blood and amniotic fluid. *Journal of Maternal-Fetal and Neonatal Medicine, 31*(21), 2898-2904. doi:10.1080/14767058.2017.1359532

Malik, S., Vinukonda, G., Vose, L. R., Diamond, D., Bhimavarapu, B. B. R., Hu, F., . . . Ballabh, P. (2013). Neurogenesis continues in the third trimester of pregnancy and is suppressed by premature birth. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 33*(2), 411-423. doi:10.1523/JNEUROSCI.4445-12.2013

Marín, O. (2012). Interneuron dysfunction in psychiatric disorders. *Nat Rev Neurosci, 13*(2), 107-120. doi:10.1038/nrn3155

Marlow, N., Wolke, D., Bracewell, M. A., & Samara, M. (2005). Neurologic and developmental disability at six years of age after extremely preterm birth. *N Engl J Med, 352*(1), 9-19. doi:10.1056/NEJMoa041367

Ment, L. R., Hirtz D Fau - Huppi, P. S., & Huppi, P. S. (2009). Imaging biomarkers of outcome in the developing preterm brain. *Lancet Neurol, 8*(1474-4465 (Electronic)), 1042-1055. doi:10.1016/S1474-4422(09)70257-1

Moster, D., Lie, R. T., & Markestad, T. (2008). Long-term medical and social consequences of preterm birth. *New England Journal of Medicine, 359*(3), 262-273. doi:10.1056/NEJMoa0706475

Mueller, M., Oppliger, B., Joerger-Messerli, M., Reinhart, U., Barnea, E., Paidas, M., . . . Schoeberlein, A. (2017). Wharton's Jelly Mesenchymal Stem Cells Protect the Immature Brain in Rats and Modulate Cell Fate. *Stem Cells Dev, 26*(4), 239-248. doi:10.1089/scd.2016.0108

Nieto-Estévez, V., Defterali, Ç., & Vicario-Abejón, C. (2016). IGF-I: A Key Growth Factor that Regulates Neurogenesis and Synaptogenesis from Embryonic to Adult Stages of the Brain. *Frontiers in Neuroscience, 10*, 52. doi:10.3389/fnins.2016.00052

Nijboer, C. H., Kooijman, E., van Velthoven, C. T., van Tilborg, E., Tiebosch, I. A., Eijkelkamp, N., . . . Heijnen, C. J. (2018). Intranasal Stem Cell Treatment as a Novel Therapy for Subarachnoid Hemorrhage. *Stem Cells Dev, 27*(5), 313-325. doi:10.1089/scd.2017.0148

Ophelders, D., Gussenhoven, R., Klein, L., Jellema, R. K., Westerlaken, R. J. J., Hütten, M. C., . . . Wolfs, T. (2020). Preterm Brain Injury, Antenatal Triggers, and Therapeutics: Timing Is Key. *Cells, 9*(8). doi:10.3390/cells9081871

Paton, M. C. B., Allison, B. J., Fahey, M. C., Li, J., Sutherland, A. E., Pham, Y., . . . McDonald, C. A. (2019). Umbilical cord blood versus mesenchymal stem cells for inflammation-induced preterm brain injury in fetal sheep. *Pediatric Research*. doi:10.1038/s41390-019-0366-z

Paul, G., & Anisimov, S. V. (2013). The secretome of mesenchymal stem cells: potential implications for neuroregeneration. *Biochimie, 95*(12), 2246-2256. doi:10.1016/j.biochi.2013.07.013

Perined. (2019). *Perinatale zorg in Nederland anno 2018: landelijke perinatale cijfers en duiding*. Utrecht Retrieved from https://assets.perined.nl/docs/fc23b860-a5ff-4ef6-b164-aedf7881cbe3.pdf

Pierrat, V., Marchand-Martin, L., Arnaud, C., Kaminski, M., Resche-Rigon, M., Lebeaux, C., . . . Ancel, P. Y. (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

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., & Nijboer, C. H. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Progress in Neurobiology, 136*(1873-5118 (Electronic)), 28-49. doi:10.1016/j.pneurobio.2015.11.002

van Velthoven, C. T., Kavelaars, A., & Heijnen, C. J. (2012). 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., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain, Behavior and Immunity, 24*(3), 387-393. doi:10.1016/j.bbi.2009.10.017

Volpe, J. J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurology, 8*(1), 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. (2019). Dysmaturation of Premature Brain: Importance, Cellular Mechanisms, and Potential Interventions. *Pediatr Neurol, 95*, 42-66. doi:10.1016/j.pediatrneurol.2019.02.016

Volpe, J. J., Kinney, H. C., Jensen, F. E., & Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *International Journal of Developmental Neuroscience, 29*(4), 423-440. doi:10.1016/j.ijdevneu.2011.02.012

Wagenaar, N., Nijboer, C. H., & van Bel, F. (2017). Repair of neonatal brain injury: bringing stem cell-based therapy into clinical practice. *Developmental medicine and child neurology, 59*(10), 997-1003. doi:10.1111/dmcn.13528

Zeger, M., Popken, G., Zhang, J., Xuan, S., Lu, Q. R., Schwab, M. H., . . . Ye, P. (2007). Insulin-like growth factor type 1 receptor signaling in the cells of oligodendrocyte lineage is required for normal in vivo oligodendrocyte development and myelination. *Glia, 55*(4), 400-411. doi:10.1002/glia.20469

2

Prevention, reduction and repair of brain injury of the preterm infant

Frank van Bel, Josine Vaes and Floris Groenendaal

Published in Frontiers in Physiology, 2019 Mar; 10, 181

ABSTRACT

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.

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; Hamilton et al. (2013); Pierrat et al. (2017); Stoll et al. (2010)). This brain injury may lead to cerebral palsy and learning difficulties, and can have major impact on the quality of life (Pierrat et al., 2017; Stoll et al., 2010).

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 (Fischer et al., 2017; Fleiss et al., 2014; Wagenaar et al., 2017). We discuss this development in relation with repair of the sequelae of severe PIVH and dWMI.

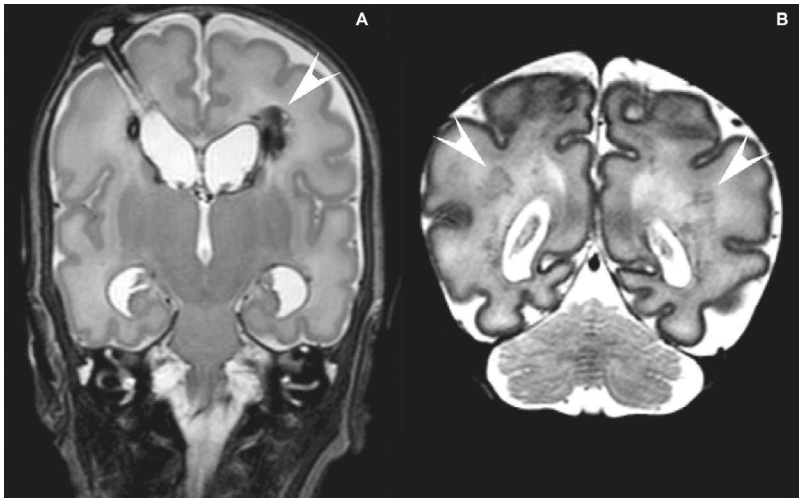


Figure 1. A. MRI of a preterm infant (gestational age 26 2/7 weeks, birthweight 965 g) with a large IVH and a large left-sided (arrowhead) and small right-sided venous infarct. Post-hemorrhagic ventricular dilatation was treated with CSF removal from a subcutaneous reservoir. T2 weighted coronal image at the age of 8 weeks after birth. **B**. MRI of a preterm infant (gestational age 32 2/7 weeks, birthweight 1670 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-to-35% of preterm infants born before 30 weeks of gestation or a birth weight less than 1,500g 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; Mukerji et al., 2015; Stoll et al., 2010). 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 (Luu et al., 2009; Sherlock et al., 2005). 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 (Ballabh, 2014; Krediet et al., 2006; Ozdemir et al., 1997). 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 & 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 (Chevallier et al., 2017; Chisholm et al., 2016; Krediet et al., 2006; 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 (Ballabh, 2014; Bilguvar et al., 2009; Harteman et al., 2011).

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 (Ballabh, 2014; Ment et al., 1995; 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 & 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 & 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 (Greenough & Ahmed, 2013; Walti et al., 1995). 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 & Inder, 2017). Phenobarbital sedation did not decrease PIVH incidence (Bedard et al., 1984; Donn et al., 1981). 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 & 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 (Ballabh, 2014; Ment et al., 1992). 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, preventing cerebral hyperperfusion as compared to placebo-treated controls (figure 2; Van Bel et al. (1995); Van Bel et al. (1993); Van Bel et al. (1994)).

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.

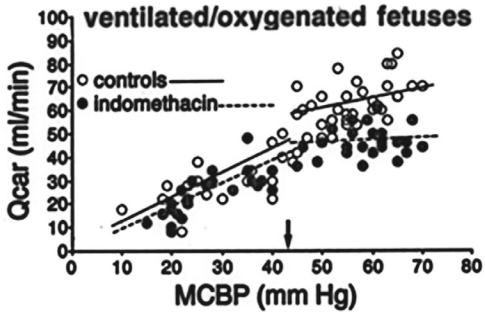


Figure 2. Individual values of Carotid blood flow (Qcar [ml/min]), representing global cerebral blood flow, as a function of (mean) carotid blood pressure (MCBP; mm Hg), representing cerebral perfusion pressure, in pretreated with indomethacin (filled circles) and non-treated ventilated preterm sheep fetuses, representing a perinatal lamb model(Van Bel et al., 1995; Van Bel et al., 1993; Van Bel et al., 1994). 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.

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 (Alderliesten et al., 2016; Skov et al., 1991; Van Bel et al., 2008; van Bel & Mintzer, 2018; Wintermark et al., 2014). 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 & 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., 2012; Rabe et al., 2008). 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 (Hellstrom et al., 2016; Juul & Pet, 2015).

EPO stimulates red cell production, cell survival and differentiation and EPO receptors are detected on endothelial, glial and neuronal cells (Chateauvieux et al., 2011; Koulnis et al., 2014; Rangarajan & Juul, 2014; van der Kooij et al., 2008). EPO has also a modulating effect on glutamate toxicity, stimulating effect on antioxidative ability and anti-inflammatory effect protecting endothelial cells from apoptotic death (Bernaudin et al., 1999; Kawakami et al., 2001; Yamaji et al., 1996). 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 (Fauchere et al., 2015; Ohls et al., 2014). 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 & Aher, 2017).

IGF-1 is an endogenous protein which exerts several actions: its positive effect on proper vascularization (Bach, 2015; Hellstrom et al., 2001) 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 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.

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 (<6h) 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)

WHITE MATTER INJURY IN THE VERY AND EXTREMELY PRETERM INFANT

Extremely preterm born infants (of ELGANs) carry a substantial risk of diffuse white matter injury or abnormal white matter development (Chau et al., 2013; Volpe, 2009). 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; Paton et al., 2017; Strunk et al., 2014), 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 & 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 (Bennet et al., 2018b; Galinsky et al., 2017), 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 & Dinan, 2012). Nutritional components such as fatty acids and protein may stimulate brain growth and neurodevelopment (Coviello et al., 2018; Uauy & Mena, 2015). 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 & 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 (Hagberg et al., 2015; Strunk et al., 2014). 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 (Chateauvieux et al., 2011; Rangarajan & Juul, 2014; van der Kooij et al., 2008).

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 to 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 & Pet, 2015). Given its positive effect on neurogenesis and angiogenesis a more prolonged course of appropriately (high) dosed rhEPO (up to 2500 IU/kg daily) may further optimize clinical outcome of the preterm infant (Chateauvieux et al., 2011; Rangarajan & Juul, 2014; van der Kooij et al., 2008).

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 (Cai et al., 2011; Cao et al., 2003; Hansen-Pupp et al., 2011; O'Kusky & Ye, 2012; Pang et al., 2010). Moreover, genetic studies in mice display lower total brain volumes and severe hypomyelination following IGF-1 knockout (O'Kusky & 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 (Fleiss et al., 2014; Uccelli et al., 2008). 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) (Bennet et al., 2018a; Kizil et al., 2015; Ophelders et al., 2016). 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 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 (Gronbach et al., 2018; Niimi & Levison, 2018; Vaes et al., 2019; Wagenaar et al., 2017).

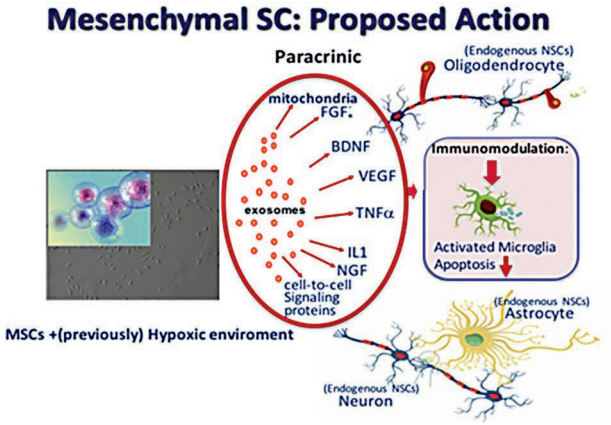


Figure 3. Proposed actions of mesenchymal stem cells (MSC) when present in a (previously) hypoxic-ischemic environment: production of vesicles (exosomes) of various growth factors, (anti-inflammatory) cytokines, signaling proteins and mitochondria which give rise to recovery of affected neurons and to proliferation of endogenous paraventricular-situated neural stem cells to form oligodendrocytes, astrocytes and neurons.

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; Drommelschmidt et al., 2017; Jadasz et al., 2013; Jellema et al., 2013; Li et al., 2016). 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.

REFERENCES

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Ahn, J. Y., & 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*(1), 145-156. doi:10.3727/096368916×692861

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Yoo, H. S., Lee, J. H., . . . Park, W. S. (2013). Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. *Stroke, 44*(2), 497-504. doi:10.1161/strokeaha.112.679092

Alderliesten, T., De Vis, J. B., Lemmers, P. M. A., van Bel, F., Benders, M., Hendrikse, J., & Petersen, E. T. (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

Alderliesten, T., Lemmers, P. M., Smarius, J. J., van de Vosse, R. E., Baerts, W., & van Bel, F. (2013). Cerebral oxygenation, extraction, and autoregulation in very preterm infants who develop peri-intraventricular hemorrhage. *J Pediatr, 162*(4), 698-704.e692. doi:10.1016/j.jpeds.2012.09.038

Anand, K. J., Barton, B. A., McIntosh, N., Lagercrantz, H., Pelausa, E., Young, T. E., & Vasa, R. (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*(4), 331-338.

Bach, L. A. (2015). Endothelial cells and the IGF system. *J Mol Endocrinol, 54*(1), R1-13. doi:10.1530/jme-14-0215

Baenziger, O., Stolkin, F., Keel, M., von Siebenthal, K., Fauchere, J. C., Das Kundu, S., . . . Wolf, M. (2007). The influence of the timing of cord clamping on postnatal cerebral oxygenation in preterm neonates: a randomized, controlled trial. *Pediatrics, 119*(3), 455-459. doi:10.1542/peds.2006-2725

Ballabh, P. (2014). Pathogenesis and prevention of intraventricular hemorrhage. *Clin Perinatol, 41*(1), 47-67. doi:10.1016/j.clp.2013.09.007

Banker, B. Q., & Larroche, J. C. (1962). Periventricular leukomalacia of infancy. A form of neonatal anoxic encephalopathy. *Arch Neurol, 7*, 386-410.

Bayer, K. (2016). Delayed Umbilical Cord Clamping in the 21st Century: Indications for Practice. *Adv Neonatal Care, 16*(1), 68-73. doi:10.1097/anc.0000000000000247

Bedard, M. P., Shankaran, S., Slovis, T. L., Pantoja, A., Dayal, B., & Poland, R. L. (1984). Effect of prophylactic phenobarbital on intraventricular hemorrhage in high-risk infants. *Pediatrics, 73*(4), 435-439.

Bennet, L., Dhillon, S., Lear, C. A., van den Heuij, L., King, V., Dean, J. M., . . . Gunn, A. J. (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., & Gunn, A. J. (2018b). Time and sex dependent effects of magnesium sulphate on post-asphyxial seizures in preterm fetal sheep. *J Physiol, 596*(23), 6079-6092. doi:10.1113/jp275627

Benson, J. W., Drayton, M. R., Hayward, C., Murphy, J. F., Osborne, J. P., Rennie, J. M., . . . Cooke, R. W. (1986). Multicentre trial of ethamsylate for prevention of periventricular haemorrhage in very low birthweight infants. *Lancet, 2*(8519), 1297-1300.

Beresford, M. W., & 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*(6), 973-978. doi:10.1203/00006450-200212000-00025

Bernaudin, M., Marti, H. H., Roussel, S., Divoux, D., Nouvelot, A., MacKenzie, E. T., & Petit, E. (1999). A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J Cereb Blood Flow Metab, 19*(6), 643-651. doi:10.1097/00004647-199906000-00007

Bierstone, D., Wagenaar, N., Gano, D. L., Guo, T., Georgio, G., Groenendaal, F., . . . Miller, S. P. (2018). Association of Histologic Chorioamnionitis With Perinatal Brain Injury and Early Childhood Neurodevelopmental Outcomes Among Preterm Neonates. *JAMA Pediatr, 172*(6), 534-541. doi:10.1001/jamapediatrics.2018.0102

Bilguvar, K., DiLuna, M. L., Bizzarro, M. J., Bayri, Y., Schneider, K. C., Lifton, R. P., . . . Ment, L. R. (2009). COL4A1 mutation in preterm intraventricular hemorrhage. *J Pediatr, 155*(5), 743-745. doi:10.1016/j.jpeds.2009.04.014

Brion, L. P., Bell, E. F., & Raghuveer, T. S. (2003). Vitamin E supplementation for prevention of morbidity and mortality in preterm infants. *Cochrane Database Syst Rev*(4), Cd003665. doi:10.1002/14651858.cd003665

Brownfoot, F. C., Gagliardi, D. I., Bain, E., Middleton, P., & Crowther, C. A. (2013). Different corticosteroids and regimens for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst Rev*(8), Cd006764. doi:10.1002/14651858.CD006764.pub3

Cai, Z., Fan, L. W., Lin, S., Pang, Y., & 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., . . . Guan, J. (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*(6), 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., & Park, W. S. (2014). Mesenchymal stem cells for bronchopulmonary dysplasia: phase 1 dose-escalation clinical trial. *J Pediatr, 164*(5), 966-972.e966. doi:10.1016/j.jpeds.2013.12.011

Chateauvieux, S., Grigorakaki, C., Morceau, F., Dicato, M., & Diederich, M. (2011). Erythropoietin, erythropoiesis and beyond. *Biochem Pharmacol, 82*(10), 1291-1303. doi:10.1016/j.bcp.2011.06.045

Chau, V., Synnes, A., Grunau, R. E., Poskitt, K. J., Brant, R., & Miller, S. P. (2013). Abnormal brain maturation in preterm neonates associated with adverse developmental outcomes. *Neurology, 81*(24), 2082-2089. doi:10.1212/01.wnl.0000437298.43688.b9

Chen, A., Siow, B., Blamire, A. M., Lako, M., & Clowry, G. J. (2010). Transplantation of magnetically labeled mesenchymal stem cells in a model of perinatal brain injury. *Stem Cell Res, 5*(3), 255-266. doi:10.1016/j.scr.2010.08.004

Chen, M. Y., Lie, P. C., Li, Z. L., & 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*(5), 629-640. doi:10.1016/j.exphem.2009.02.003

Chevallier, M., Debillon, T., Pierrat, V., Delorme, P., Kayem, G., Durox, M., . . . Ancel, P. Y. (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*(5), 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., & Penn, A. A. (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., . . . Johnston, M. V. (2008). Neural stem cells reduce brain injury after unilateral carotid ligation. *Pediatr Neurol, 38*(2), 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., . . . Kurtzberg, J. (2014). Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *J Pediatr, 164*(5), 973-979.e971. doi:10.1016/j.jpeds.2013.11.036

Coviello, C., Keunen, K., Kersbergen, K. J., Groenendaal, F., Leemans, A., Peels, B., . . . Benders, M. (2018). Effects of early nutrition and growth on brain volumes, white matter microstructure, and neurodevelopmental outcome in preterm newborns. *Pediatr Res, 83*(1-1), 102-110. doi:10.1038/pr.2017.227

Crowther, C. A., Middleton, P. F., Voysey, M., Askie, L., Duley, L., Pryde, P. G., . . . Doyle, L. W. (2017). Assessing the neuroprotective benefits for babies of antenatal magnesium sulphate: An individual participant data meta-analysis. *PLoS Med, 14*(10), e1002398. doi:10.1371/journal.pmed.1002398

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci, 13*(10), 701-712. doi:10.1038/nrn3346

de Vries, L. S., van Haastert, I. C., Rademaker, K. J., Koopman, C., & Groenendaal, F. (2004). Ultrasound abnormalities preceding cerebral palsy in high risk preterm infants. *Journal of Pediatrics, 144*, 815-820.

Dent, C. L., Spaeth, J. P., Jones, B. V., Schwartz, S. M., Glauser, T. A., Hallinan, B., . . . Kurth, C. D. (2005). Brain magnetic resonance imaging abnormalities after the Norwood procedure using regional cerebral perfusion. *J Thorac Cardiovasc Surg, 130*(6), 1523-1530. doi:10.1016/j.jtcvs.2005.07.051

Donega, V., Nijboer, C. H., van Tilborg, G., Dijkhuizen, R. M., Kavelaars, A., & 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., . . . Heijnen, C. J. (2015). Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse. *Pediatr Res, 78*(5), 520-526. doi:10.1038/pr.2015.145

Donn, S. M., Roloff, D. W., & Goldstein, G. W. (1981). Prevention of intraventricular haemorrhage in preterm infants by phenobarbitone. A controlled trial. *Lancet, 2*(8240), 215-217.

Doyle, L. W., Cheong, J. L., Ehrenkranz, R. A., & 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.CD001146.pub5

Drommelschmidt, K., Serdar, M., Bendix, I., Herz, J., Bertling, F., Prager, S., . . . Felderhoff-Muser, U. (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., . . . Miller, S. P. (2018). Early Procedural Pain Is Associated with Regionally-Specific Alterations in Thalamic Development in Preterm Neonates. *J Neurosci, 38*(4), 878-886. doi:10.1523/jneurosci.0867-17.2017

Ersdal, H. L., Linde, J., Mduma, E., Auestad, B., & Perlman, J. (2014). Neonatal outcome following cord clamping after onset of spontaneous respiration. *Pediatrics, 134*(2), 265-272. doi:10.1542/peds.2014-0467

Fauchere, J. C., Koller, B. M., Tschopp, A., Dame, C., Ruegger, C., & Bucher, H. U. (2015). Safety of Early High-Dose Recombinant Erythropoietin for Neuroprotection in Very Preterm Infants. *J Pediatr, 167*(1), 52-57.e51-53. doi:10.1016/j.jpeds.2015.02.052

Fischer, H. S., Reibel, N. J., Buhrer, C., & Dame, C. (2017). Prophylactic Early Erythropoietin for Neuroprotection in Preterm Infants: A Meta-analysis. *Pediatrics, 139*(5). doi:10.1542/peds.2016-4317

Fischer, U. M., Harting, M. T., Jimenez, F., Monzon-Posadas, W. O., Xue, H., Savitz, S. I., . . . Cox, C. S., Jr. (2009). Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev, 18*(5), 683-692. doi:10.1089/scd.2008.0253

Fleiss, B., Guillot, P. V., Titomanlio, L., Baud, O., Hagberg, H., & Gressens, P. (2014). Stem cell therapy for neonatal brain injury. *Clin Perinatol, 41*(1), 133-148. doi:10.1016/j.clp.2013.09.002

Fogarty, M., Osborn, D. A., Askie, L., Seidler, A. L., Hunter, K., Lui, K., . . . Tarnow-Mordi, W. (2018). Delayed vs early umbilical cord clamping for preterm infants: a systematic review and meta-analysis. *Am J Obstet Gynecol, 218*(1), 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., . . . Bennet, L. (2017). Magnesium sulfate reduces EEG activity but is not neuroprotective after asphyxia in preterm fetal sheep. *J Cereb Blood Flow Metab, 37*(4), 1362-1373. doi:10.1177/0271678×16655548

Gisslen, T., Alvarez, M., Wells, C., Soo, M. T., Lambers, D. S., Knox, C. L., . . . Kallapur, S. G. (2016). Fetal inflammation associated with minimal acute morbidity in moderate/late preterm infants. *Arch Dis Child Fetal Neonatal Ed, 101*(6), F513-f519. doi:10.1136/archdischild-2015-308518

Gitto, E., Reiter, R. J., Cordaro, S. P., La Rosa, M., Chiurazzi, P., Trimarchi, G., . . . Barberi, I. (2004). Oxidative and inflammatory parameters in respiratory distress syndrome of preterm newborns: beneficial effects of melatonin. *Am J Perinatol, 21*(4), 209-216. doi:10.1055/s-2004-828610

Goldberg, R. N., Joshi, A., Moscoso, P., & Castillo, T. (1983). The effect of head position on intracranial pressure in the neonate. *Crit Care Med, 11*(6), 428-430.

Greenough, A., & Ahmed, N. (2013). Perinatal prevention of bronchopulmonary dysplasia. *J Perinat Med, 41*(1), 119-126. doi:10.1515/jpm-2012-0084

Greisen, G., & Vannucci, R. C. (2001). Is periventricular leucomalacia a result of hypoxic-ischaemic injury? Hypocapnia and the preterm brain. *Biology of the Neonate, 79*, 194-200.

Groenendaal, F., & de Vries, L. S. (2001). Hypocarbia and white matter echolucencies in newborns < 28 weeks gestation [letter]. *Pediatr Res, 50*, 772-773.

Gronbach, J., Shahzad, T., Radajewski, S., Chao, C. M., Bellusci, S., Morty, R. E., . . . Ehrhardt, H. (2018). The Potentials and Caveats of Mesenchymal Stromal Cell-Based Therapies in the Preterm Infant. *Stem Cells Int, 2018*, 9652897. doi:10.1155/2018/9652897

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., & Gressens, P. (2015). The role of inflammation in perinatal brain injury. *Nat Rev Neurol, 11*(4), 192-208. doi:10.1038/nrneurol.2015.13

Hamilton, B. E., Hoyert, D. L., Martin, J. A., Strobino, D. M., & Guyer, B. (2013). Annual summary of vital statistics: 2010-2011. *Pediatrics, 131*(3), 548-558. doi:10.1542/peds.2012-3769

Hansen-Pupp, I., Hovel, H., Hellstrom, A., Hellstrom-Westas, L., Lofqvist, C., Larsson, E. M., . . . Ley, D. (2011). Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J Clin Endocrinol Metab, 96*(4), 1129-1135. doi:10.1210/jc.2010-2440

Harteman, J. C., Groenendaal, F., van, H. I., Liem, K. D., Stroink, H., Bierings, M. B., . . . de Vries, L. S. (2011). Atypical timing and presentation of periventricular haemorrhagic infarction in preterm infants: the role of thrombophilia. *Dev.Med.Child Neurol.*

Hedley-Whyte, E. T., & Hsu, D. W. (1986). Effect of dexamethasone on blood-brain barrier in the normal mouse. *Ann Neurol, 19*(4), 373-377. doi:10.1002/ana.410190411

Hellstrom, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Ramenghi, L. A., Lofqvist, C., . . . Hard, A. L. (2016). Role of Insulinlike Growth Factor 1 in Fetal Development and in the Early Postnatal Life of Premature Infants. *Am J Perinatol, 33*(11), 1067-1071. doi:10.1055/s-0036-1586109

Hellstrom, A., Perruzzi, C., Ju, M., Engstrom, E., Hard, A. L., Liu, J. L., . . . Smith, L. E. (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*(10), 5804-5808. doi:10.1073/pnas.101113998

Hinojosa-Rodríguez, M., Harmony, T., Carrillo-Prado, C., Van Horn, J. D., Irimia, A., Torgerson, C., & Jacokes, Z. (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., . . . Polglase, G. R. (2015). Cardiovascular transition at birth: a physiological sequence. *Pediatr Res, 77*(5), 608-614. doi:10.1038/pr.2015.21

Hou, X., Ding, H., Teng, Y., Zhou, C., Tang, X., Li, S., & Ding, H. (2007). Research on the relationship between brain anoxia at different regional oxygen saturations and brain damage using near-infrared spectroscopy. *Physiol Meas, 28*(10), 1251-1265. doi:10.1088/0967-3334/28/10/010

Hyttel-Sorensen, S., Greisen, G., Als-Nielsen, B., & 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

Hyttel-Sorensen, S., Pellicer, A., Alderliesten, T., Austin, T., van Bel, F., Benders, M., . . . Greisen, G. (2015). Cerebral near infrared spectroscopy oximetry in extremely preterm infants: phase II randomised clinical trial. *Bmj, 350*, g7635. doi:10.1136/bmj.g7635

Inder, T. E., Warfield, S. K., Wang, H., Huppi, P. S., & Volpe, J. J. (2005). Abnormal cerebral structure is present at term in premature infants. *Pediatrics, 115*, 286-294.

Isayama, T., Chai-Adisaksopha, C., & 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*(8), 731-739. doi:10.1001/jamapediatrics.2015.0510

Jadasz, J. J., Kremer, D., Gottle, P., Tzekova, N., Domke, J., Rivera, F. J., . . . Kury, P. (2013). Mesenchymal stem cell conditioning promotes rat oligodendroglial cell maturation. *PLoS One, 8*(8), e71814. doi:10.1371/journal.pone.0071814

Jain, N. J., Kruse, L. K., Demissie, K., & Khandelwal, M. (2009). Impact of mode of delivery on neonatal complications: trends between 1997 and 2005. *J Matern Fetal Neonatal Med, 22*(6), 491-500. doi:10.1080/14767050902769982

Jellema, R. K., Wolfs, T. G., Lima Passos, V., Zwanenburg, A., Ophelders, D. R., Kuypers, E., . . . Kramer, B. W. (2013). Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia. *PLoS One, 8*(8), e73031. doi:10.1371/journal.pone.0073031

Jensen, E. A., Foglia, E. E., & Schmidt, B. (2018). Association between prophylactic indomethacin and death or bronchopulmonary dysplasia: A systematic review and meta-analysis of observational studies. *Semin Perinatol, 42*(4), 228-234. doi:10.1053/j.semperi.2018.05.005

Juul, S. E., & Pet, G. C. (2015). Erythropoietin and Neonatal Neuroprotection. *Clin Perinatol, 42*(3), 469-481. doi:10.1016/j.clp.2015.04.004

Karemaker, R., Heijnen, C. J., Veen, S., Baerts, W., Samsom, J., Visser, G. H., . . . van Bel, F. (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*(6), 745-750. doi:10.1203/01.pdr.0000246200.76860.de

Kawakami, M., Sekiguchi, M., Sato, K., Kozaki, S., & Takahashi, M. (2001). Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. *J Biol Chem, 276*(42), 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., & de Vries, L. S. (2014a). Different patterns of punctate white matter lesions in serially scanned preterm infants. In *PLoS One* (Vol. 9, pp. e108904). United States.

Kersbergen, K. J., Leemans, A., Groenendaal, F., van der Aa, N. E., Viergever, M. A., de Vries, L. S., & Benders, M. J. (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., & Inder, T. E. (2013). New MR imaging assessment tool to define brain abnormalities in very preterm infants at term. *AJNR Am J Neuroradiol, 34*(11), 2208-2214. doi:10.3174/ajnr.A3521

Kizil, C., Kyritsis, N., & Brand, M. (2015). Effects of inflammation on stem cells: together they strive? *EMBO Rep, 16*(4), 416-426. doi:10.15252/embr.201439702

Kotowski, M., Litwinska, Z., Klos, P., Pius-Sadowska, E., Zagrodnik-Ulan, E., Ustianowski, P., . . . Machalinski, B. (2017). Autologous cord blood transfusion in preterm infants - could its humoral effect be the kez to control prematurity-related complications? A preliminary study. *J Physiol Pharmacol, 68*(6), 921-927.

Koulnis, M., Porpiglia, E., Hidalgo, D., & 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., & 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*(6), 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., . . . Stewart, D. J. (2012). Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One, 7*(10), e47559. doi:10.1371/journal.pone.0047559

Lekic, T., Flores, J., Klebe, D., Doycheva, D., Rolland, W. B., Tang, J., & Zhang, J. H. (2016). Intranasal IGF-1 Reduced Rat Pup Germinal Matrix Hemorrhage. *Acta Neurochir Suppl, 121*, 209-212. doi:10.1007/978-3-319-18497-5_37

Lemmers, P. M., Toet, M., van Schelven, L. J., & van Bel, F. (2006). Cerebral oxygenation and cerebral oxygen extraction in the preterm infant: the impact of respiratory distress syndrome. *Exp Brain Res, 173*(3), 458-467. doi:10.1007/s00221-006-0388-8

Leuchter, R. H., Gui, L., Poncet, A., Hagmann, C., Lodygensky, G. A., Martin, E., . . . Huppi, P. S. (2014). Association between early administration of high-dose erythropoietin in preterm infants and brain MRI abnormality at term-equivalent age. *Jama, 312*(8), 817-824. doi:10.1001/jama.2014.9645

Ley, D., Hansen-Pupp, I., Niklasson, A., Domellof, M., Friberg, L. E., Borg, J., . . . Hellstrom, A. (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*(1), 68-74. doi:10.1038/pr.2012.146

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., . . . Miller, S. L. (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

Luu, T. M., Ment, L. R., Schneider, K. C., Katz, K. H., Allan, W. C., & Vohr, B. R. (2009). Lasting effects of preterm birth and neonatal brain hemorrhage at 12 years of age. *Pediatrics, 123*(3), 1037-1044. doi:10.1542/peds.2008-1162

Marseglia, L., D'Angelo, G., Manti, S., Aversa, S., Reiter, R. J., Antonuccio, P., . . . Gitto, E. (2015). Oxidative Stress-Mediated Damage in Newborns with Necrotizing Enterocolitis: A Possible Role of Melatonin. *Am J Perinatol, 32*(10), 905-909. doi:10.1055/s-0035-1547328

Martinez-Biarge, M., Groenendaal, F., Kersbergen, K. J., Benders, M. J., Foti, F., Cowan, F. M., & de Vries, L. S. (2016). MRI Based Preterm White Matter Injury Classification: The Importance of Sequential Imaging in Determining Severity of Injury. *PLoS One, 11*(6), e0156245. doi:10.1371/journal.pone.0156245

McPherson, C., & Inder, T. (2017). Perinatal and neonatal use of sedation and analgesia. *Semin Fetal Neonatal Med, 22*(5), 314-320. doi:10.1016/j.siny.2017.07.007

McPherson, C., & Wambach, J. A. (2018). Prevention and Treatment of Respiratory Distress Syndrome in Preterm Neonates. *Neonatal Netw, 37*(3), 169-177. doi:10.1891/0730-0832.37.3.169

Ment, L. R., Oh, W., Ehrenkranz, R. A., Philip, A. G., Duncan, C. C., & Makuch, R. W. (1995). Antenatal steroids, delivery mode, and intraventricular hemorrhage in preterm infants. *Am J Obstet Gynecol, 172*(3), 795-800.

Ment, L. R., Stewart, W. B., Ardito, T. A., Huang, E., & Madri, J. A. (1992). Indomethacin promotes germinal matrix microvessel maturation in the newborn beagle pup. *Stroke, 23*(8), 1132-1137.

Mercer, J. S., Erickson-Owens, D. A., Vohr, B. R., Tucker, R. J., Parker, A. B., Oh, W., & Padbury, J. F. (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., . . . Edwards, A. D. (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., . . . Nagamura-Inoue, T. (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., & Shah, P. S. (2015). Periventricular/Intraventricular Hemorrhage and Neurodevelopmental Outcomes: A Meta-analysis. *Pediatrics, 136*(6), 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., . . . Anderson, P. J. (2018). Neurobehavioral Outcomes 11 Years After Neonatal Caffeine Therapy for Apnea of Prematurity. *Pediatrics, 141*(5). doi:10.1542/peds.2017-4047

Musilova, I., Andrys, C., Drahosova, M., Zednikova, B., Hornychova, H., Pliskova, L., . . . Kacerovsky, M. (2018). Late preterm prelabor rupture of fetal membranes: fetal inflammatory response and neonatal outcome. *Pediatr Res, 83*(3), 630-637. doi:10.1038/pr.2017.300

Nelin, T. D., Pena, E., Giacomazzi, T., Lee, S., Logan, J. W., Moallem, M., . . . Nelin, L. D. (2017). Outcomes following indomethacin prophylaxis in extremely preterm infants in an all-referral NICU. *J Perinatol, 37*(8), 932-937. doi:10.1038/jp.2017.71

Neubauer, A. P., Voss, W., Wachtendorf, M., & Jungmann, T. (2010). Erythropoietin improves neurodevelopmental outcome of extremely preterm infants. *Ann Neurol, 67*(5), 657-666. doi:10.1002/ana.21977

Ng, E., Taddio, A., & 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., & Levison, S. W. (2018). Pediatric brain repair from endogenous neural stem cells of the subventricular zone. *Pediatr Res, 83*(1-2), 385-396. doi:10.1038/pr.2017.261

Norman, M., Piedvache, A., Borch, K., Huusom, L. D., Bonamy, A. E., Howell, E. A., . . . Zeitlin, J. (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*(7), 678-686. doi:10.1001/jamapediatrics.2017.0602

O'Kusky, J., & Ye, P. (2012). Neurodevelopmental effects of insulin-like growth factor signaling. *Front Neuroendocrinol, 33*(3), 230-251. doi:10.1016/j.yfrne.2012.06.002

O'Shea, T. M., Allred, E. N., Kuban, K. C., Dammann, O., Paneth, N., Fichorova, R., . . . Leviton, A. (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*(3), 395-401.e394. doi:10.1016/j.jpeds.2011.08.069

Oh, K. J., Park, J. Y., Lee, J., Hong, J. S., Romero, R., & 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*(1), 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., . . . Lowe, J. R. (2014). Cognitive outcomes of preterm infants randomized to darbepoetin, erythropoietin, or placebo. *Pediatrics, 133*(6), 1023-1030. doi:10.1542/peds.2013-4307

Ohlsson, A., & 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

Onland, W., Offringa, M., Cools, F., De Jaegere, A. P., Rademaker, K., Blom, H., . . . van Kaam, A. H. (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., . . . Kramer, B. W. (2016). Mesenchymal Stromal Cell-Derived Extracellular Vesicles Protect the Fetal Brain After Hypoxia-Ischemia. *Stem Cells Transl Med, 5*(6), 754-763. doi:10.5966/sctm.2015-0197

Ozdemir, A., Brown, M. A., & Morgan, W. J. (1997). Markers and mediators of inflammation in neonatal lung disease. *Pediatr Pulmonol, 23*(4), 292-306.

Pang, Y., Zheng, B., Campbell, L. R., Fan, L. W., Cai, Z., & Rhodes, P. G. (2010). IGF-1 can either protect against or increase LPS-induced damage in the developing rat brain. *Pediatr Res, 67*(6), 579-584. doi:10.1203/PDR.0b013e3181dc240f

Papile, L. A., Burstein, J., Burstein, R., & Koffler, H. (1978). Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. *Journal of Pediatrics, 92*, 529-534.

Park, W. S., Ahn, S. Y., Sung, S. I., Ahn, J. Y., & Chang, Y. S. (2017). Mesenchymal Stem Cells: The Magic Cure for Intraventricular Hemorrhage? *Cell Transplant, 26*(3), 439-448. doi:10.3727/096368916×694193

Park, W. S., Sung, S. I., Ahn, S. Y., Sung, D. K., Im, G. H., Yoo, H. S., . . . Chang, Y. S. (2016). Optimal Timing of Mesenchymal Stem Cell Therapy for Neonatal Intraventricular Hemorrhage. *Cell Transplant, 25*(6), 1131-1144. doi:10.3727/096368915×689640

Paton, M. C. B., McDonald, C. A., Allison, B. J., Fahey, M. C., Jenkin, G., & 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., & 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*(21), 1353-1357. doi:10.1056/nejm198505233122104

Perlman, J. M., Wyllie, J., Kattwinkel, J., Wyckoff, M. H., Aziz, K., Guinsburg, R., . . . Velaphi, S. (2015). Part 7: Neonatal Resuscitation: 2015 International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science With Treatment Recommendations (Reprint). *Pediatrics, 136 Suppl 2*, S120-166. doi:10.1542/peds.2015-3373D

Pierrat, V., Marchand-Martin, L., Arnaud, C., Kaminski, M., Resche-Rigon, M., Lebeaux, C., . . . Ancel, P. Y. (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., . . . Greisen, G. (2017). Early biomarkers of brain injury and cerebral hypo- and hyperoxia in the SafeBoosC II trial. *PLoS One, 12*(3), e0173440. doi:10.1371/journal.pone.0173440

Practice, C. o. O. (2017). Committee Opinion No. 684: Delayed Umbilical Cord Clamping After Birth. *Obstet Gynecol, 129*(1), e5-e10. doi:10.1097/aog.0000000000001860

Qiu, J., Zhao, L., Yang, Y., Zhang, J. H., Feng, Y., & Cheng, R. (2018). Effects of fentanyl for pain control and neuroprotection in very preterm newborns on mechanical ventilation. *J Matern Fetal Neonatal Med*, 1-7. doi:10.1080/14767058.2018.1471593

Qu, R., Li, Y., Gao, Q., Shen, L., Zhang, J., Liu, Z., . . . Chopp, M. (2007). Neurotrophic and growth factor gene expression profiling of mouse bone marrow stromal cells induced by ischemic brain extracts. *Neuropathology, 27*(4), 355-363.

Rabe, H., Diaz-Rossello, J. L., Duley, L., & 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*(8), Cd003248. doi:10.1002/14651858.CD003248.pub3

Rabe, H., Reynolds, G., & Diaz-Rossello, J. (2008). A systematic review and meta-analysis of a brief delay in clamping the umbilical cord of preterm infants. *Neonatology, 93*(2), 138-144. doi:10.1159/000108764

Rangarajan, V., & Juul, S. E. (2014). Erythropoietin: emerging role of erythropoietin in neonatal neuroprotection. *Pediatr Neurol, 51*(4), 481-488. doi:10.1016/j.pediatrneurol.2014.06.008

Roberts, D., Brown, J., Medley, N., & 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., & Soll, R. (2012). Prophylactic versus selective use of surfactant in preventing morbidity and mortality in preterm infants. *Cochrane Database Syst Rev*(3), Cd000510. doi:10.1002/14651858.CD000510.pub2

Romantsik, O., Calevo, M. G., & 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., . . . Wright, L. L. (2001). Long-term effects of indomethacin prophylaxis in extremely-low-birth-weight infants. *N Engl J Med, 344*(26), 1966-1972. doi:10.1056/nejm200106283442602

Schmidt, B., Roberts, R. S., Davis, P., Doyle, L. W., Barrington, K. J., Ohlsson, A., . . . Tin, W. (2007). Long-term effects of caffeine therapy for apnea of prematurity. *N Engl J Med, 357*(19), 1893-1902. doi:10.1056/NEJMoa073679

Shepherd, E., Salam, R. A., Middleton, P., Makrides, M., McIntyre, S., Badawi, N., & Crowther, C. A. (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., & 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*(11), 909-916. doi:10.1016/j.earlhumdev.2005.07.007

Skov, L., Pryds, O., & Greisen, G. (1991). Estimating cerebral blood flow in newborn infants: comparison of near infrared spectroscopy and 133Xe clearance. *Pediatr Res, 30*(6), 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., . . . Higgins, R. D. (2010). Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics, 126*, 443-456.

Stolp, H. B., Ball, G., So, P. W., Tournier, J. D., Jones, M., Thornton, C., & Edwards, A. D. (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*(1), 4011. doi:10.1038/s41598-018-22295-9

Strunk, T., Inder, T., Wang, X., Burgner, D., Mallard, C., & Levy, O. (2014). Infection-induced inflammation and cerebral injury in preterm infants. *Lancet Infect Dis, 14*(8), 751-762. doi:10.1016/s1473-3099(14)70710-8

Thewissen, L., Caicedo, A., Lemmers, P., Van Bel, F., Van Huffel, S., & 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., & 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*(10), e011643. doi:10.1136/bmjopen-2016-011643

Tokida, Y., Aratani, Y., Morita, A., & 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*(30), 18123-18129.

Uauy, R., & Mena, P. (2015). Long-chain polyunsaturated fatty acids supplementation in preterm infants. *Curr Opin Pediatr, 27*(2), 165-171. doi:10.1097/mop.0000000000000203

Uccelli, A., Moretta, L., & Pistoia, V. (2008). Mesenchymal stem cells in health and disease. *Nat Rev Immunol, 8*(9), 726-736. doi:10.1038/nri2395

Vaes, J. E. G., Vink, M. A., de Theije, C. G. M., Hoebeek, F. E., Benders, M., & Nijboer, C. H. A. (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

Van Bel, F., Bartelds, B., Teitel, D. F., & Rudolph, A. M. (1995). Effect of indomethacin on cerebral blood flow and oxygenation in the normal and ventilated fetal lamb. *Pediatric Research, 38*, 243-250.

Van Bel, F., Klautz, R. J., Steendijk, P., Schipper, I. B., Teitel, D. F., & 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.

Van Bel, F., Lemmers, P., & Naulaers, G. (2008). Monitoring neonatal regional cerebral oxygenation saturation in clinical practice: Value and pitfalls. *Neonatology*.

van Bel, F., & Mintzer, J. P. (2018). Monitoring cerebral oxygenation of the immature brain: a neuroprotective strategy? *Pediatr Res, 84*(2), 159-164. doi:10.1038/s41390-018-0026-8

Van Bel, F., Roman, C., Klautz, R. J., Teitel, D. F., & Rudolph, A. M. (1994). Relationship between brain blood flow and carotid arterial flow in the sheep fetus. *Pediatr Res, 35*, 329-333.

Van Bel, F., Van de Bor, M., Stijnen, T., Baan, J., & 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.

van de Looij, Y., Lodygensky, G. A., Dean, J., Lazeyras, F., Hagberg, H., Kjellmer, I., . . . Sizonenko, S. V. (2012). High-field diffusion tensor imaging characterization of cerebral white matter injury in lipopolysaccharide-exposed fetal sheep. *Pediatr Res, 72*(3), 285-292. doi:10.1038/pr.2012.72

van der Kooij, M. A., Groenendaal, F., Kavelaars, A., Heijnen, C. J., & 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.

van Haastert, I. C., Groenendaal, F., Uiterwaal, C. S., Termote, J. U., van der Heide-Jalving, M., Eijsermans, M. J., . . . de Vries, L. S. (2011). Decreasing incidence and severity of cerebral palsy in prematurely born children. *J.Pediatr., 159*, 86-91.

van Tilborg, E., Achterberg, E. J. M., van Kammen, C. M., van der Toorn, A., Groenendaal, F., Dijkhuizen, R. M., . . . Nijboer, C. H. A. (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*(1), 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., . . . Nijboer, C. H. (2018b). Origin and dynamics of oligodendrocytes in the developing brain: Implications for perinatal white matter injury. *Glia, 66*(2), 221-238. doi:10.1002/glia.23256

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2011). Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. *Brain Behav Immun, 25*(7), 1342-1348. doi:10.1016/j.bbi.2011.03.021

Villamor-Martinez, E., Fumagalli, M., Mohammed Rahim, O., Passera, S., Cavallaro, G., Degraeuwe, P., . . . 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

Vohr, B., & Ment, L. R. (1996). Intraventricular hemorrhage in the preterm infant. *Early Hum Dev, 44*(1), 1-16.

Volpe, J. J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol, 8*(1), 110-124. doi:10.1016/s1474-4422(08)70294-1

Wagenaar, N., Nijboer, C. H., & van Bel, F. (2017). Repair of neonatal brain injury: bringing stem cell-based therapy into clinical practice. *Dev Med Child Neurol, 59*(10), 997-1003. doi:10.1111/dmcn.13528

Walti, H., Paris-Llado, J., Breart, G., & 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*(8), 913-921.

Wiest, D. B., Chang, E., Fanning, D., Garner, S., Cox, T., & Jenkins, D. D. (2014). Antenatal pharmacokinetics and placental transfer of N-acetylcysteine in chorioamnionitis for fetal neuroprotection. *J Pediatr, 165*(4), 672-677.e672. doi:10.1016/j.jpeds.2014.06.044

Wintermark, P., Hansen, A., Warfield, S. K., Dukhovny, D., & 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 Pt 1*, 287-293. doi:10.1016/j.neuroimage.2013.04.072

Wyllie, J., Bruinenberg, J., Roehr, C. C., Rudiger, M., Trevisanuto, D., & 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., . . . Ballabh, P. (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*(7), 1482-1500. doi:10.1002/jnr.21618

Yamaji, R., Okada, T., Moriya, M., Naito, M., Tsuruo, T., Miyatake, K., & Nakano, Y. (1996). Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur J Biochem, 239*(2), 494-500.

Yao, A. C., Moinian, M., & Lind, J. (1969). Distribution of blood between infant and placenta after birth. *Lancet, 2*(7626), 871-873.

Zwicker, J. G., Miller, S. P., Grunau, R. E., Chau, V., Brant, R., Studholme, C., . . . Tam, E. W. (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

3

The potential of stem cell therapy to repair white matter injury in preterm infants: lessons learned from experimental models

Josine E.G. Vaes, Marit A. Vink, Caroline G.M. de Theije, Freek E. Hoebeek, Manon J.N.L. Benders, Cora H.A. Nijboer

Published in Frontiers in Physiology, 2019 May; 10, 540

ABSTRACT

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.

INTRODUCTION

Preterm birth is defined as birth before 37 weeks of gestation, and is relatively common with global prevalence rates ranging between 8-10%. 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 & 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 (Johnson et al., 2009; Larroque et al., 2008; Linsell et al., 2018; MacKay et al., 2010; Moster et al., 2008). A smaller percentage of this population (5%-10%) is believed to suffer from major motor problems, such as cerebral palsy (Johnson et al., 2009; Larroque et al., 2008).

Preterm WMI is thought to be the result of myelination failure during white matter development in the third trimester (Back et al., 2001; Khwaja & 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 & 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 (Back, 2017; Lee, 2017; Volpe, 2017; Volpe et al., 2011; Zaghloul & 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, 2017; Back & Miller, 2014). 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 oligodendrocytes capable of myelin production. Oligodendrocytes 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) oligodendrocyte 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 oligodendrocytes (pre-OLs) that progress to the final stage of 4) mature myelinating OLs (Back et al., 2001; Emery, 2010; van Tilborg et al., 2018; Volpe et al., 2011). 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 & 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 (Jakovcevski et al., 2009; Kessaris et al., 2006; 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; Back et al., 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 (Bennet et al., 2018; van Tilborg et al., 2016). 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 (Deng, 2010; Khwaja & Volpe, 2008; Volpe, 2009). 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 & 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 & Miller, 2014; Bennet et al., 2018; van Tilborg et al., 2016). 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 & 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 (Fyfe et al., 2014; Viscardi et al., 2004). 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., 2015; 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 lead 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 & 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 (Back, 2015; Robinson et al., 2006; Segovia et al., 2008). 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 (Kassis et al., 2011; Liang et al., 2014; Paton et al., 2017; van Velthoven et al., 2010a). 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 & 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 (Oppliger et al., 2017; Zhang et al., 2016). 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, bioluminence 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 & 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 (Gutierrez-Fernandez et al., 2013a; Hayakawa & Lo, 2016; Jiang et al., 2006; Mifsud et al., 2014). 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. (2013) showed an incline in Olig2 protein levels, an oligodendrocyte 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., 2013b). 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 (Cunningham et al., 2018; Dulamea, 2015; 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 & 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; Correale et al., 2017; Garg & Smith, 2015; 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 (Cohen, 2013; Genc et al., 2018; Gharibi et al., 2015; Jadasz et al., 2012; Laroni et al., 2013; Morando et al., 2012; Rivera & Aigner, 2012; Xiao et al., 2015). 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 oligodendrocyte 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 (Bai et al., 2009; Cristofanilli et al., 2011; Kassis et al., 2008; Liu et al., 2013; Liu et al., 2009; Zappia et al., 2005; Zhang et al., 2005). 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 (Bai et al., 2009; Jaramillo-Merchan et al., 2013; Kassis et al., 2008; Liu et al., 2013; Zhang et al., 2005). 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 oligodendrocyte 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 and colleagues concluded that the lack of myelin regeneration following MSC therapy was related to the intact blood brain barrier (BBB), limiting MSC migration towards the lesion site following intravenous or intranasal administration. Cruz-Martinez and colleagues 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 (Scolding et al., 2017; von

Bahr et al., 2012). 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 & 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 & 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, oligodendrocyte 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 and colleagues reported that human AD-derived MSCs differentiated into MBP-expressing oligodendrocytes following intracranial transplantation in their rat model of inflammatory HIE. In addition, the MSCs were shown to 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 white matter injury 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 (Ding et al., 2017; Donega et al., 2014b; Gu et al., 2016; Gu et al., 2015; van Velthoven et al., 2010a). 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 (32ºC during 4h) 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 (Ahn et al., 2018a; Park et al., 2015). In their P7 rat model of HIE the combination of hypothermia, started 6 hours after HIE induction (32ºC during 24h), 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 hour period of hypothermia (32ºC, started 3 hours 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 three 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, 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 hours postnatally 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).

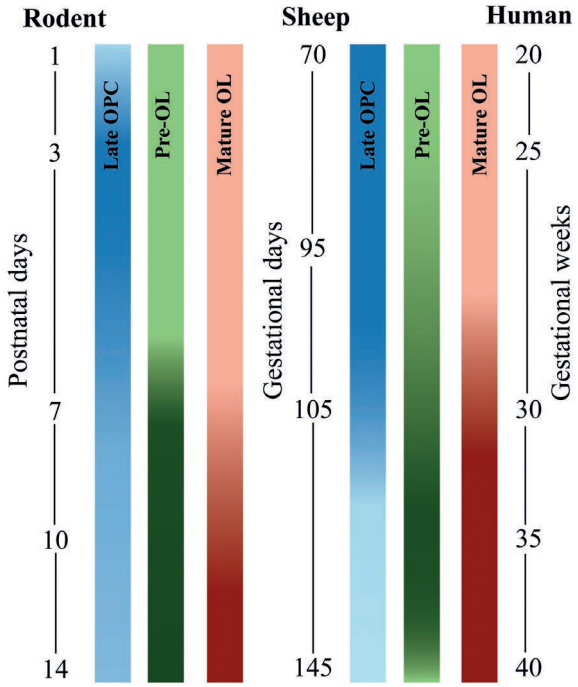


Figure 1. Developmental timeline comparing oligodendrocyte (OL) stage-specific development in different species. Blue bars depict late oligodendrocyte precursor cells (OPC), green bars depict pre-myelinating precursors (pre-OLs) and red bars depict mature OLs. From left to right: rodent, sheep and human. The postnatal window in rodent OL development between postnatal day (P)1-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).

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 & 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., 2017; van Velthoven et al., 2013). 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; Park et al., 2017; Payne et al., 2013). 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-conditioned medium. 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-conditioned medium administration, conclusions on the possible (limited) effects of MSC-conditioned medium 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 hours 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 (15mg/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 minutes 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 could not be reversed by MSC therapy. Interestingly, the authors were able to identify two phenotypes of mature oligodendrocytes. In vehicle-treated dWMI animals they found an increase in oligodendrocytes 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; Li et al., 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 hour 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 hours 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 hours 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 hours 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) modelled 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. Six hours 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 white matter injury 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 oligodendrocyte 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., 2013b; Otero-Ortega et al., 2015; Park et al., 2013), 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 (Kalaszczynska & Ferdyn, 2015; Scruggs et al., 2013; Stolzing et al., 2008). 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 towards 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 towards 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 (Jellema et al., 2013; Kurtz, 2008). 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 (Park et al., 2018; Sarmah et al., 2018; van Velthoven et al., 2010b; 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 cribiform plate and migrate towards 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 (Donega et al., 2015; Nijboer et al., 2018; Oppliger et al., 2016; van Velthoven et al., 2010b). 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 (Donega et al., 2014a; Nessler et al., 2013). 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 hours after injury induction) versus late (>7 days after injury induction) MSC treatment (Kim et al., 2012; Park et al., 2016; Wang et al., 2014). 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 & 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 & Weiss, 2012; Jin et al., 2015; Lee, 2017; Stewart et al., 2013). 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), four-day i.p. treatment with supernatant of WJ-MSCs pre-treated with IFN-γ did result in a 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) white matter injury, 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 (Kim et al., 2016; Tang et al., 2014).

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 tumorgenicity. 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 (Park et al., 2018; Schäfer et al., 2016). 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-conditioned medium (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 72h) 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 hours, rat NSCs differentiated towards 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 24h) 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 towards 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 stainings, 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. 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 animal 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.

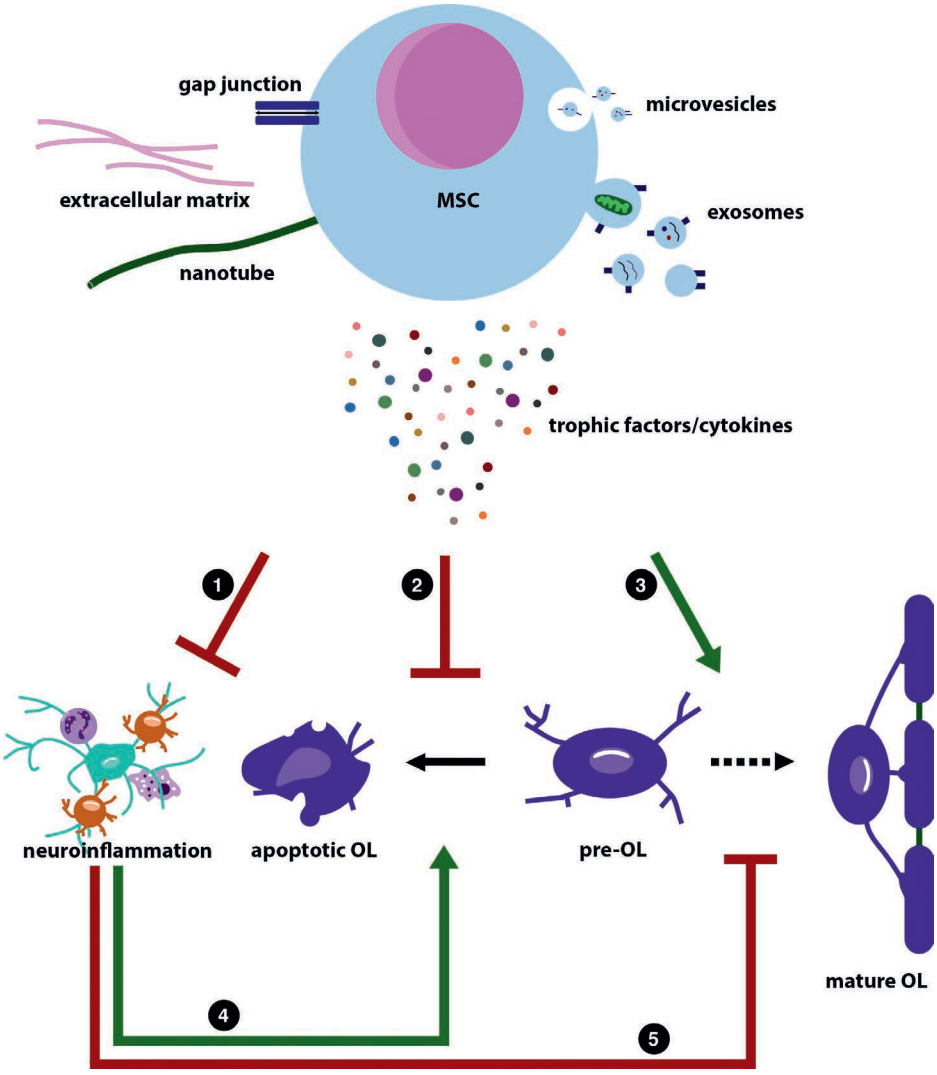


Figure 2. Illustration summarizing the possible working mechanisms of mesenchymal stem cells (MSCs). This illustration is based on the literature discussed in this review. MSCs are believed to exert their regenerative potential through reciprocal transport via gap junctions (blue), production of extracellular 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 neighbouring 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 (astrocyte in green; microglia in orange; neutrophil left top; macrophage right bottom). The MSCs' secretome can also (2) directly inhibit apoptosis of pre-myelinating oligodendrocyte (pre-OL; irregular shaped purple cell), whereas it can also (3) directly stimulate (green

stimulatory arrow) pre-OL differentiation towards 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.

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 towards 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 & Coles, 2002; Gutierrez-Fernandez et al., 2013a; 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 (Buser et al., 2012; Volpe et al., 2011). 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.

Acknowledgements

This work was supported by Brain Foundation Netherlands.

REFERENCES

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Ahn, J.-Y., & 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*(1), 145-156. doi:10.3727/096368916×692861

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., & Park, W. S. (2018a). Hypothermia broadens the therapeutic time window of mesenchymal stem cell transplantation for severe neonatal hypoxic ischemic encephalopathy. *Sci Rep, 8*(1), 7665. doi:10.1038/s41598-018-25902-x

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Yoo, H. S., Im, G. H., . . . Park, W. S. (2015). Optimal Route for Mesenchymal Stem Cells Transplantation after Severe Intraventricular Hemorrhage in Newborn Rats. *PLoS One, 10*(7), 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., . . . Park, W. S. (2013). Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. *Stroke, 44*(2), 497-504. doi:10.1161/strokeaha.112.679092

Ahn, S. Y., Chang, Y. S., Sung, S. I., & Park, W. S. (2018b). Mesenchymal Stem Cells for Severe Intraventricular Hemorrhage in Preterm Infants: Phase I Dose-Escalation Clinical Trial. *Stem Cells Transl Med*. doi:10.1002/sctm.17-0219

Back, S. A. (2015). Brain Injury in the Preterm Infant: New Horizons for Pathogenesis and Prevention. *Pediatr Neurol, 53*(3), 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 Neuropathologica, 134*(3), 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. (2001). Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci, 21*(1529-2401 (Electronic)), 1302-1312. doi:10.1523/JNEUROSCI.21-04-01302.2001

Back, S. A., & Miller, S. P. (2014). Brain injury in premature neonates: A primary cerebral dysmaturation disorder? *Annals of Neurology, 75*(4), 469-486. doi:10.1002/ana.24132

Back, S. A., Riddle, A., Dean, J., & Hohimer, A. R. (2012). The instrumented fetal sheep as a model of cerebral white matter injury in the premature infant. *Neurotherapeutics, 9*(2), 359-370. doi:10.1007/s13311-012-0108-y

Back, S. A., Riddle, A., & 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., . . . Miller, R. H. (2012). Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. *Nat Neurosci, 15*(6), 862-870. doi:10.1038/nn.3109

Bai, L., Lennon, D. P., Eaton, V., Maier, K., Caplan, A. I., Miller, S. D., & Miller, R. H. (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*(11), 1192-1203. doi:10.1002/glia.20841

Bedini, G., Bersano, A., Zanier, E. R., Pischiutta, F., & Parati, E. A. (2018). Mesenchymal Stem Cell Therapy in Intracerebral Haemorrhagic Stroke. *Curr Med Chem, 25*(19), 2176-2197. doi:10.2174/0929867325666180111101410

Bennet, L., Dhillon, S., Lear, C. A., van den Heuij, L., King, V., Dean, J. M., . . . Gunn, A. J. (2018). Chronic inflammation and impaired development of the preterm brain. *Journal of Reproductive Immunology, 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., . . . Kinney, H. C. (2008). Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia. *Brain Pathology, 18*(2), 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., . . . Lawn, J. E. (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. *The Lancet, 379*(9832), 2162-2172. doi:10.1016/S0140-6736(12)60820-4

Bradl, M., & Lassmann, H. (2010). Oligodendrocytes: biology and pathology. *Acta Neuropathol, 119*(1), 37-53. doi:10.1007/s00401-009-0601-5

Brouwer, A. J., van Stam, C., Uniken Venema, M., Koopman, C., Groenendaal, F., & 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*(3), 210-216. doi:10.1159/000331797

Brown, M. K., & DiBlasi, R. M. (2011). Mechanical Ventilation of the Premature Neonate. *Respiratory Care, 56*(9), 1298-1313. doi:10.4187/respcare.01429

Buser, J. R., Maire, J., Riddle, A., Gong, X., Nguyen, T., Nelson, K., . . . Back, S. A. (2012). Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. *Annals of Neurology, 71*(1), 93-109. doi:10.1002/ana.22627

Chen, A., Dimambro, N., & 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 Research, 1201*, 187-195. doi:10.1016/j.brainres.2008.01.066

Chen, A., Siow, B., Blamire, A. M., Lako, M., & Clowry, G. J. (2010). Transplantation of magnetically labeled mesenchymal stem cells in a model of perinatal brain injury. *Stem Cell Res, 5*(3), 255-266. doi:10.1016/j.scr.2010.08.004

Chen, X., Li, Y., Wang, L., Katakowski, M., Zhang, L., Chen, J., . . . Chopp, M. (2002). Ischemic rat brain extracts induce human marrow stromal cell growth factor production. *Neuropathology, 22*(4), 275-279. doi:10.1046/j.1440-1789.2002.00450.x

Ciccarelli, O., Barkhof, F., Bodini, B., De Stefano, N., Golay, X., Nicolay, K., . . . Miller, D. H. (2014). Pathogenesis of multiple sclerosis: insights from molecular and metabolic imaging. *Lancet Neurol, 13*(8), 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*(1-2), 43-49. doi:10.1016/j.jns.2012.12.009

Compston, A., & Coles, A. (2002). Multiple sclerosis. *The Lancet, 359*(9313), 1221-1231. doi:10.1016/s0140-6736(02)08220-x

Corcelli, M., Hawkins, K., Vlahova, F., Hunjan, A., Dowding, K., De Coppi, P., . . . Guillot, P. V. (2018). Neuroprotection of the hypoxic-ischemic mouse brain by human CD117(+)CD90(+) CD105(+) amniotic fluid stem cells. *Sci Rep, 8*(1), 2425. doi:10.1038/s41598-018-20710-9

Correale, J., Gaitan, M. I., Ysrraelit, M. C., & Fiol, M. P. (2017). Progressive multiple sclerosis: from pathogenic mechanisms to treatment. *Brain, 140*(3), 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., . . . Kurtzberg, J. (2014). Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *J Pediatr, 164*(5), 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., & Back, S. A. (2003). Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Exp Neurol, 181*(2), 231-240. doi:10.1016/s0014-4886(03)00032-3

Cristofanilli, M., Harris, V. K., Zigelbaum, A., Goossens, A. M., Lu, A., Rosenthal, H., & Sadiq, S. A. (2011). Mesenchymal stem cells enhance the engraftment and myelinating ability of allogeneic oligodendrocyte progenitors in dysmyelinated mice. *Stem Cells Dev, 20*(12), 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., . . . Martinez, S. (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., & Allan, S. M. (2018). The therapeutic potential of the mesenchymal stem cell secretome in ischaemic stroke. *Journal of Cerebral Blood Flow and Metabolism, 38*(8), 1276-1292. doi:10.1177/0271678×18776802

Danielyan, L., Schafer, R., von Ameln-Mayerhofer, A., Buadze, M., Geisler, J., Klopfer, T., . . . Frey, W. H., 2nd. (2009). Intranasal delivery of cells to the brain. *European Journal of Cell Biology, 88*(6), 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., & Arnalich-Montiel, F. (2012). Immunosuppressive properties of mesenchymal stem cells: advances and applications. *Curr Mol Med, 12*(5), 574-591. doi:10.2174/156652412800619950

de Vries, L. S., Benders, M. J. N. L., & Groenendaal, F. (2013). Imaging the premature brain: ultrasound or MRI? *Neuroradiology, 55*(2), 13-22. doi:10.1007/s00234-013-1233-y

Deng, W. (2010). Neurobiology of injury to the developing brain. *Nature Reviews Neurology, 6*(1759-4766 (Electronic)), 328-336. doi:10.1038/nrneurol.2010.53

Ding, H., Zhang, H., Ding, H., Li, D., Yi, X., Ma, X., . . . Ju, X. (2017). Transplantation of placenta-derived mesenchymal stem cells reduces hypoxic-ischemic brain damage in rats by ameliorating the inflammatory response. *Cell Mol Immunol, 14*(8), 693-701. doi:10.1038/cmi.2015.99

Doeppner, T. R., Herz, J., Gorgens, A., Schlechter, J., Ludwig, A. K., Radtke, S., . . . Hermann, D. M. (2015). Extracellular Vesicles Improve Post-Stroke Neuroregeneration and Prevent Postischemic Immunosuppression. *Stem Cells Transl Med, 4*(10), 1131-1143. doi:10.5966/sctm.2015-0078

Donega, V., Nijboer, C. H., Braccioli, L., Slaper-Cortenbach, I., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2014a). Intranasal administration of human MSC for ischemic brain injury in the mouse: in vitro and in vivo neuroregenerative functions. *PLoS One, 9*(11), e112339-e112339. doi:10.1371/journal.pone.0112339

Donega, V., Nijboer, C. H., van Tilborg, G., Dijkhuizen, R. M., Kavelaars, A., & Heijnen, C. J. (2014b). Intranasally administered mesenchymal stem cells promote a regenerative niche for repair of neonatal ischemic brain injury. *Experimental Neurology, 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., . . . Heijnen, C. J. (2015). Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse. *Pediatric Research, 78*(5), 520-526. doi:10.1038/pr.2015.145

Douglas-Escobar, M., & 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., & Weiss, M. D. (2015). Hypoxic-ischemic encephalopathy: A review for the clinician. *JAMA Pediatrics, 169*(4), 397-403. doi:10.1001/jamapediatrics.2014.3269

Drommelschmidt, K., Serdar, M., Bendix, I., Herz, J., Bertling, F., Prager, S., . . . Felderhoff-Muser, U. (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*(1-2), 1-11. doi:10.1016/j.jns.2015.03.014

El Omar, R., Beroud, J., Stoltz, J. F., Menu, P., Velot, E., & Decot, V. (2014). Umbilical cord mesenchymal stem cells: the new gold standard for mesenchymal stem cell-based therapies? *Tissue Eng Part B Rev, 20*(5), 523-544. doi:10.1089/ten.TEB.2013.0664

Emery, B. (2010). Regulation of oligodendrocyte differentiation and myelination. *Science, 330*(6005), 779-782. doi:10.1126/science.1190927

Favrais, G., van de Looij, Y., Fleiss, B., Ramanantsoa, N., Bonnin, P., Stoltenburg-Didinger, G., . . . Gressens, P. (2011). Systemic inflammation disrupts the developmental program of white matter. *Annals of Neurology, 70*(4), 550-565. doi:10.1002/ana.22489

Freeman, M. R., & Rowitch, D. H. (2013). Evolving concepts of gliogenesis: a look way back and ahead to the next 25 years. *Neuron, 80*(1097-4199 (Electronic)), 613-623. doi:10.1016/j.neuron.2013.10.034

Fyfe, K. L., Yiallourou, S. R., Wong, F. Y., & Horne, R. S. (2014). The development of cardiovascular and cerebral vascular control in preterm infants. *Sleep Med Rev, 18*(4), 299-310. doi:10.1016/j.smrv.2013.06.002

Garg, N., & Smith, T. W. (2015). An update on immunopathogenesis, diagnosis, and treatment of multiple sclerosis. *Brain Behav, 5*(9), e00362. doi:10.1002/brb3.362

Genc, B., Bozan, H. R., Genc, S., & Genc, K. (2018). Stem Cell Therapy for Multiple Sclerosis. *Adv Exp Med Biol*. doi:10.1007/5584_2018_247

Gharibi, T., Ahmadi, M., Seyfizadeh, N., Jadidi-Niaragh, F., & Yousefi, M. (2015). Immunomodulatory characteristics of mesenchymal stem cells and their role in the treatment of multiple sclerosis. *Cell Immunol, 293*(2), 113-121. doi:10.1016/j.cellimm.2015.01.002

Gluckman, P., Klempt, N., Guan, J., Mallard, C., Sirimanne, E., Dragunow, M., . . . Nikolics, K. (1992). A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun, 182*(2), 593-599. doi:10.1016/0006-291x(92)91774-k

Gu, Y., He, M., Zhou, X., Liu, J., Hou, N., Bin, T., . . . Chen, J. (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., . . . Chen, J. (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*(1), 65. doi:10.1186/s13041-015-0157-3

Gutierrez-Fernandez, M., Rodriguez-Frutos, B., Ramos-Cejudo, J., Otero-Ortega, L., Fuentes, B., & Diez-Tejedor, E. (2013a). Stem cells for brain repair and recovery after stroke. *Expert Opin Biol Ther, 13*(11), 1479-1483. doi:10.1517/14712598.2013.824420

Gutierrez-Fernandez, M., Rodriguez-Frutos, B., Ramos-Cejudo, J., Teresa Vallejo-Cremades, M., Fuentes, B., Cerdan, S., & Diez-Tejedor, E. (2013b). 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*(1), 11. doi:10.1186/scrt159

Hagberg, H., Gressens, P., & Mallard, C. (2012). Inflammation during fetal and neonatal life: implications for neurologic and neuropsychiatric disease in children and adults. *Ann Neurol, 71*(4), 444-457. doi:10.1002/ana.22620

Hayakawa, K., & Lo, E. H. (2016). Brain-peripheral cell crosstalk in white matter damage and repair. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1862*(5), 901-908. doi:10.1016/j.bbadis.2015.08.006

Herz, J., Koster, C., Reinboth, B. S., Dzietko, M., Hansen, W., Sabir, H., . . . Felderhoff-Muser, U. (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., & Van Gool, S. W. (2013). Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. *Immunol Cell Biol, 91*(1), 32-39. doi:10.1038/icb.2012.64

Jadasz, J. J., Aigner, L., Rivera, F. J., & Kury, P. (2012). The remyelination Philosopher's Stone: stem and progenitor cell therapies for multiple sclerosis. *Cell Tissue Res, 349*(1), 331-347. doi:10.1007/s00441-012-1331-x

Jadasz, J. J., Kremer, D., Gottle, P., Tzekova, N., Domke, J., Rivera, F. J., . . . Kury, P. (2013). Mesenchymal stem cell conditioning promotes rat oligodendroglial cell maturation. *PLoS One, 8*(8), e71814. doi:10.1371/journal.pone.0071814

Jakovcevski, I., Filipovic, R., Mo, Z., Rakic, S., & 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., . . . Martinez, S. (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., . . . Kramer, B. W. (2013). Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia. *PLoS One, 8*(8), e73031. doi:10.1371/journal.pone.0073031

Jiang, Q., Zhang, Z. G., Ding, G. L., Silver, B., Zhang, L., Meng, H., . . . Chopp, M. (2006). MRI detects white matter reorganization after neural progenitor cell treatment of stroke. *NeuroImage, 32*(3), 1080-1089. doi:10.1016/j.neuroimage.2006.05.025

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

Johnson, S., Fawke, J., Hennessy, E., Rowell, V., Thomas, S., Wolke, D., & Marlow, N. (2009). Neurodevelopmental disability through 11 years of age in children born before 26 weeks of gestation. *Pediatrics, 124*(2), e249-257. doi:10.1542/peds.2008-3743

Kalaszczynska, I., & 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., . . . Karussis, D. (2008). Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. *Arch Neurol, 65*(6), 753-761. doi:10.1001/archneur.65.6.753

Kassis, I., Vaknin-Dembinsky, A., & Karussis, D. (2011). Bone marrow mesenchymal stem cells: agents of immunomodulation and neuroprotection. *Curr Stem Cell Res Ther, 6*(1), 63-68. doi:10.2174/157488811794480762

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., & Richardson, W. D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat Neurosci, 9*(2), 173-179. doi:10.1038/nn1620

Khwaja, O., & Volpe, J. J. (2008). Pathogenesis of cerebral white matter injury of prematurity. *Archives of Disease in Childhood: Fetal & Neonatal Edition, 93*(2), F153-161. doi:10.1136/adc.2006.108837

Kim, E. H., Kim, D. H., Kim, H. R., Kim, S. Y., Kim, H. H., & Bang, O. Y. (2016). Stroke Serum Priming Modulates Characteristics of Mesenchymal Stromal Cells by Controlling the Expression miRNA-20a. *Cell Transplant, 25*(8), 1489-1499. doi:10.3727/096368916×690430

Kim, E. S., Ahn, S. Y., Im, G. H., Sung, D. K., Park, Y. R., Choi, S. H., . . . Park, W. S. (2012). Human umbilical cord blood–derived mesenchymal stem cell transplantation attenuates severe brain injury by permanent middle cerebral artery occlusion in newborn rats. *Pediatric Research, 72*, 277. doi:10.1038/pr.2012.71

Kirton, A., & deVeber, G. (2009). Advances in perinatal ischemic stroke. *Pediatr Neurol, 40*(3), 205-214. doi:10.1016/j.pediatrneurol.2008.09.018

Knuesel, I., Chicha, L., Britschgi, M., Schobel, S. A., Bodmer, M., Hellings, J. A., . . . Prinssen, E. P. (2014). Maternal immune activation and abnormal brain development across CNS disorders. *Nat Rev Neurol, 10*(11), 643-660. doi:10.1038/nrneurol.2014.187

Kobolak, J., Dinnyes, A., Memic, A., Khademhosseini, A., & 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., . . . Hamada, H. (2005). Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. *Mol Ther, 11*(1), 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), 1-7. doi:10.15283/ijsc.2008.1.1.1

Lakshminrusimha, S., Manja, V., Mathew, B., & Suresh, G. K. (2015). Oxygen targeting in preterm infants: a physiological interpretation. *J Perinatol, 35*(1), 8-15. doi:10.1038/jp.2014.199

Laroni, A., Novi, G., Kerlero de Rosbo, N., & Uccelli, A. (2013). Towards clinical application of mesenchymal stem cells for treatment of neurological diseases of the central nervous system. *J Neuroimmune Pharmacol, 8*(5), 1062-1076. doi:10.1007/s11481-013-9456-6

Larroque, B., Ancel, P. Y., Marret, S., Marchand, L., Andre, M., Arnaud, C., . . . Kaminski, M. (2008). Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study. *The Lancet, 371*(9615), 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., . . . Gutierrez-Fernandez, M. (2018). Therapeutic potential of extracellular vesicles derived from human mesenchymal stem cells in a model of progressive multiple sclerosis. *PLoS One, 13*(9), e0202590. doi:10.1371/journal.pone.0202590

Le Blon, D., Guglielmetti, C., Hoornaert, C., Quarta, A., Daans, J., Dooley, D., . . . Ponsaerts, P. (2016). Intracerebral transplantation of interleukin 13-producing mesenchymal stem cells limits microgliosis, oligodendrocyte loss and demyelination in the cuprizone mouse model. *J Neuroinflammation, 13*(1), 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*(5), 449-455. doi:10.4132/jptm.2017.07.25

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Allison, B. J., . . . Miller, S. L. (2017). Term vs. preterm cord blood cells for the prevention of preterm brain injury. *Pediatr Res, 82*(6), 1030-1038. doi:10.1038/pr.2017.170

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., . . . Miller, S. L. (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., . . . Miller, S. L. (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., & Lian, Q. (2014). Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant, 23*(9), 1045-1059. doi:10.3727/096368913×667709

Lin, H. Y., Liou, C. W., Chen, S. D., Hsu, T. Y., Chuang, J. H., Wang, P. W., . . . Chuang, Y. C. (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., & Marlow, N. (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*(4), 363-370. doi:10.1136/archdischild-2017-313414

Liu, A. M., Lu, G., Tsang, K. S., Li, G., Wu, Y., Huang, Z. S., . . . Poon, W. S. (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*(2), 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., . . . Xu, Y. (2013). Human umbilical cord stem cells ameliorate experimental autoimmune encephalomyelitis by regulating immunoinflammation and remyelination. *Stem Cells Dev, 22*(7), 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., . . . Li, H. L. (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*(1), 37-44. doi:10.1111/j.1365-2249.2009.03995.x

Lu, Z., Hu, X., Zhu, C., Wang, D., Zheng, X., & Liu, Q. (2009). Overexpression of CNTF in Mesenchymal Stem Cells reduces demyelination and induces clinical recovery in experimental autoimmune encephalomyelitis mice. *J Neuroimmunol, 206*(1-2), 58-69. doi:10.1016/j.jneuroim.2008.10.014

Lv, B., Li, F., Han, J., Fang, J., Xu, L., Sun, C., . . . Jiang, X. (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., & Pell, J. P. (2010). Gestational age at delivery and special educational need: retrospective cohort study of 407,503 schoolchildren. *PLoS Med, 7*(6), e1000289. doi:10.1371/journal.pmed.1000289

Mahrouf-Yorgov, M., Augeul, L., Da Silva, C. C., Jourdan, M., Rigolet, M., Manin, S., . . . Rodriguez, A. M. (2017). Mesenchymal stem cells sense mitochondria released from damaged cells as danger signals to activate their rescue properties. *Cell Death Differ, 24*(7), 1224-1238. doi:10.1038/cdd.2017.51

Mifsud, G., Zammit, C., Muscat, R., Di Giovanni, G., & Valentino, M. (2014). Oligodendrocyte Pathophysiology and Treatment Strategies in Cerebral Ischemia. *CNS Neuroscience & Therapeutics, 20*(7), 603-612. doi:doi:10.1111/cns.12263

Mitew, S., Hay, C. M., Peckham, H., Xiao, J., Koenning, M., & 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., . . . Uccelli, A. (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*(1), 3. doi:10.1186/scrt94

Morioka, C., Komaki, M., Taki, A., Honda, I., Yokoyama, N., Iwasaki, K., . . . Morita, I. (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., & Markestad, T. (2008). Long-term medical and social consequences of preterm birth. *New England Journal of Medicine, 359*(3), 262-273. doi:10.1056/NEJMoa0706475

Mueller, M., Oppliger, B., Joerger-Messerli, M., Reinhart, U., Barnea, E., Paidas, M., . . . Schoeberlein, A. (2017). Wharton's Jelly Mesenchymal Stem Cells Protect the Immature Brain in Rats and Modulate Cell Fate. *Stem Cells Dev, 26*(4), 239-248. doi:10.1089/scd.2016.0108

Mukai, T., Mori, Y., Shimazu, T., Takahashi, A., Tsunoda, H., Yamaguchi, S., . . . Nagamura-Inoue, T. (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., . . . Okada, T. (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., . . . Stangel, M. (2013). Effects of murine and human bone marrow-derived mesenchymal stem cells on cuprizone induced demyelination. *PLoS One, 8*(7), e69795. doi:10.1371/journal.pone.0069795

Nijboer, C. H., Kooijman, E., van Velthoven, C. T., van Tilborg, E., Tiebosch, I. A., Eijkelkamp, N., . . . Heijnen, C. J. (2018). Intranasal Stem Cell Treatment as a Novel Therapy for Subarachnoid Hemorrhage. *Stem Cells Dev, 27*(5), 313-325. doi:10.1089/scd.2017.0148

Ophelders, D. R., Wolfs, T. G., Jellema, R. K., Zwanenburg, A., Andriessen, P., Delhaas, T., . . . Kramer, B. W. (2016). Mesenchymal Stromal Cell-Derived Extracellular Vesicles Protect the Fetal Brain After Hypoxia-Ischemia. *Stem Cells Transl Med, 5*(6), 754-763. doi:10.5966/sctm.2015-0197

Oppliger, B., Joerger-Messerli, M., Mueller, M., Reinhart, U., Schneider, P., Surbek, D. V., & Schoeberlein, A. (2016). Intranasal Delivery of Umbilical Cord-Derived Mesenchymal Stem Cells Preserves Myelination in Perinatal Brain Damage. *Stem Cells and Development, 25*(16), 1234-1242. doi:10.1089/scd.2016.0027

Oppliger, B., Joerger-Messerli, M. S., Simillion, C., Mueller, M., Surbek, D. V., & 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*(7), 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., . . . Diez-Tejedor, E. (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., . . . Gutierrez-Fernandez, M. (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., & Mohanty, S. (2018). Regenerative abilities of mesenchymal stem cells through mitochondrial transfer. *J Biomed Sci, 25*(1), 31. doi:10.1186/s12929-018-0429-1

Panda, S., Dohare, P., Jain, S., Parikh, N., Singla, P., Mehdizadeh, R., . . . Ballabh, P. (2018). Estrogen Treatment Reverses Prematurity-Induced Disruption in Cortical Interneuron Population. *J Neurosci, 38*(34), 7378-7391. doi:10.1523/jneurosci.0478-18.2018

Park, D., Lee, S. H., Bae, D. K., Yang, Y. H., Yang, G., Kyung, J., . . . Kim, Y. B. (2013). Transplantation of Human Adipose Tissue-Derived Mesenchymal Stem Cells Restores the Neurobehavioral Disorders of Rats With Neonatal Hypoxic-Ischemic Encephalopathy. *Cell Med, 5*(1), 17-28. doi:10.3727/215517913×658936

Park, W. S., Ahn, S. Y., Sung, S. I., Ahn, J. Y., & Chang, Y. S. (2017). Mesenchymal Stem Cells: The Magic Cure for Intraventricular Hemorrhage? *Cell Transplant, 26*(3), 439-448. doi:10.3727/096368916×694193

Park, W. S., Ahn, S. Y., Sung, S. I., Ahn, J. Y., & Chang, Y. S. (2018). Strategies to enhance paracrine potency of transplanted mesenchymal stem cells in intractable neonatal disorders. *Pediatr Res, 83*(1-2), 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., . . . Chang, Y. S. (2016). Optimal Timing of Mesenchymal Stem Cell Therapy for Neonatal Intraventricular Hemorrhage. *Cell Transplant, 25*(6), 1131-1144. doi:10.3727/096368915×689640

Park, W. S., Sung, S. I., Ahn, S. Y., Yoo, H. S., Sung, D. K., Im, G. H., . . . Chang, Y. S. (2015). Hypothermia augments neuroprotective activity of mesenchymal stem cells for neonatal hypoxic-ischemic encephalopathy. *PLoS One, 10*(3), e0120893. doi:10.1371/journal.pone.0120893

Paton, M. C. B., Allison, B. J., Fahey, M. C., Li, J., Sutherland, A. E., Pham, Y., . . . McDonald, C. A. (2019). Umbilical cord blood versus mesenchymal stem cells for inflammation-induced preterm brain injury in fetal sheep. *Pediatric Research*. doi:10.1038/s41390-019-0366-z

Paton, M. C. B., Allison, B. J., Li, J., Fahey, M. C., Sutherland, A. E., Nitsos, I., . . . Miller, S. L. (2018). Human Umbilical Cord Blood Therapy Protects Cerebral White Matter from Systemic LPS Exposure in Preterm Fetal Sheep. *Dev Neurosci, 40*(3), 258-270. doi:10.1159/000490943

Paton, M. C. B., McDonald, C. A., Allison, B. J., Fahey, M. C., Jenkin, G., & Miller, S. L. (2017). Perinatal Brain Injury As a Consequence of Preterm Birth and Intrauterine Inflammation: Designing Targeted Stem Cell Therapies. *Frontiers in Neuroscience, 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., & Wilson-Costello, D. E. (2013). Neurodevelopmental outcomes of extremely low-gestational-age neonates with low-grade periventricular-intraventricular hemorrhage. *JAMA Pediatr, 167*(5), 451-459. doi:10.1001/jamapediatrics.2013.866

Perrone, S., Tataranno, L. M., Stazzoni, G., Ramenghi, L., & 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., & 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*(3), 351-358. doi:10.1089/scd.2009.0049

Redondo-Castro, E., Cunningham, C., Miller, J., Martuscelli, L., Aoulad-Ali, S., Rothwell, N. J., . . . Pinteaux, E. (2017). Interleukin-1 primes human mesenchymal stem cells towards an anti-inflammatory and pro-trophic phenotype in vitro. *Stem Cell Res Ther, 8*(1), 79. doi:10.1186/s13287-017-0531-4

Rezaie, P., & Dean, A. (2002). Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. *Neuropathology, 22*(3), 106-132. doi:10.1046/j.1440-1789.2002.00438.x

Rivera, F. J., & Aigner, L. (2012). Adult mesenchymal stem cell therapy for myelin repair in multiple sclerosis. *Biol Res, 45*(3), 257-268. doi:10.4067/s0716-97602012000300007

Rivera, F. J., Couillard-Despres, S., Pedre, X., Ploetz, S., Caioni, M., Lois, C., . . . Aigner, L. (2006). Mesenchymal stem cells instruct oligodendrogenic fate decision on adult neural stem cells. *Stem Cells, 24*(10), 2209-2219. doi:10.1634/stemcells.2005-0614

Robinson, S., Li, Q., Dechant, A., & 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

Salmaso, N., Jablonska, B., Scafidi, J., Vaccarino, F. M., & Gallo, V. (2014). Neurobiology of premature brain injury. *Nature Neuroscience, 17*(3), 341-346. doi:10.1038/nn.3604

Sarmah, D., Agrawal, V., Rane, P., Bhute, S., Watanabe, M., Kalia, K., . . . Bhattacharya, P. (2018). Mesenchymal Stem Cell Therapy in Ischemic Stroke: A Meta-analysis of Preclinical Studies. *Clin Pharmacol Ther, 103*(6), 990-998. doi:10.1002/cpt.927

Schäfer, R., Spohn, G., & Baer, P. C. (2016). Mesenchymal Stem/Stromal Cells in Regenerative Medicine: Can Preconditioning Strategies Improve Therapeutic Efficacy. *Transfusion Medicine and Hemotherapy, 43*(4), 256-267. doi:10.1159/000447458

Scolding, N. J., Pasquini, M., Reingold, S. C., & Cohen, J. A. (2017). Cell-based therapeutic strategies for multiple sclerosis. *Brain, 140*(11), 2776-2796. doi:10.1093/brain/awx154

Scruggs, B. A., Semon, J. A., Zhang, X., Zhang, S., Bowles, A. C., Pandey, A. C., . . . Bunnell, B. A. (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*(10), 797-807. doi:10.5966/sctm.2013-0026

Segovia, K. N., McClure, M., Moravec, M., Luo, N. L., Wan, Y., Gong, X., . . . Back, S. A. (2008). Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann Neurol, 63*(4), 520-530. doi:10.1002/ana.21359

Silbereis, J. C., Huang, E. J., Back, S. A., & Rowitch, D. H. (2010). Towards improved animal models of neonatal white matter injury associated with cerebral palsy. *Dis Model Mech, 3*(11-12), 678-688. doi:10.1242/dmm.002915

Singh, D. K., Ling, E. A., & Kaur, C. (2018). Hypoxia and myelination deficits in the developing brain. *Int J Dev Neurosci*. doi:10.1016/j.ijdevneu.2018.06.012

Steffenhagen, C., Dechant, F. X., Oberbauer, E., Furtner, T., Weidner, N., Kury, P., . . . Rivera, F. J. (2012). Mesenchymal stem cells prime proliferating adult neural progenitors toward an oligodendrocyte fate. *Stem Cells Dev, 21*(11), 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., . . . Schwartz, N. E. (2016). Clinical Outcomes of Transplanted Modified Bone Marrow-Derived Mesenchymal Stem Cells in Stroke: A Phase 1/2a Study. *Stroke, 47*(7), 1817-1824. doi:10.1161/strokeaha.116.012995

Stewart, A., Tekes, A., Huisman, T. A., Jennings, J. M., Allen, M. C., Northington, F. J., . . . Graham, E. M. (2013). Glial fibrillary acidic protein as a biomarker for periventricular white matter injury. *Am J Obstet Gynecol, 209*(1), 27.e21-27. 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., . . . Higgins, R. D. (2015). Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm Neonates, 1993-2012. *Jama, 314*(10), 1039-1051. doi:10.1001/jama.2015.10244

Stolzing, A., Jones, E., McGonagle, D., & Scutt, A. (2008). Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech Ageing Dev, 129*(3), 163-173. doi:10.1016/j.mad.2007.12.002

Sugiyama, Y., Sato, Y., Kitase, Y., Suzuki, T., Kondo, T., Mikrogeorgiou, A., . . . Hayakawa, M. (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., & Wei, L. (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., . . . Yang, G. Y. (2014). Melatonin Pretreatment Improves the Survival and Function of Transplanted Mesenchymal Stem Cells after Focal Cerebral Ischemia. *Cell Transplant, 23*(10), 1279-1291. doi:10.3727/096368913×667510

Thompson, A. J., Baranzini, S. E., Geurts, J., Hemmer, B., & Ciccarelli, O. (2018). Multiple sclerosis. *The Lancet, 391*(10130), 1622-1636. doi:10.1016/s0140-6736(18)30481-1

Tibrewal, M., Cheng, B., Dohare, P., Hu, F., Mehdizadeh, R., Wang, P., . . . Ballabh, P. (2018). Disruption of Interneuron Neurogenesis in Premature Newborns and Reversal with Estrogen Treatment. *J Neurosci, 38*(5), 1100-1113. doi:10.1523/jneurosci.1875-17.2017

Toyoshima, A., Yasuhara, T., & Date, I. (2017). Mesenchymal Stem Cell Therapy for Ischemic Stroke. *Acta Med Okayama, 71*(4), 263-268. doi:10.18926/amo/55302

Uccelli, A., Frassoni, F., & Mancardi, G. (2007). Stem cells for multiple sclerosis: promises and reality. *Regen Med, 2*(1), 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., . . . Nijboer, C. H. (2018). Origin and dynamics of oligodendrocytes in the developing brain: Implications for perinatal white matter injury. *Glia, 66*(2), 221-238. doi:10.1002/glia.23256

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., & Nijboer, C. H. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Progress in Neurobiology, 136*(1873-5118 (Electronic)), 28-49. doi:10.1016/j.pneurobio.2015.11.002

van Velthoven, C. T., Braccioli, L., Willemen, H. L., Kavelaars, A., & Heijnen, C. J. (2014). Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage. *Mol Ther, 22*(3), 645-654. doi:10.1038/mt.2013.260

van Velthoven, C. T., Dzietko, M., Wendland, M. F., Derugin, N., Faustino, J., Heijnen, C. J., . . . Vexler, Z. S. (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*(5), 1225-1236. doi:10.1002/jnr.23954

van Velthoven, C. T., Kavelaars, A., & 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., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010a). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain, Behavior and Immunity, 24*(3), 387-393. doi:10.1016/j.bbi.2009.10.017

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010b). Nasal administration of stem cells: a promising novel route to treat neonatal ischemic brain damage. *Pediatric Research, 68*(5), 419-422. doi:10.1203/PDR.0b013e3181f1c289

van Velthoven, C. T., Kavelaars, A., van Bel, F., & 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*(28), 9603-9611. doi:10.1523/jneurosci.1835-10.2010

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2011). Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. *Brain, Behavoir and Immunity, 25*(7), 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., . . . Ferriero, D. M. (2013). Mesenchymal stem cell transplantation attenuates brain injury after neonatal stroke. *Stroke, 44*(5), 1426-1432. doi:10.1161/strokeaha.111.000326

van Velthoven, C. T., van de Looij, Y., Kavelaars, A., Zijlstra, J., van Bel, F., Huppi, P. S., . . . Heijnen, C. J. (2012b). Mesenchymal stem cells restore cortical rewiring after neonatal ischemia in mice. *Ann Neurol, 71*(6), 785-796. doi:10.1002/ana.23543

Verney, C., Pogledic, I., Biran, V., Adle-Biassette, H., Fallet-Bianco, C., & 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*(3), 251-264. doi:10.1097/NEN.0b013e3182496429

Viscardi, R. M., Muhumuza, C. K., Rodriguez, A., Fairchild, K. D., Sun, C. C., Gross, G. W., . . . Hasday, J. D. (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*(6), 1009-1017. doi:10.1203/01.pdr.0000127015.60185.8a

Volkman, R., & Offen, D. (2017). Concise Review: Mesenchymal Stem Cells in Neurodegenerative Diseases. *Stem Cells, 35*(8), 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 Neurology, 8*(1), 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., & Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *International Journal of Developmental Neuroscience, 29*(4), 423-440. doi:10.1016/j.ijdevneu.2011.02.012

von Bahr, L., Batsis, I., Moll, G., Hagg, M., Szakos, A., Sundberg, B., . . . Le Blanc, K. (2012). Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. *Stem Cells, 30*(7), 1575-1578. doi:10.1002/stem.1118

Wagenaar, N., de Theije, C. G. M., de Vries, L. S., Groenendaal, F., Benders, M., & Nijboer, C. H. A. (2018). Promoting neuroregeneration after perinatal arterial ischemic stroke: neurotrophic factors and mesenchymal stem cells. *Pediatric Research, 83*(1-2), 372-384. doi:10.1038/pr.2017.243

Wang, L. Q., Lin, Z. Z., Zhang, H. X., Shao, B., Xiao, L., Jiang, H. G., . . . Jin, K. L. (2014). Timing and dose regimens of marrow mesenchymal stem cell transplantation affect the outcomes and neuroinflammatory response after ischemic stroke. *CNS Neuroscience & Therapeutics, 20*(4), 317-326. doi:10.1111/cns.12216

Wei, N., Yu, S. P., Gu, X., Taylor, T. M., Song, D., Liu, X.-F., & Wei, L. (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*(6), 977-991. doi:10.3727/096368912X657251

Wei, Z. Z., Gu, X., Ferdinand, A., Lee, J. H., Ji, X., Ji, X. M., . . . Wei, L. (2015). Intranasal delivery of bone marrow mesenchymal stem cells improved neurovascular regeneration and rescued neuropsychiatric deficits after neonatal stroke in rats. *Cell Transplant, 24*(3), 391-402. doi:10.3727/096368915×686887

Xiao, J., Yang, R., Biswas, S., Qin, X., Zhang, M., & Deng, W. (2015). Mesenchymal stem cells and induced pluripotent stem cells as therapies for multiple sclerosis. *Int J Mol Sci, 16*(5), 9283-9302. doi:10.3390/ijms16059283

Xiao, Y., Geng, F., Wang, G., Li, X., Zhu, J., & 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

Xue, P., Wang, M., & 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., . . . Wang, X. (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., & Ahmed, M. (2017). Pathophysiology of periventricular leukomalacia: What we learned from animal models. *Neural Regen Res, 12*(11), 1795-1796. doi:10.4103/1673-5374.219034

Zappia, E., Casazza, S., Pedemonte, E., Benvenuto, F., Bonanni, I., Gerdoni, E., . . . Uccelli, A. (2005). Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood, 106*(5), 1755-1761. doi:10.1182/blood-2005-04-1496

Zeddou, M., Briquet, A., Relic, B., Josse, C., Malaise, M. G., Gothot, A., . . . Beguin, Y. (2010). The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. *Cell Biol Int, 34*(7), 693-701. doi:10.1042/cbi20090414

Zhang, H. L., Xie, X. F., Xiong, Y. Q., Liu, S. M., Hu, G. Z., Cao, W. F., & Wu, X. M. (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., . . . Chopp, M. (2005). Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. *Experimental Neurology, 195*(1), 16-26. doi:10.1016/j.expneurol.2005.03.018

Zhang, Z., Li, Z., Deng, W., He, Q., Wang, Q., Shi, W., . . . Xu, X. (2016). Ectoderm mesenchymal stem cells promote differentiation and maturation of oligodendrocyte precursor cells. *Biochem Biophys Res Commun, 480*(4), 727-733. doi:10.1016/j.bbrc.2016.10.115

Zhao, J., Chen, Y., Xu, Y., & Pi, G. (2013). Effect of intrauterine infection on brain development and injury. *International Journal of Developmental Neuroscience, 31*(7), 543-549. doi:10.1016/j.ijdevneu.2013.06.008

Zhu, L. H., Bai, X., Zhang, N., Wang, S. Y., Li, W., & 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

4

The impact of trophic and immunomodulatory factors on oligodendrocyte maturation: potential treatments for encephalopathy of prematurity

Josine E.G. Vaes*, Myrna J.V. Brandt*, Nikki Wanders, Manon J.N.L. Benders, Caroline G.M. de Theije, Pierre Gressens, Cora H. Nijboer

* Both authors contributed equally to this manuscript and share the first authorship.

Published in GLIA, 2021 Jun, 69, 6

ABSTRACT

Encephalopathy of Prematurity (EoP) is a major cause of morbidity in preterm neonates, causing neurodevelopmental adversities that can lead to lifelong impairments. Preterm birth-related insults, such as cerebral oxygen fluctuations and perinatal inflammation, are believed to negatively impact brain development, leading to a range of brain abnormalities. Diffuse white matter injury (dWMI) is a major hallmark of EoP and characterized by widespread hypomyelination, the result of disturbances in oligodendrocyte lineage development. At present, there are no treatment options available, despite the enormous burden of EoP on patients, their families, and society. Over the years, research in the field of neonatal brain injury and other white matter pathologies has led to the identification of several promising trophic factors and cytokines that contribute to the survival and maturation of oligodendrocytes, and/or dampening neuroinflammation. In this review, we discuss the current literature on selected factors and their therapeutic potential to combat EoP, covering a wide range of *in vitro*, pre-clinical and clinical studies. Furthermore, we offer a future perspective on the translatability of these factors into clinical practice.

INTRODUCTION

Worldwide, approximately 10% of live-born babies is born preterm, i.e. before 37 weeks of gestation. Preterm birth can be subdivided into extremely preterm (< 28 weeks), very preterm (28 - <32 weeks) and moderate or late preterm birth (32 - <37 weeks; Blencowe et al., 2012) and is associated with multiple neurodevelopmental morbidities, ranging from motor problems and cognitive impairments to an increased risk of psychiatric disorders. The risk of neurological consequences of preterm birth is inversely correlated with gestational age, meaning that extreme preterm infants are most at risk (Deng, 2010; Larroque et al., 2008; Linsell et al., 2018; MacKay et al., 2010; Moster et al., 2008).

Neurodevelopmental morbidities after preterm birth are thought to arise from *Encephalopathy of Prematurity* (EoP), an umbrella term used to describe the brain abnormalities that result from impeded brain development due to preterm birth-related complications (Volpe, 2009a). The most prominent hallmark of EoP is white matter injury (WMI), however, neuronal and axonal deficits, such as GABAergic interneuron maldevelopment, have received growing attention over the years (Panda et al., 2018; Stolp et al., 2019; Volpe, 2009b). Preterm WMI is a collective term referring to a spectrum of pathological changes in the developing white matter and is often classified based on neuropathological findings, such as (small) necrotic lesions (Back, 2017; Volpe, 2017). As a result of advances in supportive care, diffuse white matter injury (dWMI), characterized by global hypomyelination without focal necrosis, is currently the most prevalent form of preterm WMI with reported prevalence rates up to 80% in all affected preterm neonates (Back, 2017; Back & Miller, 2014; Schneider & Miller, 2019). Histopathological findings in dWMI display injured immature oligodendrocytes, along with astrocytosis and microgliosis. Consequently, a deficit of mature myelinating oligodendrocytes is observed, leading to a reduction in axonal myelination (Buser et al., 2012; Lee, 2017; Schneider & Miller, 2019; van Tilborg et al., 2016; Volpe et al., 2011). Due to the key role of dWMI in EoP, this review will focus primarily on potential treatments aimed at restoration of *white matter* development.

Although treatment options for dWMI in preterm infants are currently limited, research in the last decade has created larger understanding of the pathophysiology underlying myelination failure, pinpointing impaired oligodendrocyte maturation as an critical target for therapeutic intervention. Experimental research has identified

several growth factors and cytokines that play essential roles in healthy white matter development or that boost myelination in other (adult) white matter pathologies, such as multiple sclerosis (MS), (neonatal) stroke and traumatic brain injury (TBI). Despite the evident differences in pathophysiology - most of the mentioned diseases are characterized by demyelination of existing white matter tracts while dWMI is associated with impaired myelin formation-, there are some overlapping characteristics, such as insufficient oligodendrocyte maturation and the occurrence of neuroinflammation. Thus, knowledge from previous research in the above-mentioned pathologies could aid in the refinement and identification of potential therapeutic strategies to restore white matter development in (extreme) preterm infants. Using a wide range of *in vivo* and *in vitro* studies, this review aims to integrate the current knowledge on a selection of trophic and immunomodulatory factors that boost oligodendrocyte maturation and white matter development, leading to the identification of potent therapeutic targets to combat preterm dWMI.

DEVELOPMENTAL WHITE MATTER (PATHO)PHYSIOLOGY

During normal human brain development, the formation of myelin sheaths by oligodendrocytes starts relatively late, at >32 weeks of gestation (Back et al., 2001). Before oligodendrocytes are able to produce myelin, the proliferation, migration and maturation of oligodendrocyte precursors has to be completed. This typically occurs in four stages: (1) oligodendrocyte precursor cells (OPCs) originate from differentiated neural stem cells (NSCs) in the ventral forebrain; (2) OPCs migrate throughout the brain and proliferate to increase their numbers; (3) at the site of destination, OPCs differentiate into pre-myelinating oligodendrocytes (pre-OLs), which are still non-myelinating cells until (4) differentiation-repressive factors are lifted and pre-OLs differentiate into post-mitotic, mature oligodendrocytes that produce myelin to enwrap axons (van Tilborg et al., 2016). Not all OPCs reach this end stage during brain development: a homeostatic pool of OPCs populates the brain to maintain regenerative capacity after oligodendrocyte damage throughout adulthood. Under physiological circumstances, oligodendrocyte maturation is aided by microglia and astrocytes, through the release of essential nutrients, proteins and cytokines (Hammond et al., 2018; Traiffort et al., 2020; Volpe, 2019). Interestingly, interneurons, other cells at risk in EoP pathophysiology, have been reported to regulate oligodendrocyte lineage development, by emitting pro-differentiation cues through transient synaptic input and secreted factors (Benamer et al., 2020; Zonouzi et al., 2015).

The particular vulnerability of white matter in prematurely born infants results from the fact that (extreme) preterm birth coincides with the initiation of oligodendrocyte lineage development. Especially between 24 and 30 weeks of gestation, the brain contains a large population of OPCs and pre-OLs, which are particularly vulnerable to insults associated with preterm birth (van Tilborg et al., 2016; Volpe, 2019). Two major preterm birth-related insults known to affect maturation of pre-OLs to myelinating oligodendrocytes are inflammation and oxygen fluctuations. Moreover, encountering multiple hits is reported to aggravate neonatal brain damage and worsen neurodevelopmental outcome (Brehmer et al., 2012; Volpe, 2019; Volpe et al., 2011).

Inflammation is estimated to occur in 65-79% of very low birth weight or extremely preterm infants, either caused by perinatal immune activation (such as intrauterine infection or maternal fever) or postnatal inflammation/infections (such as neonatal sepsis or necrotizing enterocolitis (NEC) of the immature bowel; Cappelletti et al., 2016; Volpe et al., 2011). After extremely preterm birth, oxygen fluctuations due to immature lungs and cardiovascular system (hypoxia) and/or the need for ventilation (hyperoxia) are also detrimental to the immature brain (Brehmer et al., 2012).

Inflammation induces the release of pro-inflammatory cytokines into the circulation which subsequently reach the immature brain (Li et al., 2017). This process triggers microglia, the resident immune cells of the brain, to shift to a pro-inflammatory (M1) phenotype, stimulating additional release of pro-inflammatory cytokines by these cells in the brain parenchyma (Li et al., 2017). The pro-inflammatory microglial shift may be sustained by multiple hits during pregnancy and following preterm birth (Li et al., 2017; Volpe, 2019). Pre-OLs possess a high amount of cytokine receptors, and are therefore particularly sensitive to the release of these cytokines in the immature brain, which causes apoptosis (Goldstein et al., 2016). Activated microglia cause further harm to immature oligodendrocytes by reducing their trophic factor support, and releasing excessive amounts of glutamate which causes excitotoxicity (Vaes et al., 2019; van Tilborg et al., 2016; Volpe et al., 2011). Similarly, astrocytes respond to inflammation by increased reactivity, during which astrocytes release growth factors that stimulate OPC proliferation but impair oligodendrocyte maturation (Back, 2017; Shiow et al., 2017; van Tilborg et al., 2016). As will become apparent in this review, neuroinflammation and myelination are complexly linked, since cytokines that are secreted during inflammation have a dual role in oligodendrocyte development (Goldstein et al., 2016).

Oxygen fluctuations such as hypoxia and/or hyperoxia can directly and indirectly induce apoptosis and necrosis, and contribute to accumulation of reactive oxygen species (ROS) in the brain (Brill et al., 2017; Li et al., 2017; Scheuer et al., 2015). Oligodendrocyte precursors are sensitive to the release of ROS from activated microglia, as they lack enzymes needed to counteract oxidative stress (Back, 2017; van Tilborg et al., 2016). Similarly, oligodendrocyte precursors express a large amount of glutamate receptors, which increases their susceptibility to the release of excess glutamate by microglia and the halted or reversed uptake of glutamate by astrocytes (Back, 2017; van Tilborg et al., 2016). However, astrocytes may also shift to a protective type A2 activation state in response to hypoxic conditions, during which they release neurotrophic factors that stimulate proliferation and survival of multiple cell types, among which oligodendrocytes and their precursors (Liddelow & Barres, 2017).

Together, preterm-birth related insults such as inflammation and oxygen fluctuations lead to a reduced number of mature oligodendrocytes and consequent myelin insufficiency in the preterm brain. It is under current debate whether survival of oligodendrocytes precursors is impaired as a result of neuroinflammation and oxygen fluctuations, or whether these hits cause arrested maturation of pre-OLs (Back, 2017; van Tilborg et al., 2016; Volpe, 2019). In fact, both cell death and arrested maturation of oligodendrocyte precursors may contribute to dWMI, as accumulation of ROS and cytokines leads to apoptosis particularly in pre-OLs, and oxygen fluctuations induce the upregulation of trophic factors that sustain OPC proliferation and survival, keeping them in an immature state (Liddelow & Barres, 2017; van Tilborg et al., 2016). Thus, reducing the impact of detrimental insults such as inflammation and oxygen fluctuations, plus providing trophic or immunomodulatory factors that stimulate oligodendrocyte maturation and myelination are two important strategies to reduce preterm dWMI.

THE ROLE OF GROWTH FACTORS AND CYTOKINES IN OLIGODENDROCYTE MATURATION AND NEUROINFLAMMATION

Although dWMI can lead to life-long neurological impairments, no therapeutic options to promote white matter development in the preterm brain are currently available. Future therapies would preferably stimulate oligodendrocyte lineage survival and maturation, increasing the proportion of mature, myelin-producing oligodendrocytes. Stimulation of differentiation could be achieved through growth factors and cytokines

that directly affect the oligodendrocyte lineage, boosting maturation and/or survival of oligodendrocyte precursors, or indirectly by modulating neuroinflammation, providing a more favorable intracerebral milieu for myelination (Figure 1). In this review we integrate the current knowledge on the role of promising growth factors and cytokines in healthy white matter development and oligodendrocyte maturation and neuroinflammation after injury from 1) *in vitro* and 2) *in vivo* animal models and 3) clinical studies, in order to identify potential therapeutic targets for preterm dWMI.

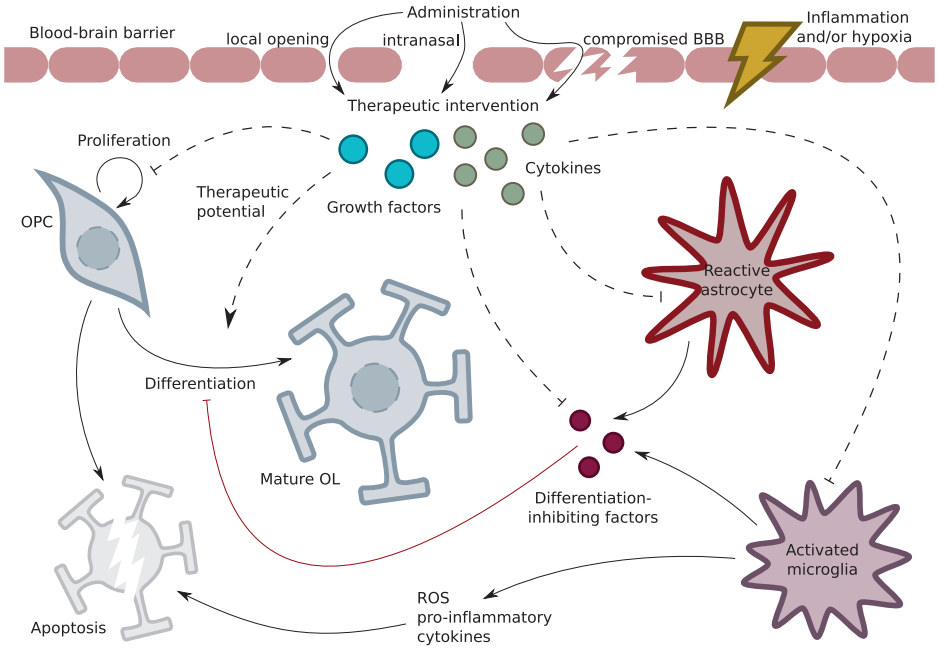


Figure 1. The underlying pathophysiology of EoP and proposed mechanism of therapeutic intervention. Preterm birth-related issues such as inflammation and hypoxia/hyperoxia (yellow flash) are thought to lead to oligodendrocyte (OL) death (apoptosis) and/or a maturational arrest of the oligodendrocyte lineage, through the activation of microglia (purple cell) and astrocyte (red cell) reactivity. Microglia release reactive oxygen species (ROS) and pro-inflammatory cytokines, which leads to the death of vulnerable oligodendrocyte precursors (OPCs). Reactive astrocytes respond to inflammation and/or oxygen fluctuations by reducing their uptake of glutamate and increasing the release of proliferative factors (red dots), that inhibit the differentiation of oligodendrocyte precursor cells (OPCs). The proposed therapeutic effect of growth factor- and cytokine-based treatments is indicated in dotted lines. This review summarizes growth factors (blue dots) and cytokines (green dots) that have the potential to stimulate oligodendrocyte maturation/differentiation and increase survival of these cells directly, while simultaneously inhibiting the harmful neuroimmune response caused by microglia and astrocytes. Several routes of administration are proposed, such as intranasal through a local opening in, or through the compromised blood-brain-barrier (BBB) in the acute phase after brain injury.

Insulin-like growth factor 1

A compelling quantity of experimental studies has provided evidence for the essential role of insulin-like growth factor 1 (IGF-1) in normal fetal white matter development and in regeneration following cerebral injury (D'Ercole & Ye, 2008; Guan et al., 2003; Huang & Dreyfus, 2016). The effects of IGF-1 are mediated by activation of the type I IGF receptor (IGF1R), broadly expressed on different cell types in the central nervous system (CNS), among which all cells of the oligodendrocyte lineage (D'Ercole & Ye, 2008; Zeger et al., 2007). IGF1R activation is thought to trigger the PI3kinase-Akt (PI3k/Akt) pathway, mitogen-activated protein kinase (MAPK) activation and mammalian target of rapamycin (mTOR) activation, driving mitogenesis, maturation and survival of oligodendrocytes (see Box 2; O'Kusky & Ye, 2012; Palacios et al., 2005; Wrigley et al., 2017).

Over the years, a wide range of *in vitro* studies have provided evidence of a direct effect of IGF-1 on proliferation and differentiation of healthy oligodendrocyte lineage cells, ultimately resulting in myelination (Barres et al., 1992; Masters et al., 1991; McMorris et al., 1986; Mozell & McMorris, 1991; Roth et al., 1995; Wilson et al., 2003). However, a study using OPCs derived from *human* fetal tissue did not find a proliferative response following IGF-1 administration, suggesting the mitogenic effect of IGF-1 may be more pronounced in rodents (Wilson et al., 2003). Other than its role in healthy oligodendrocyte development, multiple *in vitro* studies show that IGF-1 promotes differentiation and myelin production, and inhibits oligodendrocyte cell death when exposed to different WMI-associated stimuli (e.g. hypoxia or inflammation; Ness et al., 2004; Pang et al., 2007; Wood et al., 2007; Ye & D'Ercole, 1999).

In vivo studies underline the prominent role of IGF-1 in white matter development, as well as in injury repair. Transgenic mice lacking IGF-1 display a dramatic reduction in size of white matter structures, myelination and oligodendrocyte numbers (Beck et al., 1995; Ye et al., 2002; Zeger et al., 2007). In line with these findings, mice overexpressing IGF-1 show excessive oligodendrocyte numbers and myelin content (Carson et al., 1993; Popken et al., 2004; Ye et al., 1995). Exogenous IGF-1 treatment in animal models of neonatal brain injury has shown beneficial effects on oligodendrocyte differentiation, survival and myelination. The effectiveness of IGF-1 therapy in *term* hypoxic/ischemic encephalopathy (HIE) has been studied in both rodent and larger animal models using different routes of administration. Lin et al. (2009b) showed a significant improvement in myelination of the subcortical white matter after intrana-

sal IGF-1 treatment in a rat model of *near-term* HIE. Moreover, the authors report a mitogenic effect of IGF-1 administration based on an increase in proliferating NG2+ cells, a marker for OPCs. In a similar rat model, intracerebral IGF-1 treatment induced activation of Akt and pGSK3β, inhibiting activation of caspases, thereby reducing brain injury (Brywe et al., 2005). These results were confirmed in sheep models of *near-term* HIE, using intracerebral IGF-1 administration (Cao et al., 2003; Guan et al., 2001). In a rodent model of severe preterm WMI, where intracerebral LPS was injected, intranasal IGF-1 therapy was shown to reduce pre-oligodendrocyte (O4+) and mature oligodendrocyte (CC1+) loss, which resulted in myelin recovery (Cai et al., 2011). In these studies, the reported increases in total oligodendrocyte numbers after IGF-1 treatment were attributed to prevention of oligodendrocyte death, with a less prominent role for oligodendrocyte proliferation. Conversely, a previous study from this group showed conflicting outcomes of IGF-1 treatment, detecting both recovery and exacerbation of injury. Co-administration of intracerebral LPS and IGF-1 in a low dose reduced oligodendrocyte loss and myelin deficits, while higher doses of IGF-1 led to intracerebral hemorrhage and exacerbation of brain damage (Pang et al., 2010). These damaging effects were not observed following intranasal treatment with IGF-1 nor in any of the other aforementioned studies, implying that caution is only advised when locally injecting (very high) doses of IGF-1 in an acute inflammatory environment. However, intracerebral injection is a clinically unfeasible route of administration in the instable preterm infant. Although these models do not represent the commonly observed pattern of WMI in human preterm infants (i.e. dWMI) and/or use a rather invasive administration method of IGF-1, IGF-1 generally seems to have beneficial effects on the oligodendrocyte lineage.

Results from a handful *in vitro* studies indicate an immunoregulatory effect of IGF-1 directly on astrocytes and microglia, implying an indirect role of IGF-1 on pre-OL differentiation and survival by contributing to a more favorable environment by reducing microglia and astrocyte activation (Dodge et al., 2008; Genis et al., 2014; Grinberg et al., 2013). Grinberg et al. (2013) demonstrated a reduction in microglial ROS and tumor necrosis factor α (TNF-α) production following IGF-1 supplementation in rat hippocampal slice cultures. Astrocytic ROS and TNF-α production however were not reduced by IGF-1. In contrast, Genis et al. (2014) did demonstrate a decrease in ROS levels following IGF-1 treatment in astrocytic cultures, protecting the brain against oxidative injury.

Evidence for the immunomodulatory properties of IGF-1 obtained from *in vivo* studies seems inconclusive. In a sheep model *near-term* HIE intracerebral IGF-1 administration led to increased proliferation of (reactive) astrocytes and microglia. While reactive (micro)glia are traditionally associated with dWMI pathophysiology, the authors propose that the increased numbers of reactive glial cells after IGF-1 treatment are associated with improvement of white matter repair. It is suggested that the neuroprotective properties of reactive glia might be the result of paracrine signaling (Cao et al., 2003; Guan et al., 2001). Similar observations of potential pro-regenerative glial subtypes, particularly for astrocytes, have been reported in other studies (Du et al., 2017a; Liddelow & Barres, 2017; Zhou & Spittau, 2018). The possible role of reactive glia subtypes in perinatal brain injury remains unclear, though in the majority of studies (micro)glia activation is linked to exacerbation of brain injury and a poorer outcome (Baud & Saint-Faust, 2019; Del Bigio & Becker, 1994; Olivier et al., 2005; Verney et al., 2012). Intranasal IGF-1 administration in a rodent model of severe LPS-induced preterm WMI reduced microglia activation and peripheral immune cell infiltration, even though pro-inflammatory IL-1β and TNF-α concentrations remained unchanged. The authors hypothesize that the direct anti-inflammatory effect of IGF-1 is likely limited and that the observed attenuation of microglia activation and peripheral immune cell infiltration could be the result of reduced oligodendrocyte apoptosis (Cai et al., 2011). As mentioned previously, another study by this group showed exacerbation of brain injury after local co-injection of a high dose of IGF-1 and LPS. Even though a low dose of IGF-1 did provide protection against oligodendrocyte loss and myelination deficits, it failed to attenuate LPS-induced micro- and astrogliosis and was associated with an increase in peripheral immune cell infiltration. These effects were more pronounced in the higher doses of IGF-1, leading to profuse leukocyte infiltration and subsequent exacerbation of brain injury. The authors suggest that IGF-1 likely negatively affects blood-brain-barrier integrity while upregulating chemotactic signaling during episodes of acute inflammation (Pang et al., 2010).

Glial cells are an important source of local IGF-1 production during brain development. Endogenous microglial IGF-1 secretion was shown to be hampered *in vitro* following glutamate treatment in a primary microglial culture obtained from hypoxic rats, mimicking glutamate excitotoxicity in WMI (Sivakumar et al., 2010). These findings are supported by *in vivo* evidence demonstrating a decrease in IGF-1 gene expression in the ipsilateral hemisphere following an hypoxic-ischemic insult in near-term rats (Lee et al., 1996). Interestingly, this decrease in local IGF-1 secretion seems to be

distinctive for the neonatal period, as hypoxia/ischemia in older animals leads to a local upregulation IGF-1 (Gluckman et al., 1992; Lee et al., 1992). These studies emphasize the potential need for IGF-1 supplementation following perinatal hits (Lee et al., 1996; Sivakumar et al., 2010).

Interestingly, evidence from human studies indicated a reduction in circulatory IGF-1 in the first weeks following extreme preterm birth due to inadequate endogenous IGF-1 production. This relative IGF-1 deficiency during a developmental time-window analogous to the third trimester of pregnancy has been associated with a poorer neurodevelopmental outcome, as well as with retinopathy of prematurity (ROP) severity and respiratory complications (Hansen-Pupp et al., 2013; Hellstrom et al., 2016). These observations, along with the promising preclinical data instigated the first feasibility and pharmacokinetics studies using IGF-1 administration in neonates. Even though intravenously administered IGF-1 (including IGF binding protein 3; either from fresh frozen plasma or provided by a pharmaceutical company) was shown to successfully elevate serum IGF1/IGFBP3 concentrations in human extreme preterm infants, IGF-1 half-life was shown to be extremely short (<1 hr; Ley et al., 2013; Lofqvist et al., 2009). Continuous intravenous infusion during a mean of two weeks was deemed safe and feasible, however one-third of all included infants did not reach target serum IGF-1 levels. Additional studies are needed to determine the optimal dosing regimen and to assess treatment efficacy (Hansen-Pupp et al., 2017). Even though IGF-1 has been shown to cross the (intact) blood-brain-barrier (BBB) by active transport, the proportion of the poly-peptide that reaches CNS following intravenous injection is likely limited (Pan & Kastin, 2000; Reinhardt & Bondy, 1994; Thorne et al., 2004). An adult rat study comparing intravenous and intranasal administration of IGF-1 showed significantly higher CNS concentrations following intranasal treatment, with similar blood and peripheral tissue levels. Intranasally administered IGF-1 was shown to bypass the BBB, entering the CNS via the olfactory system and trigeminal nerve (Thorne et al., 2004). Thus, while continuous intravenous infusion of IGF-1 would pose a substantial clinical burden with a limited supply to the brain, intranasal IGF-1 administration might offer a less invasive, rapid and direct route to target the CNS (Cai et al., 2011; Lin et al., 2009b; Liu et al., 2001).

Box 1. Jagged-1 and Notch in EoP

Although the Notch receptor and its ligand Jagged-1 have previously been associated with OPC proliferation thereby regulating the timing of differentiation, Jagged-1 and Notch may be more complexly linked to oligodendrocyte development. Subtle increases in Notch and Sonic hedgehog (Shh) signaling may skew the OPC fate to differentiation instead of recruitment into the homeostatic NG2+ OPC pool, as was demonstrated in the spinal cord of zebrafish larvae (Ravanelli et al., 2018). In EoP, neuroinflammation and oxygen fluctuations upregulate the expression of Jagged-1 by astrocytes, thereby activating the Notch-pathway which can contribute to the oligodendrocyte maturational arrest (van Tilborg et al., 2016; Yuan & Yu, 2010). This was also shown in a mouse model of neonatal hyperoxia, in which WMI could be rescued by blocking the Notch-signaling pathway using a γ-secretase inhibitor (Du et al., 2017b). However, a recent *in vivo* study showed the downregulation of Notch-1 and Hes-1 proteins in the corpus callosum of rat pups subjected to fetal inflammation, which corresponded with reduced maturation of OPCs into mature oligodendrocytes and myelination (Ying et al., 2018). Together, these results indicate that oligodendrocyte development is tightly regulated by the Notch-pathway, and that subtle imbalances in Notch pathway activation caused by EoP lead to failure of myelination.

Epidermal growth factor family

Epidermal growth factor and transforming growth factor alpha

The epidermal growth factor (EGF) family consists of several factors including EGF, heparin-binding EGF (hb-EGF) and transforming growth factor alpha (TGF-α), that are involved in the proliferation and survival of many cell types, among which cells of the oligodendrocyte lineage (Oyagi & Hara, 2012; Yang et al., 2017). EGF-family members and the EGF receptor (EGFR) are upregulated in the CNS during development and in response to injury, for example after hypoxia-ischemia (Aguirre et al., 2007; Ferrer et al., 1996; Kornblum et al., 1997; Oyagi & Hara, 2012). The EGFR signals through multiple intracellular pathways, such as PI3K/Akt, RAS/ERK and JAK/STAT (see Box 2; Jorissen et al., 2003).

In vitro studies indicate that during normal white matter development, EGF interacts with the mitogens platelet-derived growth factor AA (PDGF-AA) and basic fibroblast growth factor (bFGF) to skew glial precursor cells towards OPC cell fate, and to enhance survival and proliferation of OPCs (Yang et al., 2017; Yang et al., 2016). However,

when OPCs are cultured with EGF in the absence of PDGF-AA, EGF promotes differentiation into mature (myelin basic protein (MBP)-expressing) oligodendrocytes, indicating a role for EGF in both proliferation and differentiation (Yang et al., 2017). Since cerebral PDGF-levels decrease during third trimester development (Van Heyningen et al., 2001), the role of EGF shifts to promote oligodendrocyte differentiation. In contrast, when PDGF is upregulated in response to e.g. pro-inflammatory cytokines (Gard et al., 1995; Silberstein et al., 1996), EGF may halt oligodendrocyte differentiation and promote proliferation of the extensive OPC pool that is already present in preterm brain injury, making it a less feasible therapeutic candidate to treat preterm WMI.

In vivo rodent experiments confirm the potential of EGF to stimulate both OPC proliferation and differentiation. In a transgenic mouse model, overexpression of EGFR in CNPase+ cells (i.e. pre-OLs) led to increased proliferation of OPCs, mature oligodendrocyte numbers, MBP expression, and myelinated axons (Aguirre et al., 2007). Conversely, hypoactive EGFR signaling in a mouse mutant reduced the number of OPCs (NG2+) and mature (CC1+) oligodendrocytes and myelination during development, supporting the *in vitro* evidence that EGF plays a role in both proliferation and maturation. Results from several preclinical studies indicate a protective role of EGF in animal models of white matter pathologies. EGFR-overexpressing neonatal mice were less susceptible to developing dWMI after subjection to chronic hypoxia, whilst an EGFR-antagonist reduced the number of OPCs, mature oligodendrocytes and production of myelin (Scafidi et al., 2014). This had previously been shown in adult EGFR-overexpressing mice recovering from focal demyelination (Aguirre et al., 2007). Moreover, intranasal treatment with exogenous hb-EGF following neonatal chronic hypoxia reduced apoptosis of mature oligodendrocytes, preserved axonal myelination and improved behavioral outcome, through a reduction of Notch-signaling (see Box 1; Scafidi et al., 2014). Interestingly, in a rabbit model of neonatal intraventricular hemorrhage, EGF levels were reduced, indicating a deficit in endogenous EGF production after injury (Vinukonda et al., 2016). Intraventricular injection of EGF increased OPC proliferation, oligodendrocyte maturation, and astrogliosis (Vinukonda et al., 2016).

In an *in vitro* model of HIE, in which OPCs were deprived of oxygen and glucose, treatment with EGF-family member TGF-α significantly reduced apoptosis of OPCs and mature oligodendrocytes through STAT3 signaling (see Box 2), but had no direct effect on oligodendrocyte differentiation or myelination (Dai et al., 2019). This may indicate that TGF-α might preferably be given shortly after WMI is induced to reduce

apoptosis of oligodendrocyte precursors. Consistent with *in vitro* findings, EGF-family member TGF-α also protected OPCs and mature oligodendrocytes against apoptosis in adult ischemic stroke, while TGF-α knock-out mice displayed more extensive white matter lesions compared to wildtype mice (Dai et al., 2019). Together, these studies indicate the therapeutic potential of EGF-family members in protection of oligodendrocyte-lineage cells against apoptosis and stimulating OPC proliferation and differentiation after WMI.

Besides their involvement in oligodendrocyte development and survival, EGF-family members modulate neuroinflammatory processes that contribute to the pathophysiology of EoP. It has been shown *in vitro* that shedding of hb-EGF by astrocytes can be induced by inflammatory cytokines such as IFN-γ and TNF-α, inducing proliferation of microglia, enhancing their phagocytotic capacity and increasing monocyte migration (Martín et al., 2012; Schenk et al., 2013). TGF-α and EGF are known to promote astrogliosis through the EGFR (Kuhn et al., 1997; Rabchevsky et al., 1998; Weickert & Blum, 1995). Expression of hb-EGF by reactive astrocytes has also been demonstrated in active MS lesions, which may trigger further inflammatory events in the lesioned area (Schenk et al., 2013). Single treatment with an anti-EGF antibody was therefore beneficial in an experimental autoimmune encephalomyelitis (EAE) mouse model, by shifting NSC differentiation towards neurons and oligodendrocytes instead of astrocytes (Amir-Levy et al., 2014). Simultaneous activation of *both* mitogen receptors (bFGF and PDGF) *and* EGFR further induces neuroinflammation and oligodendrocyte apoptosis in response to pathogens (Parthasarathy & Philipp, 2017), suggesting that supplementation of EGF should not coincide with extensive FGFR and PDGFR activation.

Further research is warranted to design therapies that balance the potential beneficial effect of EGF-family members on oligodendrocyte survival and maturation while avoiding excessive astrogliosis which may exacerbate neuroinflammation, for example by targeting the EGFR on oligodendrocytes specifically. Clinical trials involving the exogenous administration of EGF, TGF-α or hb-EGF for the treatment of preterm WMI have not been conducted yet to the best of our current knowledge (clinicaltrials.gov).

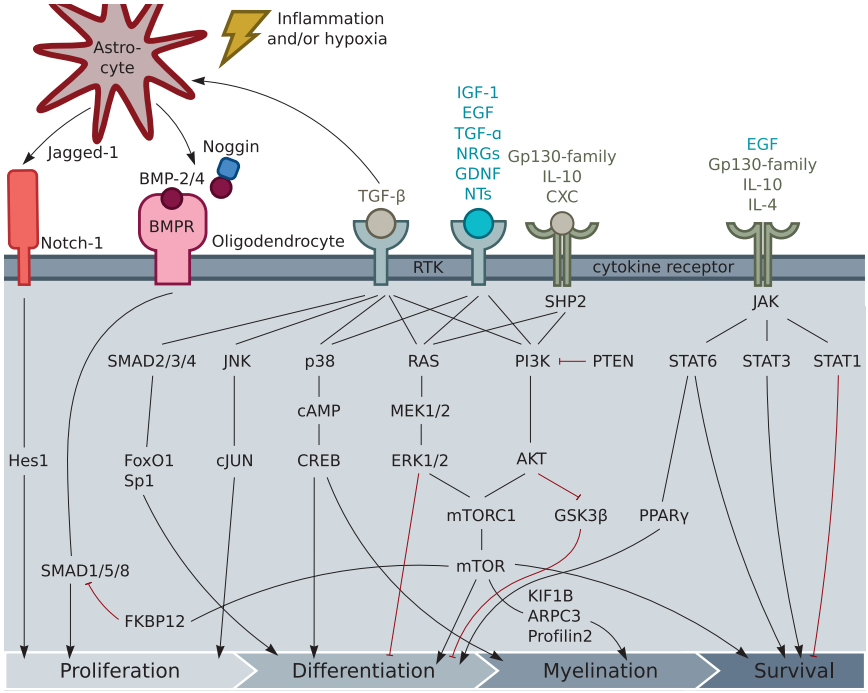


Figure 2. Proposed model of intracellular pathways that mediate the different stages of oligodendrocyte development. Astrocytes (red cell), triggered by inflammation and/or hypoxia (yellow flash), produce factors that stimulate oligodendrocyte precursor cell (OPC) proliferation, which contributes to the maturational arrest of oligodendrocytes in preterm WMI. In contrast, several factors discussed in this review either directly or indirectly stimulate oligodendrocyte maturation/differentiation, myelination and survival, through shared intracellular pathways. IGF-1, insulin-like growth factor 1; EGF, epidermal growth factor; TGF, transforming growth factor (α and β); NRGs, neuregulins; GDNF, glial cell-line derived neurotrophic factor; NTs, neurotrophins; Gp130, glycoprotein 130; IL, interleukin (4 and 10); BMP, bone morphogenetic protein; BMPR, BMP receptor; RTK, receptor tyrosine kinase; JAK, janus kinase; STAT, signal transducer and activator of transcription proteins; JNK, c-Jun N-terminal kinase; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; cAMP, cyclic adenosine monophosphate; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; FoxO1, forkhead box protein O1; Sp1, specificity protein 1; CREB, cAMP response element-binding protein, mTORC1, mTOR complex 1; GSK3β, glycogen synthase kinase 3 β; PPARγ, peroxisome proliferator-activated receptor γ.

Box 2. Shared intracellular pathways targeting oligodendrocyte survival and maturation

Several trophic and immunomodulatory factors discussed in this review activate shared intracellular pathways downstream of their specific receptors, such as PI3K/Akt, MAPK pathways and JAK/STAT, that are associated with oligodendrocyte survival and maturation (Figure 2). Next to supplementing growth factors and/or cytokines that activate these pathways, a potent treatment strategy for EoP could be to directly target these pathways on a molecular level.

The PI3K/Akt pathway drives oligodendrocyte survival, differentiation and maturation (Ishii et al., 2019; Wrigley et al., 2017). Specifically, the downstream activation of mTOR promotes oligodendrocyte survival through inhibition of pro-apoptotic pathways (O'Kusky & Ye, 2012; Wrigley et al., 2017) and stimulates oligodendrocyte differentiation and myelination (Gaesser & Fyffe-Maricich, 2016). Recently, it has been discovered that the pro-differentiating effect of mTOR is elicited by inhibition of BMP signaling, which suppresses the expression of Olig1/2 (see section 3.3.2; Ornelas et al., 2020; Song et al., 2018). Furthermore, mTOR is involved in oligodendrocyte differentiation by regulating morphological complexity and proper axon ensheathment through downstream targets ARPC3 and profilin2, myelin gene expression, and Mbp RNA transport through mTOR target KIF1B (Musah et al., 2020). Increased expression and signaling of the PI3K/Akt/mTOR pathway was observed for up to two weeks after injury in a mouse model of preterm hypoxia-ischemia (Wang et al., 2020b). mTOR is likely not the only target of the PI3k/Akt pathway that influences oligodendrocyte development, as OPC-specific inactivation of PTEN, an Akt-inhibitor, led to enhanced differentiation in OPCs independently of mTOR-deletion (González-Fernández et al., 2018). Ablation of GSK3β, a downstream target of Akt that is phosphorylated after PTEN activation, similarly led to increased OPC differentiation, making it a likely mediator of mTOR-independent OPC differentiation (González-Fernández et al., 2018).

The rat sarcoma/extracellular signal-regulated kinases (RAS/ERK) MAP kinase pathway is activated after growth factors (e.g. IGF-1, EGF) bind specific tyrosine kinase receptors, and is involved in all stages of oligodendrocyte lineage progression, but most prominently during myelination (Gaesser & Fyffe-Maricich, 2016; Gonsalvez et al., 2016). Although *in vitro* evidence has revealed a role for RAS/ERK in oligodendrocyte differentiation, this has not been conclusively demonstrated *in vivo* (reviews by Gaesser & Fyffe-Maricich, 2016; Gonsalvez

et al., 2016). Moreover, a recent study by Suo et al. (2019) demonstrated that inhibition of MEK, the direct regulator of ERK1/2, promoted oligodendrocyte differentiation from NPC-derived OPCs *in vitro* and *in vivo* in an EAE model. This has led to the hypothesis that RAS/ERK is not directly involved in OPC differentiation, but does come into play during the process of myelination (Ishii et al., 2019). The RAS/ERK pathway is hypothesized to determine proper myelin thickness relative to axon diameter *in vivo* through ERK1/2 activation (Gaesser & Fyffe-Maricich, 2016; Ishii et al., 2012), and in myelin maintenance throughout adulthood (Ishii et al., 2019). Furthermore, the RAS/ERK pathway has been shown to converge with the PI3K/Akt/mTOR pathway to promote myelination during development and after demyelinating injury at the level of mTORC1, as RAS/ERK by itself could not promote sufficient myelination in mTOR-deficient mice (Ishii et al., 2019). The potential pleiotropic role of the RAS/ERK pathway in both proliferation and myelination could help explain the involvement of some OPC mitogens, such as bFGF (see section 3.8) during myelination in mature oligodendrocytes.

Another MAP kinase, c-JUN N-terminal kinase (JNK), has been described to inhibit differentiation of OPCs by promoting proliferation (van Tilborg et al., 2016). The JNK pathway is activated after EoP-associated injury, such as neuroinflammation or oxygen fluctuations, which makes it a salient target for intervention after preterm birth (Bhat et al., 2007; van Tilborg et al., 2016). Therapeutic inhibition of the JNK pathway rescues myelination after an inflammatory stimulus *in vitro* and in a rat model of preterm dWMI, potentially through inhibiting OPC proliferation which induces differentiation (van Tilborg et al., unpublished results of our group).

Activation of the MAP kinase p38 has also been associated with oligodendrocyte development (Bhat et al., 2007; Chew et al., 2010). p38 inhibition leads to reduced expression of oligodendrocyte signature genes such as O1 and O4, and reduced myelin production *in vitro* (Bhat et al., 2007). Furthermore, p38MAPK induces phosphorylation of the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB; Bhat et al., 2007), which induces gene transcription that has been proposed to underlie stimulation of oligodendrocyte differentiation and mitogenesis, for example after innervation of the IGFR (Palacios et al., 2005; Wrigley et al., 2017). Next to its function during normal development, the p38 MAPK pathway has been associated with upregulation after inflammation (Bhat et al., 2007). OPCs from p38a specific knock-

out mice failed to myelinate after differentiation *in vitro*, and brains of these mouse mutants showed abnormalities in myelin microstructure (Chung et al., 2015). The p38 MAPK has also been shown to interact with other MAP kinases, such as through inhibition of c-JUN phosphorylation (Chew et al., 2010).

Numerous cytokines, including the interleukin-family of which IL-4, IL-6, IL-10 and IL-11 discussed in this review, activate the JAK/STAT-pathway downstream of their specific receptors (Liu et al., 2015). Activation of STAT1 is linked to oligodendrocyte apoptosis (Liu et al., 2015), while STAT3 activation promotes oligodendrocyte survival (Zhang et al., 2011). Furthermore, STAT3 is implicated in myelinogenesis during postnatal brain development, but a mouse model with specific STAT3 ablation in oligodendrocytes showed that it is not essential for developmental myelination (Steelman et al., 2016). However, when STAT3 activation was ablated in oligodendrocytes, maturation and remyelinating capacity after focal demyelination was impaired (Steelman et al., 2016). STAT6 promotes oligodendrocyte differentiation through activation of PPARγ (Zhang et al., 2019).

Neuregulins

Neuregulins (NRGs) are members of the EGF-family that have been classically linked to myelination through their secretion by neuronal axons (e.g. Taveggia et al., 2008). OPCs and oligodendrocytes also express NRGs, which may give them the capacity to self-regulate their development (Calaora et al., 2001; Raabe et al., 1997). NRGs signal through different homo- and heterodimers of the ErbB receptor tyrosine kinases that are related to the EGFR (i.e. ErbB1) and can activate PI3K/Akt and MAPK pathways intracellularly (see Box 2; Canoll et al., 1999). NRG1 is the most widely studied NRG, and has been extensively studied in the context of peripheral myelination.

It has been demonstrated *in vitro* that NRG1 possesses isoform-dependent effects on OPCs and oligodendrocytes (Raabe et al., 1997). Specifically, Type II NRG1 glial growth factor 2 (GGF2) promotes proliferation in OPCs through ErbB3, as OPCs differentiate after soluble ErbB3 is administered to neutralize GGF2 (Calaora et al., 2001; Canoll et al., 1999). In contrast, type I NRG1 isoforms induce differentiation and reduce apoptosis (Raabe et al., 1997). ErbB4, which binds multiple NRGs, was found to be particularly involved in promoting oligodendrocyte maturation and myelination (Lai & Feng, 2004; Sussman et al., 2005). More recently, it has been discovered that type I isoforms of NRG1 promote a shift in oligodendrocyte phenotype which increases

their responsiveness to glutamate through generation of NMDA receptors (Lundgaard et al., 2013). This enables oligodendrocytes to respond to environmental triggers to enhance their myelin production. NRG1 type III was also found to be involved specifically in myelination, as oligodendrocytes co-cultured with type III deficient DRGs formed significantly less myelin sheaths (Taveggia et al., 2008).

In vivo evidence supports the role of NRGs in myelin formation. Knockout of the nardilysin (Nrd1) gene, an important regulator of NRG1 shedding, resulted in hypomyelination in the CNS and peripheral nervous system (PNS), while neuronal Nrd1 overexpression induced NRG1 type I and III availability and consequent hypermyelination, while the availability of NRG1 type II was not investigated (Ohno et al., 2009). Similarly, NRG1 type III knockout mice showed hypomyelination throughout development and adulthood (Ohno et al., 2009; Taveggia et al., 2008). In both studies, numbers of OPCs and mature oligodendrocytes were not altered between mutants and wildtypes, suggesting that NRG1 types I and III are specifically involved in myelination and do not play a prominent role in oligodendrocyte lineage progression. In mutant mice with oligodendrocyte-specific impairment in NRG1/ErbB signaling, oligodendrocyte morphology was altered, leading to smaller cells and less myelin production per cell (Roy et al., 2007). NRGs may therefore be a promising therapeutic agent to enhance myelination capacity of mature oligodendrocytes, perhaps in combination with a therapeutic agent that triggers oligodendrocyte survival or maturation. To our knowledge, *in vivo* studies in which NRGs are administered as a therapeutic in models of EoP have not yet been conducted.

Microglia have been shown to release NRG1 types I and III and NRG3 after stimulation with LPS *in vitro* (Ikawa et al., 2017). *In vivo*, NRG expression was enhanced in microglia and astrocytes after injury (Ikawa et al., 2017; Tokita et al., 2001). This may be a protective response, as exogenous NRG1β treatment partly reduced microglial and astrocytic activation in mice exposed to LPS, leading to reduced inflammatory cytokine production and improved neuronal survival (Xu et al., 2017). However, there have also been some reports indicating antagonism of NRG1 may be beneficial in neuroinflammatory conditions, by reducing the trophic effect of NRG1 on microglia (e.g. Allender et al., 2018). Thus, although NRGs have been reported to be mainly beneficial in the context of neuroinflammation, the effects of NRGs on pro-inflammatory microglia must be examined further to rule out exacerbation of inflammation in EoP. Although not the primary focus of our review, NRGs/ErbBs have also been

associated with interneuron migration and development (see Mei & Nave, 2014 for a review), possibly indicating a complex interaction between NRGs, interneurons and oligodendrocytes in EoP.

In humans, genetically caused disturbances in the NRG/ErbB balance have been associated with several neuropsychiatric disorders, such as schizophrenia, depression and bipolar disorder (see review by Mei & Nave, 2014), and have been directly linked to an impaired social performance in children with ASD (Ikawa et al., 2017). Interestingly, NRG1 is endogenously upregulated in human umbilical endothelial cells after preterm birth, and a SNP increasing its availability is linked to improved neurodevelopmental outcome after preterm WMI (Hoffmann et al., 2010). Considering this and its role in myelination described above, NRG1 has been put forward as a protective agent in preterm WMI (see review by Dammann et al., 2008). However, more recent evidence points to NRG1 isoform-dependent effects that must be taken into consideration. Further pre-clinical research regarding the effectiveness of NRGs in EoP is warranted to substantiate the initiation of clinical trials.

Transforming growth factor beta superfamily

The transforming growth factor beta (TGF-β) superfamily consists of at least 30 cytokines, and can be subdivided into TGF-β-type and bone morphogenetic protein (BMP) type-proteins, that play a role in a wide array of physiological processes, among which oligodendrocyte development and gliosis (see Weiss & Attisano, 2013 for a review).

Transforming growth factor beta-type proteins

There are three isoforms of TGF-β that are postulated to play different roles in the central nervous system during development and disease (Dobolyi et al., 2012; Stoll et al., 2004). TGF-β1 has been the most studied in the context of oligodendrocyte development. Receptors for TGF-β (TGFBR1/activing-like kinase 5 receptor (ALK-5) and TGFBR2) are present on cells of the oligodendrocyte lineage, as well as astrocytes (e.g. Gómez Pinto et al., 2018; Hamaguchi et al., 2019), and canonically activates the SMAD pathway, as well as the PI3k/Akt, RAS/ERK, JNK and p38 MAPK pathways discussed in Box 2 (Heldin & Moustakas, 2016).

TGF-β1 has been shown to increase proliferation of OPCs and differentiation to mature oligodendrocytes *in vitro* (Gómez Pinto et al., 2018; McKinnon et al., 1993).

The mitogenic effect of TGF-β1 on OPCs is likely to be indirect and mediated through astrocytes, which secrete Jagged-1 upon stimulation with TGF-β1 (Gómez Pinto et al., 2018; Zhang et al., 2010). Through activation of the Notch-1 receptor on OPCs, Notch ligands such as Jagged-1 stimulate OPC proliferation to prevent early differentiation, thereby regulating the timing of oligodendrocyte lineage progression (van Tilborg et al., 2016; Wang et al., 1998). Jagged-1 is also secreted by adult oligodendrocytes, possibly to signal to OPCs that the region is sufficiently myelinated (Wang et al., 1998). However, the relationship between the Notch pathway and oligodendrocyte development is likely more complex, which is highlighted in Box 1. OPCs also express TGF-β receptors, and direct TGF-β1 stimulation is believed to exert a maturational effect (Gómez Pinto et al., 2018; Hamaguchi et al., 2019), likely after Jagged-1 expression by astrocytes is downregulated during normal development (e.g. Wang et al., 1998).

In a series of loss- and gain-of-function experiments, Palazuelos et al. (2014) demonstrated that TGF-β signaling is critical for OPC differentiation *in vivo*, by mediating cell cycle withdrawal through SMAD2/3/4 and downstream FoxO1 and Sp1 activation. It is therefore perhaps surprising that the TGF-β pathway was found to be upregulated in a rat model of neonatal HI brain injury at preterm-equivalent age in humans (Sun et al., 2010). Although TGF-β is able to stimulate differentiation of oligodendrocytes, the upregulation of TGF-β after HI injury may have adverse effects on EoP pathology through Jagged-1 expression by astrocytes. Therefore, studies in HI-injured neonatal rats have aimed to target the TGF-β pathway by antagonizing the activin-like kinase 5 receptor (ALK-5 or TGFBR1), that mediates the effect of TGF-β on astrocytes. In a model of preterm injury, the ALK-5 antagonist SB505124 decreased microgliosis and astrogliosis, improved oligodendrogenesis and myelination, and promoted autophagy of potentially toxic debris (Guardia Clausi & Levison, 2017; Kim et al., 2017). However, when hypoxic-ischemic injury was induced at a later postnatal age in mice (p9 instead of p6), administration of the ALK-5 antagonist exacerbated hippocampal injury and functional outcome (Kim et al., 2017). It can be speculated that astrogliosis caused by TGF-β/ALK-5 signaling after preterm birth negatively impacts oligodendrocyte maturation through Jagged-1 expression, while astrogliosis is necessary for demarcating lesions in hypoxic-ischemic injury at term (Kim et al., 2017) or in adult stroke (Cekanaviciute et al., 2014). Thus, *in vitro* and *in vivo* evidence suggests that TGF-β can have a dual role in the developing brain by inducing oligodendrocyte maturation directly through TGF-β receptors on OPCs on the one hand, but on the other hand

triggering astrocytes to produce Jagged-1 that preserves OPC immaturity by inducing proliferation (e.g. Wang et al., 1998). TGF-β-based therapies to combat EoP should therefore be aimed at oligodendrocyte-lineage cells specifically, for example by using NG2+-targeted nanoparticles (Rittchen et al., 2015), or TGF-β should be given in combination with a Jagged-1 antagonist.

Besides its effect on oligodendrocytes and astrocytic scar formation, TGF-β is also involved in microglia homeostasis and activation (Bohlen et al., 2019). TGF-β is often mentioned as an anti-inflammatory cytokine, either dampening the pro-inflammatory response, or skewing microglial activation to the neuroprotective M2-phenotype (Dobolyi et al., 2012). Administration of TGF-β1 has been shown to specifically induce microglial apoptosis *in vitro* (Xiao et al., 1997). In addition, TGF-β2 administration reduced spontaneous myelin phagocytosis by microglia *in vitro*, while it had no effect on the amount of microglia that were recruited (Stoll et al., 2004). Substantive evidence demonstrating the protective potential of TGF-β isoforms against excitotoxicity is also available (Dobolyi et al., 2012).

Together, these results underline a potential protective effect for TGF-β in EoP. As of yet, no clinical trials using TGF-β or ALK-5 antagonists have been registered in the clinical trials database (clinicaltrials.gov).

Bone morphogenetic proteins and Noggin

Bone morphogenetic proteins (BMPs) form the second major protein class of the TGF-β superfamily, and BMP receptors are present on oligodendrocytes during all developmental stages (See et al., 2004). Intracellularly, BMPs activate the SMAD pathway as well as the MAPK p38 pathway (see Box 2; Eixarch et al., 2018; Shijo et al., 2018). Through SMAD1/5, BMPs downregulate the expression of Olig1/2 (Song et al., 2018).

In vitro studies illustrate the inhibitory role of BMPs on oligodendrocyte maturation. It has been shown that BMP-4 applied to primary oligodendrocytes or brain slices irreversibly inhibits OPC differentiation into myelinating cells (Morell et al., 2015; Reid et al., 2012; See et al., 2004). Similarly, BMP-2 cooperates with mitogen PDGF to restrict oligodendrocyte differentiation (Adachi et al., 2005). Oxidative stress has been reported to cause BMP-4 expression leading to oligodendrocyte maturational arrest (Reid et al., 2012). Since the BMP-family negatively impacts oligodendrocyte

maturation, it was hypothesized that the BMP-2/4-antagonist Noggin could protect against white matter damage. Indeed, Noggin overexpression led to increased oligodendrocyte differentiation in an *in vitro* model using neurospheres (Morell et al., 2015).

In a mouse model of late preterm hypoxic-ischemic injury, transgenic mice that overexpressed Noggin were protected against WMI, as shown by preservation of cells across the oligodendrocyte-lineage, and subsequent increased myelination (Dizon et al., 2011). In a rabbit model of intraventricular hemorrhage (IVH), treatment with Noggin similarly rescued oligodendrocyte maturational arrest, preserved myelination and decreased astrogliosis (Dummula et al., 2011).

Besides their role halting oligodendrocyte maturation, BMPs have been implicated in neuroinflammation in MS and amyotrophic lateral sclerosis (ALS) pathophysiology (Eixarch et al., 2018; Shijo et al., 2018). BMP-4 expression by astrocytes was found to be increased in a mutant rat model for ALS, and Noggin was effective at dampening the neuroimmune response, reducing astrogliosis and microglial activation (Shijo et al., 2018). In an *in vitro* model of oxygen/glucose deprivation and reperfusion, Noggin induced release of iron from microglia, aiding the process of remyelination after injury (Shin et al., 2018). BMP signaling affects differentiation of interneuron subpopulations differently, stimulating differentiation of parvalbumin interneuron differentiation, but halting somatostatin interneuron development (Mukhopadhyay et al., 2009). This suggests upregulation of BMPs in EoP may alter interneuron development as well as oligodendrocyte maturation.

In humans, elevation of BMP-4 in the brain has been associated with intraventricular hemorrhage in preterm infants (Dummula et al., 2011). Although results from pre-clinical studies show that BMP-antagonist Noggin is a promising option for the treatment of EoP, more research in pre-clinical models is warranted before clinical trials can be initiated (clinicaltrials.gov).

Glial cell line-derived neurotrophic factor family

The glial cell line-derived neurotrophic factor (GDNF) family has also recently emerged as a potential candidate for treatment of white matter pathologies. GDNF-family members have ranging affinities to the GDNF family receptor α (GFRα) subtypes, and these receptors are differentially expressed on cells of the oligodendrocyte lineage, suggesting that each GDNF- family member may play a unique role

during oligodendrocyte development (Razavi et al., 2015; Strelau & Unsicker, 1999). When bound by a GDNF-family member, the designated GFRα receptor couples with a tyrosine kinase Ret-receptor to activate the MAPK and PI3K/Akt signaling pathways (see Box 2; Duarte et al., 2012).

Not much is known about the direct effects of the GDNF-family on OPCs or mature oligodendrocytes. In an *in vitro* model of stroke using oxygen and glycose deprivation, immediate treatment with GDNF increased OPC proliferation and differentiation into myelinating oligodendrocytes which persisted for several days after the insult, while reducing oligodendrocyte apoptosis (Li et al., 2015a).

In line with the *in vitro* evidence, stereotactic intracerebral injection with GDNF in a rat model of periventricular leukomalacia showed similar beneficial effects on oligodendrocyte maturation and myelination (Li et al., 2015a). Transplantation of GDNF-overexpressing NSCs showed promising effects in a mouse model of EAE by increasing numbers of OPCs, mature oligodendrocytes and myelin content (Gao et al., 2016). However, overexpression of the GDNF-family member persephin in MSCs did not show an additive beneficial effect compared to wildtype MSCs in a mouse model of neonatal HI injury (Van Velthoven et al., 2014). More research is needed to assess the therapeutic potential of GDNF-family members in preterm dWMI.

GDNF is produced by astrocytes and microglia in response to injury (Duarte Azevedo et al., 2020) and has been found to be protective after brain ischemia through promotion of neuronal survival (see review by Duarte et al., 2012). However, sustained GDNF activation may prolong neuroinflammation (Duarte Azevedo et al., 2020).

GDNF and its family members have not yet been tested in clinical trials in preterm infants (clinicaltrials.gov), but have been clinically tested and found safe for the treatment of Parkinson's disease and neuropathic pain (e.g. Marks et al., 2016; Rolan et al., 2015; Whone et al., 2019).

Neurotrophins

The neurotrophin (NT) family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (NT-6). Each neurotrophin acts with high affinity on a preferred receptor of the tropomyosin-related kinase (Trk) family and with low affinity to the

p75 neurotrophin receptor present on oligodendrocytes (Acosta et al., 2013; Huang & Reichardt, 2001; Wong et al., 2008). Through activation of the Trk/p75 receptors, neurotrophins have been linked to activation of the PI3K/Akt, MAPK and signaling pathways (see Box 2; Cohen et al., 1996; Huang & Reichardt, 2001; Ness et al., 2002). Neurotrophins and their receptors are upregulated starting from early embryonic neurodevelopment (Bernd, 2008) and likely provide trophic support to neurons and oligodendrocytes throughout life. After CNS injury, expression of NTs and their receptors has been shown to be increased (Acosta et al., 2013; Huang & Reichardt, 2001; Ness et al., 2002).

In vitro results indicate that NTs, through their preferred receptors, may have different effects on cells of the oligodendrocyte lineage. BDNF, well-known for its trophic effect on the brain, has been shown to enhance myelination in a model where DRG neurons are co-cultured with oligodendrocytes (Xiao et al., 2010). Furthermore, it was found that the effect of BDNF on myelination was mediated by TrkB expression on oligodendrocytes, as myelination of both DRG subtypes expressing either TrkA or TrkB occurred after BDNF supplementation, and this effect was only suppressed by a TrkB-inhibitor. In an *in vitro* model of chemically-induced hypoxic injury, astrocyte-conditioned medium containing BDNF promoted OPC differentiation (Miyamoto et al., 2015). This study also showed that the beneficial effect of BDNF was halted by use of the PI3K-inhibitor LY294002 or when astrocyte-conditioned medium was depleted of BDNF with a TrkB-Fc decoy receptor (Miyamoto et al., 2015). Not much is known about the effect of NT-4/5 on oligodendrocyte maturation, but this NT shares about 95% sequence homology with BDNF and also binds to TrkB, suggesting it may have a similar beneficial effect (Dhobale, 2014). Similar to BDNF, NT-3 induced substantial axonal myelination when oligodendrocytes were co-cultured with DRG neurons, as well as survival and proliferation of OPCs and mature oligodendrocytes (Barres et al., 1994; Saini et al., 2005; Yan & Wood, 2000). In an isolated OPC culture, biofabricated sponges emitting NT-3 subtly increased differentiation of primary OPCs towards the pre-OL stage, but the population of MBP+ mature oligodendrocytes was similar to the untreated condition, indicating NT-3 only affected oligodendrocyte differentiation but not final maturation (Mekhail et al., 2015). In another experiment where DRG neurons were co-cutured with oligodendrocytes, administration of NGF indirectly reduced spontaneous myelination through TrkA present on DRG neurons, whereas NGF had no effect on myelin expression of purified OPCs, or when oligodendrocytes were co-cultured with DRG neurons lacking TrkA (Chan et al., 2004). This is

in contrast to BDNF, which directly affects myelination of oligodendrocytes (Xiao et al., 2010). NGF has also been reported to cause apoptosis of mature oligodendrocytes cultured from neonatal rat brains through p75/JNK (see Box 2; Casaccia-Bonnefil et al., 1996), but apoptosis was not seen after NGF administration to post-mitotic oligodendrocytes isolated from human tissue (Ladiwala et al., 1998).

Trophic effects of neurotrophins on myelination were largely confirmed in animal models. In BDNF+/- mice, oligodendrocyte numbers are reduced and myelination is impaired in the forebrain during the postnatal period (Nicholson et al., 2018; Vondran et al., 2010; Xiao et al., 2010). TrkB is likely a mediator of myelination through BDNF, as conditional knock-out of TrkB in oligodendrocytes restricted formation of thick myelin sheaths during development (Wong et al., 2013). In models of neonatal WMI, knockdown of BDNF expression in astrocytes reduces oligodendrogenesis and exacerbates WMI (Miyamoto et al., 2015), whereas upregulation of BDNF and TrkB through induced histone acetylation increases OPC proliferation, oligodendrocyte maturation and myelination, and improved behavioral outcome (Huang et al., 2018). In a model of neonatal hypoxic-ischemic injury, genetically modified MSCs that overexpressed BDNF did not have a superior effect on reducing white matter damage compared to empty-vector MSCs, but did decrease lesion size and motor outcome compared to empty vector-MSCs (Van Velthoven et al., 2014; Van Velthoven et al., 2013). NT-3 has been proven effective in rescuing myelination and enhancing remyelination in mouse models of MS (Fressinaud, 2005; Jean et al., 2003; Zhang et al., 2012) and spinal cord injury (SCI; Thomas et al., 2014). Finally, TrkA-agonist BNN27, which mimics the effects of NGF but with improved pharmacokinetics, protects the brain from demyelination and stimulates oligodendrocyte maturation in a cuprizone-induced mouse model of MS (Bonetto et al., 2017).

A vast amount of literature is available about the role of neurotrophins, particularly BDNF, in neuroinflammation. It is generally accepted that neurotrophin release functions as a protective mechanism following neuroinflammation, as neurotrophin insufficiency is linked to a wide array of neuropsychiatric conditions with a neuroinflammatory component (Lima Giacobbo et al., 2019). While neuroinflammation can induce neurotrophin release in astrocytes, an excess of pro-inflammatory cytokines, particularly IL-1β, can also halt neurotrophic support (Ohja et al., 2018; Tong et al., 2012). Nonetheless, NGF and BDNF have been shown to promote a neuroprotective phenotype in microglia (Lai et al., 2018; Neumann et al., 1998; Rizzi et al., 2018) and

reduce astrogliosis in *in vitro* studies (Cragnolini et al., 2009). These results support the notion that neurotrophins contribute to dampening the immune response in the injured brain.

In human studies, lowered maternal and cord blood BDNF, NGF and NT-3 levels have been reported in preterm compared to term born infants (Dhobale, 2014). However, postnatal inflammation in extremely preterm neonates has been associated with an upregulation of neurotrophins in blood (Leviton et al., 2017), which likely serves as an endogenous protective response, as increased systemic neurotrophin levels have been linked to improved cognitive outcomes at 10 years of age (Kuban et al., 2018). In sum, endogenous production of neurotrophins, particularly BDNF, has been associated with improvement of neurodevelopmental outcome following preterm birth, and exogenous neurotrophin supplementation could provide a valuable treatment option to enhance oligodendrocyte maturation and reduce neuroinflammation. Systemic administration of neurotrophins is likely complicated by restricted passage of these large molecules over the (intact) blood-brain-barrier (Kastin et al., 2003; Pardridge, 2003). Alternative strategies to ensure optimal delivery of neurotrophins are discussed in the *future perspectives*. Currently, no clinical trials have been initiated in preterm infants, but several have been planned and/or conducted for the treatment of traumatic and degenerative brain diseases (clinicaltrials.gov).

Glycoprotein 130 receptor cytokine family

Glycoprotein 130 (gp130) is a cytokine receptor subunit that interacts with several cytokine receptors like the soluble receptors for IL-6, IL-11, LIF, and CNTF, and is localized throughout the brain on both neurons and glial cells (Watanabe et al., 1996). For IL-6, the IL-6R is minimally present on human oligodendrocytes (Cannella & Raine, 2004), therefore it mainly communicates through gp130 using a process called trans-signaling, during which a cytokine binds its soluble receptor that in turn engages the transmembrane gp130 receptor (Rothaug et al., 2016). Gp130 intracellularly activates the JAK/STAT and SHP2 pathways, the latter triggering the downstream RAS/ERK and PI3K/Akt pathways (see Box 2; e.g. Zhang et al., 2011).

In vitro, gp130 ligands have been shown to promote maturation of primary OPCs, and subsequently stimulate myelination *in vitro* through activation of the STAT3 pathway (CNTF & LIF: Fischer et al., 2014; Rittchen et al., 2015; Steelman et al., 2016; IL-6/IL-6R: Valerio et al., 2002; Zhang et al., 2011; Zhang et al., 2004; IL-11: Zhang et al., 2006).

IL-11, CNTF and LIF have also been shown to increase survival of oligodendrocyte lineage cells (Steelman et al., 2016; Zhang et al., 2011; Zhang et al., 2006). Interestingly, it was shown for IL-11 that its anti-apoptotic effect is predominantly mediated by STAT3 in cells of the neural lineage such as OPCs, but can be pro-apoptotic through STAT1 signaling in CD11c+ dendritic cells (Zhang et al., 2011), indicating a differential effect of IL-11 on downstream STAT subtype involved based on cell lineage. Whether this pro-apoptotic effect of IL-11 extends to (Cd11c+) microglia has not been examined yet. In an *in vitro* model where OPCs were exposed to TNF-α, IL-11 reduced caspase cleavage, suggesting that IL-11 is protective against apoptosis even in the presence of an inflammatory stimulus (Zhang et al., 2011). Additionally, in a BV-2 microglial cell line culture, IL-11 dose-dependently inhibited myelin phagocytosis (Maheshwari et al., 2013). In a glial co-culture, activation of the TNF-α receptor TNFR2 on astrocytes led to enhanced LIF production, which consequently stimulated oligodendrocyte maturation, suggesting TNFR2 mediates a protective effect of LIF in response to TNF-α (Fischer et al., 2014).

In vivo evidence implicating gp130 ligands in oligodendrocyte maturation and myelination comes largely from animal models of MS. Knockout of IL-11Rα, LIF or CNTF in MS models exacerbated demyelination and oligodendrocyte loss compared to wild-type mice, whilst increasing glial reactivity (Butzkueven et al., 2006; Linker et al., 2002; Zhang et al., 2011).In line with the knockout studies, local overexpression of IL-11 increased mature oligodendrocytes and remyelination, and decreased microglial activation in a cuprizone-induced mouse model of MS (Maheshwari et al., 2013). LIF has also been shown to be a beneficial trophic factor for myelination during postnatal development, as LIF knockout mice showed reduced optic nerve myelination (Ishibashi et al., 2009). Therapeutic targeting of NG2+ OPCs/pre-OLs with nanoparticles containing LIF has proven to be effective in promoting remyelination after focal demyelinating injury (Rittchen et al., 2015). In contrast, LIF activation has also been shown to adversely influence oligodendrocyte maturation after perinatal hypoxic-ischemic injury through the induction of Notch signaling, which promotes NSC/OPC proliferation rather than maturation (Covey & Levison, 2007).

Although the effect of gp130-ligands has been well-established in relation to oligodendrocyte survival and maturation (e.g. D'Souza et al., 1996; Mayer et al., 1994), they are upregulated during neuroinflammation and have contrasting effects (Davis & Pennypacker, 2018; Rothaug et al., 2016; Winship & Dimitriadis, 2017). IL-11 treat-

ment decreases TNF-α expression and skews microglia towards a protective M2 phenotype in the adult rodent brain (Pusic et al., 2014). Through gp130, LIF has also been shown to increase expression of antioxidant genes in oligodendrocytes, which may decrease damage caused by oxidative stress from excessive ROS production by reactive microglia (Rowe et al., 2014). In contrast, IL-6 and CNTF/soluble CNTFRα have been shown to induce pro-inflammatory microglial activation (Lin et al., 2009a; Rothaug et al., 2016), which may exacerbate neuroinflammation during EoP.

In humans, elevated levels of IL-11 in decidua have been found after preterm compared to term birth (Cakmak et al., 2005). These increased IL-11 levels have been linked to induction of parturition in preterm neonates, as a protective response to circumvent excessive maternal inflammation (Winship & Dimitriadis, 2017). Elevated IL-11 in plasma of preterm infants is also associated with sepsis and NEC during the postnatal period, whereas it is not detectable in term-born infants (McCloy et al., 2002). Although gp130 ligands seem promising for oligodendrocyte differentiation and maturation, gp130-based therapies must be tailored to maximize their anti-inflammatory potential if they are used for the treatment of EoP. Clinical trials with CNTF, LIF, IL-6 and IL-11 in preterm infants have not yet been initiated (clinicaltrials.gov). CNTF is being tested in implants for the treatment of adult retinopathies (e.g. Chew et al., 2019).

Interleukin-4 and interleukin-10

Due to their canonical status as anti-inflammatory cytokines, much research has been conducted on IL-10 and IL-4 in neuroinflammatory pathologies (e.g. Kwilasz et al., 2015). Production of IL-10 and IL-4 by immune cells shifts the microglial phenotype to enhance recovery (Kwilasz et al., 2015), thereby counteracting the effects of pro-inflammatory cytokines such as IL-1β, TNF-α and granulocyte-macrophage colony stimulating factor (GM-CSF; Butovsky et al., 2006; Hashimoto et al., 2001). All in all these anti-inflammatory cytokines contribute to a more favorable environment for neuronal and oligodendrocyte survival (Yang et al., 2009). Although protection by IL-4 and IL-10 is believed to be largely mediated through their role in dampening adverse inflammatory events, IL-4 and IL-10 can also directly target their respective receptors present on cells of the oligodendrocyte-lineage (Molina-Holgado et al., 2001; Zanno et al., 2019). Intracellularly, IL-10 activates the JAK/STAT and PI3k/Akt pathways (see Box 2; Zhou et al., 2009) while it is known IL-4 targets PPARγ in OPCs, potentially through STAT6 (Zanno et al., 2019; Zhang et al., 2019).

In primary OPCs and oligodendrocytes, IL-4 and IL-10 are able to increase survival under pro-inflammatory conditions, by decreasing the expression of iNOS and production of NO by oligodendrocyte and co-cultured glial cells (Molina-Holgado et al., 2001; Paintlia et al., 2006). IL-10R activation in neurons enhances survival under excitotoxic conditions through PI3K/Akt and JAK/STAT3 pathway activation (Zhou et al., 2009), which might also hold true for oligodendrocytes. Aside from their anti-apoptotic effects, IL-10 and IL-4 promote neurogenesis and oligodendrogenesis (Butovsky et al., 2006; Yang et al., 2009). Specifically, oligodendrogenesis is instigated via IGF-1 production by IL-4-conditioned microglia (Butovsky et al., 2006). The studies on direct effects of IL-4 on OLs are conflicting as some studies find no direct effects of IL-4 on cells of the oligodendrocyte-lineage *in vitro* (Butovsky et al., 2006; Psachoulia et al., 2016), others report inhibition of differentiation of these cells (Zanno et al., 2019) or stimulation of OPC differentiation through transcription factor PPARγ (Zhang et al., 2019).

Treatment with IL-10 *in vivo* has shown promise in several models of neonatal brain injury. For instance, IL-10 decreased white matter lesion size after neonatal excitotoxic brain injury in mice (Mesples et al., 2003). A study by Pang et al. (2005) showed that IL-10 decreased apoptosis and enhanced presence of pre-OLs, mature oligodendrocytes, and MBP density in a rat model of intrauterine infection. Microglial activation and astrogliosis were also reduced after IL-10 treatment (Pang et al., 2005). NSCs modified to overexpress IL-10 rescued demyelination in a model for MS, by suppressing the infiltration and stimulating the apoptosis of peripheral immune cells and causing a more favorable intracerebral milieu (Yang et al., 2009). IL-10 overexpression also favored differentiation of NSCs into neurons or oligodendrocytes instead of astrocytes *in vitro* and *in vivo*, thereby reducing astrogliosis and stimulating remyelination.

It has been shown that IL-4 may elicit a different response in neonates versus adults in *in vivo* experimental models. In a rat model of intra-uterine growth restriction (IUGR), IL-4 was significantly increased in the brain of growth-restricted pups, and treatment with a neutralizing IL-4 antibody directly after birth improved oligodendrocyte maturation and white matter development, by decreasing the Th2 neuroinflammatory response (Zanno et al., 2019). In contrast, IL-4-based therapies show great promise in regenerating white matter in models of adult inflammatory brain injury (Zhang et al., 2019).

Preterm infants that homozygously carry the IL-10 "high producer" allele were less vulnerable to development of periventricular WMI as assessed by ultrasound echodensities, and showed a better neurodevelopmental outcome at 2 years of age (Dördelmann et al., 2006). In conclusion, IL-10 and IL-4 protect oligodendrocytes against inflammatory conditions, either by increasing survival or by halting neuroinflammatory processes. Although both IL-10 and IL-4 seem to positively affect remyelination in adults, only IL-10 is deemed safe in neonatal models. To our knowledge, IL-4 and IL-10 have not been explored in clinical trials of preterm brain injury (clinicaltrials.gov).

CXC chemokine family

The CXC-family is a subclass of chemotactic cytokines (i.e. chemokines) that are expressed in the CNS, and their receptors have been found on cells of the oligodendrocyte lineage, as well as other CNS cell types (Bajetto et al., 2002; Banisadr et al., 2005). CXCRs have been reported to activate the RAS/ERK and PI3K/Akt pathways in oligodendrocytes (Tian et al., 2018).

CXCL1 was found to promote OPC proliferation in rat isolated OPC cultures (Robinson et al., 1998) and human preterm fetal brain slices (Filipovic & Zecevic, 2008), but only in conjunction with astrocytes and/or PDGF, suggesting that CXCL1 acts together with other factors to mediate the proliferative effect on oligodendrocytes (Bradl & Lassmann, 2010). The direct effect of CXCL1 on oligodendrocytes was studied in transgenic mice, in which knocking out its receptor CXCR2 led to a reduced number of mature oligodendrocytes and consequent hypomyelination, but an increased population of OPCs (Padovani-Claudio et al., 2006). In a study by Wang et al. (2020a), it was shown that a CXCR2 antagonist was effective in stimulating OPC proliferation and differentiation in a cuprizone-induced mouse model of MS. Only a small subpopulation of oligodendrocytes expresses CXCR2 in human fetal and adult MS tissue (Filipovic et al., 2003; Filipovic & Zecevic, 2008), therefore CXCL1 acts mainly on human oligodendrocytes through other cell types such as astrocytes (Bradl & Lassmann, 2010).

CXCL12, another member of the CXC-family, exerts different effects on OPCs and oligodendrocytes through its two receptors CXCR4 and CXCR7 (Kremer et al., 2016; Maysami et al., 2006). Initially, CXCL12 stimulates migration of OPCs through CXCR4 that activates downstream MEK/ERK and PI3K/Akt pathways (Tian et al., 2018). During normal development, CXCR4 is downregulated and CXCR7 is upregulated (Dziembowska et al., 2005; Göttle et al., 2010), thereby switching the role of CXCL12 to

promote differentiation and maturation through CXCR7 in later stages of development (Göttle et al., 2010; Kremer et al., 2016). Although downregulated during development, CXCR4 was also found to be crucial for oligodendrocyte maturation in a mouse model of cuprizone-induced demyelination, where blockade of the CXCR4 receptor using either a pharmacological or genetic (lentiviral) approach, decreased the number of mature oligodendrocytes, while increasing the pre-oligodendrocyte population (Patel et al., 2010).

Next to direct effects of CXC-chemokines on cells of the oligodendrocyte-lineage, they could indirectly affect EoP through their role in inflammatory processes. For example, CXCL1 and CXCL5 can contribute to neuroinflammation through their mutual receptor CXCR2. In a rat model of hypoxic-ischemic injury, antagonizing CXCR2 attenuated microglial activation which rescued myelination and BBB integrity, whereas supplementing CXCL5 caused the opposite effect (Wang et al., 2016). Antagonizing CXCR2 similarly reduced neuroinflammation and preserved white matter microstructure in a mouse model of chorioamnionitis (Yellowhair et al., 2019). CXCL12 secretion by microglia is increased in response to hypoxia *in vitro* and in neonatal and adult rodents after hypoxic-ischemic injury (Kaur et al., 2009; Li et al., 2015b), but CXCL12 expression is downregulated in brain endothelial cells *in vitro* after stimulation with LPS or TNF-α (Silwedel et al., 2018). In contrast to CXCL1 and CXCL5, decreased CXCL12 levels caused by neuroinflammation may cause additional harm, as was shown in an EAE model where antagonism of CXCL12 exacerbated WMI (Miljkovic et al., 2011).

In humans, upregulation of certain CXCs have been implicated in several CNS diseases with an inflammatory hallmark, such as MS, Alzheimer's disease, and ischemic stroke (Mirabelli-Badenier et al., 2011), and are also associated with preterm birth (Aminzadeh et al., 2012; Keelan et al., 2004; Malamitsi-Puchner et al., 2006). CXC-family members have not yet been used in clinical trials for the prevention of EoP (clinicaltrials.gov).

Other factors

Several other (growth) factors have been implicated in oligodendrocyte lineage development and survival, but evidence underlining their potential efficacy and working mechanisms to combat EoP are still limited. Factors such as bFGF, PDGF, granulocyte-colony stimulating factor (G-CSF) and hepatocyte growth factor (HGF) are primarily known as (OPC) mitogens (Armstrong et al., 2002; PDGF: Baron et al.,

2000; Frost et al., 2003; bFGF: Goddard et al., 2001; Hill et al., 2013; Hu et al., 2008; Mela & Goldman, 2013; Mitew et al., 2014; Ohya et al., 2007; Sil et al., 2018; Wilson et al., 2003; HGF: Yan & Rivkees, 2002), which keep OPCs in the cell cycle and restrict their differentiation. Concomitant upregulation of several of these factors after brain injury may help sustain the immature OPC population (Armstrong et al., 2002; Furusho et al., 2015) and thus inhibit myelination. However, some of these factors could contribute to oligodendrocyte maturation and subsequent myelination, either through a direct effect on oligodendrocytes or through facilitating a better intracerebral milieu.

bFGF and PDGF are significantly reduced in preterm compared to term umbilical cord blood (Gródecka-Szwajkiewicz et al., 2020). Both bFGF and PDGF are known to stimulate OPC proliferation, but bFGF may exert this effect through inhibition of maturation, keeping OPCs in the cell cycle, while PDGF induces both proliferation and maturation (Murtie et al., 2005). However, more recent studies with selective knockout mice have implicated FGFR1/2 in proper myelin formation, axonal ensheathment and in remyelinating capacity after injury (Furusho et al., 2012; Furusho et al., 2015). FGFR3 is present on OPCs and is downregulated after hypoxic-ischemic injury (Furusho et al., 2012; Qu et al., 2015). Prolonged i.p. treatment with bFGF ameliorated WMI in a HI rat model, through increasing the number of myelinated axons and thickness of the myelin sheath, while upregulating FGFR3 expression in pre-OLs (Qu et al., 2015). bFGF therapy has also shown some efficacy in restoring myelination in mouse models of MS (Dehghan et al., 2012; Furusho et al., 2012). Consistent with *in vitro* evidence, PDGF-A was found to be important for oligodendrocyte maturation as endogenous production was reduced after hyperoxia in neonatal rats, corresponding to a reduction in mature oligodendrocytes and hypomyelination (Scheuer et al., 2015). Similarly, PDGF-α +/- mice had a reduced number of mature oligodendrocytes in the corpus callosum after 6-weeks recovery from cuprizone treatment to mimic MS (Murtie et al., 2005). Although primarily known as OPC mitogens, novel roles for bFGF and PDGF in oligodendrocyte maturation and myelination that have been discovered more recently may warrant further investigation into these factors for the treatment of EoP.

An upregulation in G-CSF levels in umbilical cord blood and amniotic fluid has been associated with preterm brain injury (Lu et al., 2018; Lu et al., 2016). Increased levels of G-CSF may be a protective response, as some results have indicated efficacy of G-CSF against hallmarks of EoP, such as neuroinflammation (Jellema et al., 2013;

Kadota et al., 2012; Peng, 2017; Song et al., 2016), excitotoxicity (Neubauer et al., 2016) and apoptosis through activation of the JAK/STAT, PI3K/Akt and ERK pathways (see Box 2; Kim et al., 2008; Yata et al., 2007). Consequently, improved oligodendrocyte maturation and myelination after G-CSF treatment was observed in an ovine model of preterm brain injury (Jellema et al., 2013) and in traumatic nerve injuries (Kadota et al., 2012; Song et al., 2016). Conflicting results have been obtained in rodent models of preterm brain injury, ranging from adverse effects of G-CSF on brain injury and apoptosis, to positive or absent effects after delayed administration (Keller et al., 2006; Neubauer et al., 2016; Schlager et al., 2011). Together, these results indicate some positive effects of G-CSF on apoptosis and inflammation exist, but timing seems crucial to its efficacy in EoP. Enteral administration of G-CSF has already been utilized in preterm infants to induce feeding tolerance and reduce the risk of NEC (El-Ganzoury et al., 2014), but studies on the effect of G-CSF on the preterm brain have not been conducted yet (clinicaltrials.gov). However, G-CSF has been tested for the treatment of several other neurological diseases, such as Parkinson's disease and cerebral palsy (Koh et al., 2018; Tsai et al., 2017).

c-Met receptor ligands HGF and CD82 are also involved in oligodendrocyte lineage progression. HGF mostly contributes to OPC proliferation and migration, keeping OPCs in an immature state and decreasing after birth (Mela & Goldman, 2013; Ohya et al., 2007; Yan & Rivkees, 2002). In contrast, expression of the c-Met antagonist CD82 emerges later in postnatal brain development and is involved in oligodendrocyte maturation and myelination (Mela & Goldman, 2009, 2013). However, no *in vivo* evidence is available on efficacy of CD82 administration in models of white matter pathologies.

Table 1. Factors promoting each stage of oligodendrocyte lineage progression and survival, and their effects on neuroinflammation

	Proliferation	Differentiation	Myelin sheath formation	OL survival	Inflammation
IGF-1	●	●●		●●	○
EGF-family					
EGF	(●)	●●		○	pro
TGF-α				●●	○
NRGs	● (GGF2)	●	●●	●	○
TGF-β family					
TGF-β	(●)	●●			anti
Noggin		●●			
GDNF	●●	●●	●●	●●	○
Neurotrophins		●●	●●		anti
Gp130 family					
IL-6		●			pro
IL-11		●●		●	anti
CNTF		●		●	pro
LIF	○	●	●●	●	anti
IL-4		○		●	anti
IL-10		●●		●●	anti
CXCL1/5	(●)	●			pro
CXCL12	●	●			anti
Other factors					
bFGF	●●		●●		
PDGF	●●	●●			
G-CSF		○	○	○	anti
HGF	●●				
CD82		●	●		

● = only *in vitro* evidence available; ●● = *in vitro* and *in vivo* evidence available; ○ = inconclusive; (●) = indirect effect (i.e. through interaction with other factors or cell types such as astrocytes)

OL = oligodendrocyte, IGF-1 = insulin-like growth factor 1, EGF = epidermal growth factor, TGF = transforming growth factor (α and β), NRGs = neuregulins, GDNF = glial cell-line derived neurotrophic factor, Gp130 = glycoprotein 130, IL = interleukin (6, 4, 10 and 11), CNTF = ciliary neurotrophic factor, LIF = leukemia inhibitory factor, CXCL = CXC ligand, bFGF = basic fibroblast growth factor, PDGF = platelet-derived growth factor, G-CSF = granulocyte colony-stimulating factor, HGF = hepatocyte growth factor, CD82 = cluster of differentiation 82, GGF2 = glial growth factor-2.

FUTURE PERSPECTIVES

In this review, we have illustrated that a wide range of growth factors and cytokines can impact oligodendrocyte lineage maturation and microglia/astrocyte activation during healthy brain development and after injurious conditions like dWMI (Table 1). For some factors, such as IGF-1, a large body of evidence supporting its beneficial potential in dWMI is available from both *in vitro* and *in vivo* models of (neonatal) WMI. Other factors like GDNF and CNTF, have been implicated in healthy oligodendrocyte development, or increased expression of factors like IL-11 and EGF, has been shown as an (inadequate) endogenous protective response following (diffuse) WMI but currently lack vigorous evidence in models of preterm dWMI. Moreover, for some of these factors, like LIF, NGF, TGF-α and GDNF, evidence seems inconclusive as some studies describe beneficial properties where others describe detrimental effects of the factor on oligodendrocyte lineage development or neuroinflammation. With the current knowledge, many challenges still lie ahead when translating these experimental in vitro and in vivo studies on trophic and immunomodulatory factors to the field of EoP and clinical application further down the line.

One of these challenges is the interpretation of data obtained in experimental rodent models of oligodendrocyte development to the human developing brain and to interpret data of other (adult) white matter pathologies to preterm dWMI. Even though rodent and human white matter development share similarities, such as the origin and migratory pattern of OPCs and the caudal-to-rostral pattern of myelin formation, there are also some apparent dissimilarities (van Tilborg et al., 2018). For example, while rodent oligodendrocytes widely express CXCR2, the receptor that mediates the pro-differentiation effect of CXCL1, CXCR2 expression in human oligodendrocytes is scarce (Filipovic & Zecevic, 2008). Similarly, the pro-differentiating effect of bFGF was shown to be limited in human OPCs (Wilson et al., 2003), making these factors less attractive for clinical application. Thus, potential differences between species should be considered carefully when translating a promising factor from experimental studies to the clinic. To facilitate translationability from rodent studies to humans, van Tilborg et al. (2018) proposed the use of human iPSC-derived OPCs to complement the limited supply of fetal human tissue, as an alternative to study the efficacy of these factors on human oligodendrocyte lineage development. iPSCs could also be used to generate cerebral organoids, providing a more complex structure to study the therapeutic potential of trophic factors, including the interaction

with other glial cell types (Kim et al., 2019; Madhavan et al., 2018; Ormel et al., 2018). Moreover, when interpreting data from other (adult) animal models, differences in the pathophysiology of preterm dWMI compared to other (adult) white matter diseases should be considered. In preterm dWMI, myelin formation is hampered due to a developmental oligodendrocyte *maturation arrest*, while in neonatal stroke or HIE, loss of white matter volume is accompanied by pronounced oligodendrocyte apoptosis. In addition, preterm dWMI is caused by impaired a priori myelination, whereas in adult models of stroke or other neurodegenerative diseases preexisting myelin sheaths are damaged and need to be remyelinated by the trophic therapies. Furthermore, other white matter disease models like MS have a considerable different pathophysiology in which immune system dysfunction causes demyelination (Compston & Coles, 2002; Gutierrez-Fernandez et al., 2013; Mifsud et al., 2014). Thus, to make the next step towards clinical application, additional research on the efficacy of promising factors in clinically relevant models of preterm dWMI, in combination with *in vitro* experiments using human glial cells is strongly advised.

Another challenge in clinical translationability is determination of the most optimal treatment strategy for preterm dWMI. One option would be to select the most promising candidate factor for mono-therapy, preferably an all-round factor that boosts oligodendrocyte maturation while simultaneously dampening neuroinflammation. As summarized in this review, BDNF, NRG1, IL-11, LIF, IL-10 and CXCL12 seem to possess these all-round properties, and are therefore, in our opinion, the most promising candidates for mono-therapy. Even though IGF-1 possibly does not tick both these boxes, we believe it should be considered as a mono-therapeutic option, due to the extensive amount of evidence on its role in healthy white matter development and potential to boost myelination after neonatal brain injury, gathered from multiple *in vitro and in vivo* models. Moreover, recent clinical research has deemed IGF-1 administration to extreme preterm infants to be safe (Ley et al., 2013; Lofqvist et al., 2009). Another option is administration of a cocktail of multiple promising factors, aimed at oligodendrocyte survival, differentiation and reduction of neuroinflammation. The contents and timing of such a trophic/immunomodulatory factor-cocktail could be given in a tailor-made way, following the patient's disease course, for example by administration of factors with anti-inflammatory properties during episodes of inflammation. Tailor-made treatment could also reduce safety-related issues, by preventing adverse effects that could result from an interaction with the environment, such as a high dose of IGF-1 during an episode of acute inflammation. Another strategy to pre-

vent side effects due to undesirable interactions could be targeted delivery of factors to specific cell types in the brain, using nanoparticles (McMillan et al., 2011; Rittchen et al., 2015). In recent years, natural or synthetic nanoparticles, binding or encapsulating a multitude of therapeutic agents, have been proposed as carriers for targeted delivery of drugs to the CNS surpassing the BBB (see below; Barbu et al., 2009; Nag & Delehanty, 2019; Patra et al., 2018). Additionally, nanoparticles were shown to release the drug of interest in a controlled manner, preventing high, potentially toxic drug concentrations and prolonging the therapeutic effect (Teleanu et al., 2019). Interestingly, pioneer studies have shown the potential of engineered nanoparticles expressing cell-specific ligands on their outer surface, allowing cell-specific targeting, limiting undesirable interactions with other cell types (Rittchen et al., 2015). Though promising, some hurdles for clinical application of nanoparticles remain. Ligand-specificity might prove to be challenging, especially considering potential changes in receptor expression on the surface of target cells, either in development or in response to environmental cues (i.e. inflammation; Luo et al., 2020; Mi et al., 2020). Moreover, additional challenges include reliable reproducibility in nanoparticle syntheses, the avoidance of nanoparticle clearance by macrophages using nanoparticle coatings, and the optimization of biodegradability and subsequent clearance of nanoparticles after administration (Nag & Delehanty, 2019). The proposed route of personalized medicine to ensure optimal treatment efficacy and safety for each individual patient could be supported by identification of biomarkers, for instance the low serum IGF-1 observed in (extreme) preterm infants. Aside from serum concentrations in preterm patients, analysis of genetic profiles could contribute to development of personalized medicine, for example by selecting children that lack the protective NRG1 SNP discussed earlier, or children with genetic variants that are associated with exacerbation of neuroinflammation (van Tilborg et al., 2016). Apart from factor-based therapy, other therapies that affect cerebral growth factor and cytokine concentrations during brain development have been identified, such as nutritional interventions (Hortensius et al., 2019; Keunen et al., 2015) and mesenchymal stem cell (MSC) therapy (Vaes et al., 2019). These treatments could be used in conjunction with additional trophic factor or cytokine supplementation or possibly serve as an overarching alternative. Even though stem cell therapy and/or nutritional interventions could be considered as a more desirable option, due to a more continuous secretion of trophic and/or immunomodulatory factors, these therapies limit manipulation of the exact protein cocktail composition (Hortensius et al., 2019; Keunen et al., 2015; Vaes et al., 2019). For this reason, some studies propose genetic modification of MSCs, inducing (transient)

overexpression of specific beneficial factors in a controlled manner (Vaes et al., 2019). Additional preclinical studies with back-to-back comparison of the efficacy of mono-therapy, cocktails of factors or overarching therapies like stem cell therapy in models of preterm dWMI are needed to substantiate these statements.

Treatments with a small number of trophic factors described in this review have made the first steps towards clinical application in phase I/II studies. Among things to consider in the process of clinical translation of these promising factors are the timing and mode of administration. Even though the optimal timing of treatment is still unclear, one could speculate that treatment with beneficial factors during a time window analogous to healthy white matter development, and thus prevention of myelination failure, could prove to be most effective. This strategy would entail long-term treatment during the first, vulnerable weeks of life after (extreme) preterm birth to aid white matter development. Additionally, the route of administration should be considered carefully. In animal studies, factors are often administered using intracerebral injections, intraperitoneal injection or intravenous infusion. Despite being relatively non-invasive and easy to implement, systemic administration of most of these factors is likely complicated due to loss of protein in the periphery, a relatively short half-life, and restricted passage over BBB, leading to limited supply to the CNS (Lochhead & Thorne, 2012; Thorne et al., 2004). These issues could be less pronounced following intravenous treatment in the early stages of injury, as BBB integrity has been reported to be compromised following inflammation in preterm infants (Douglas-Escobar & Weiss, 2012; Moretti et al., 2015; Yap & Perlman, 2020; Zhao et al., 2013). Moreover, CNS delivery of (larger) molecules following systemic administration, could be improved via focused ultrasound or use of nanoparticles. Focused ultrasound has been applied to transiently open the BBB in targeted areas, promoting entry of drugs from the circulation into the brain (Burgess et al., 2015; Konofagou et al., 2012). Similarly, nanoparticles were shown to induce local toxic effects, leading to BBB permeabilization, or support BBB passage through facilitation of endothelial trans- or endocytosis (Saraiva et al., 2016; Zhou et al., 2018). Aside from these options, one could speculate that loss of trophic or immunomodulatory factors in the periphery following intravenous administration could still benefit the preterm patient with multi-organ failure, by dampening systemic inflammation and aiding in development of other organs. Optimal CNS delivery could be attained by local intracerebral injection, preferably using viral vector-mediated gene transfer, inducing long-lasting expression of the transgene, avoiding repeated invasive injections (Bemelmans et al., 2006; Brizard et al., 2006;

Lim et al., 2010). Intranasal administration provides a non-invasive method of local delivery of therapeutic agents to the brain, bypassing the BBB, and therefore might prove to be the most favorable route to directly target the CNS (Hanson & Frey, 2008; Lochhead & Thorne, 2012; Thorne et al., 2004; Vaes et al., 2019).

Though not our primary focus, preterm birth-related events, i.e. hypoxia and inflammation, have been shown to disrupt the development of other immature cell types, such as cortical interneurons (Ardalan et al., 2019; Duchatel et al., 2019; Lacaille et al., 2019; Stolp et al., 2019). Interestingly, interneurons have been shown to play a role in oligodendrocyte development (Benamer et al., 2020; Zonouzi et al., 2015) and could therefore be essential for healthy white matter development. Thus, interventions aimed to target the common pathophysiological pathways are likely to positively impact multiple aspects of EoP, potentially restoring both myelination failure and interneuron deficits.

Despite the challenges yet to overcome for clinical translation, this review proposes a broad range of potential therapeutic trophic/immunomodulatory targets, known to aid myelination by directly boosting oligodendrocyte lineage development and/or by modulation of neuroinflammation. Future studies are urgently needed to refine these treatment strategies for preterm dWMI, in order to improve the neurodevelopmental prospects for these vulnerable patients.

REFERENCES

Acosta, C. M., Cortes, C., MacPhee, H., & Namaka, M. P. (2013). Exploring the role of nerve growth factor in multiple sclerosis: implications in myelin repair. *CNS & Neurological Disorders Drug Targets, 12*(8), 1242-1256. doi:10.2174/18715273113129990087

Adachi, T., Takanaga, H., Kunimoto, M., & Asou, H. (2005). Influence of LIF and BMP-2 on differentiation and development of glial cells in primary cultures of embryonic rat cerebral hemisphere. *Journal of Neuroscience Research, 79*(5), 608-615. doi:10.1002/jnr.20373

Aguirre, A., Dupree, J. L., Mangin, J. M., & Gallo, V. (2007). A functional role for EGFR signaling in myelination and remyelination. *Nature Neuroscience, 10*(8), 990-1002. doi:10.1038/nn1938

Allender, E., Deol, H., Schram, S., Maheras, K. J., Gow, A., Simpson, E. H., & Song, F. (2018). Neuregulin1 modulation of experimental autoimmune encephalomyelitis (EAE). *Journal of Neuroimmunology, 318*, 56-64. doi:10.1016/j.jneuroim.2018.02.008

Aminzadeh, F., Ghorashi, Z., Nabati, S., Ghasemshirazi, M., Arababadi, M. K., Shamsizadeh, A., . . . Hassanshahi, G. (2012). Differential Expression of CXC Chemokines CXCL10 and CXCL12 in Term and Pre-term Neonates and Their Mothers. *American Journal of Reproductive Immunology, 68*(4), 338-344. doi:10.1111/j.1600-0897.2012.01167.x

Amir-Levy, Y., Mausner-Fainberg, K., & Karni, A. (2014). Treatment with Anti-EGF Ab Ameliorates Experimental Autoimmune Encephalomyelitis via Induction of Neurogenesis and Oligodendrogenesis. *Multiple Sclerosis International, 2014*, 1-9. doi:10.1016/j.scr.2014.06.001

Ardalan, M., Chumak, T., Vexler, Z., & Mallard, C. (2019). Sex-Dependent Effects of Perinatal Inflammation on the Brain: Implication for Neuro-Psychiatric Disorders. *International Journal of Molecular Sciences, 20*(9), 2270. doi:10.3390/ijms20092270

Armstrong, R. C., Le, T. Q., Frost, E. E., Borke, R. C., & Vana, A. C. (2002). Absence of fibroblast growth factor 2 promotes oligodendroglial repopulation of demyelinated white matter. *Journal of Neuroscience, 22*(19), 8574-8585. doi:10.1523/jneurosci.22-19-08574.2002

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathologica, 134*(3), 331-349. doi:10.1007/s00401-017-1718-6

Back, S. A., L., L. N., Borenstein, N. S., Levine, J. M., Volpe, J. J., & Kinney, H. C. (2001). Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *Journal of Neuroscience, 21*(4), 1302-1312. doi:10.1523/JNEUROSCI.21-04-01302.2001

Back, S. A., & Miller, S. P. (2014). Brain injury in premature neonates: A primary cerebral dysmaturation disorder? *Annals of Neurology, 75*(4), 469-486. doi:10.1002/ana.24132

Bajetto, A., Bonavia, R., Barbero, S., & Schettini, G. (2002). Characterization of chemokines and their receptors in the central nervous system: Physiopathological implications. *Journal of Neurochemistry, 82*(6), 1311-1329. doi:10.1046/j.1471-4159.2002.01091.x

Banisadr, G., Rostène, W., Kitabgi, P., & Parsadaniantz, S. M. (2005). Chemokines and brain functions. *Current Drug Targets: Inflammation and Allergy, 4*(3), 387-399. doi:10.2174/1568010054022097

Barbu, E., Molnàr, É., Tsibouklis, J., & Górecki, D. C. (2009). The potential for nanoparticle-based drug delivery to the brain: overcoming the blood–brain barrier. *Expert Opinion on Drug Delivery, 6*(6), 553-565. doi:10.1517/17425240902939143

Baron, W., Metz, B., Bansal, R., Hoekstra, D., & De Vries, H. (2000). PDGF and FGF-2 signaling in oligodendrocyte progenitor cells: Regulation of proliferation and differentiation by multiple intracellular signaling pathways. *Molecular and Cellular Neurosciences, 15*(3), 314-329. doi:10.1006/mcne.1999.0827

Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyyodic, J. T., Richardson, W. D., & Raff, M. C. (1992). Cell-Death and Control of Cell-Survival in the Oligodendrocyte Lineage. *Cell, 70*, 31-46. doi:10.1016/0092-8674(92)90531-G

Barres, B. A., Raff, M. C., Gaese, F., Bartke, I., Dechant, G., & Barde, Y. A. (1994). A crucial role for neurotrophin-3 in oligodendrocyte development. *Nature, 367*, 371-375. doi:10.1038/246170a0

Baud, O., & Saint-Faust, M. (2019). Neuroinflammation in the Developing Brain: Risk Factors, Involvement of Microglial Cells, and Implication for Early Anesthesia. *Anesthesia and Analgesia, 128*(4), 718-725. doi:10.1213/ANE.0000000000004032

Beck, K. D., Powell-Braxton, L., Widmer, H. R., Valverde, J., & Hefti, F. (1995). Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. *Neuron, 14*(4), 717-730. doi:10.1016/0896-6273(95)90216-3

Bemelmans, A. P., Husson, I., Jaquet, M., Mallet, J., Kosofsky, B. E., & Gressens, P. (2006). Lentiviral-mediated gene transfer of brain-derived neurotrophic factor is neuroprotective in a mouse model of neonatal excitotoxic challenge. *Journal of Neuroscience Research, 83*(1), 50-60. doi:10.1002/jnr.20704

Benamer, N., Vidal, M., & Angulo, M. C. (2020). The cerebral cortex is a substrate of multiple interactions between GABAergic interneurons and oligodendrocyte lineage cells. *Neuroscience Letters, 715*, 134615. doi:10.1016/j.neulet.2019.134615

Bernd, P. (2008). The role of neurotrophins during early development. *Gene Expression, 14*(4), 241-250. doi:10.3727/105221608786883799

Bhat, N. R., Zhang, P., & Mohanty, S. B. (2007). p38 MAP kinase regulation of oligodendrocyte differentiation with CREB as a potential target. *Neurochemical Research, 32*(2), 293-302. doi:10.1007/s11064-006-9274-9

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., . . . Lawn, J. E. (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*(9832), 2162-2172. doi:10.1016/S0140-6736(12)60820-4

Bohlen, C. J., Friedman, B. A., Dejanovic, B., & Sheng, M. (2019). Microglia in Brain Development, Homeostasis, and Neurodegeneration. *Annual Review of Genetics, 53*(1), 263-288. doi:10.1146/annurev-genet-112618-043515

Bonetto, G., Charalampopoulos, I., Gravanis, A., & Karagogeos, D. (2017). The novel synthetic microneurotrophin BNN27 protects mature oligodendrocytes against cuprizone-induced death, through the NGF receptor TrkA. *Glia, 65*(8), 1376-1394. doi:10.1002/glia.23170

Bradl, M., & Lassmann, H. (2010). Oligodendrocytes: biology and pathology. *Acta Neuropathologica, 119*(1), 37-53. doi:10.1007/s00401-009-0601-5

Brehmer, F., Bendix, I., Prager, S., van de Looij, Y., Reinboth, B. S., Zimmermanns, J., . . . Gerstner, B. (2012). Interaction of Inflammation and Hyperoxia in a Rat Model of Neonatal White Matter Damage. *PloS One, 7*(11), 1-13. doi:10.1371/journal.pone.0049023

Brill, C., Scheuer, T., Bührer, C., Endesfelder, S., & Schmitz, T. (2017). Oxygen impairs oligodendroglial development via oxidative stress and reduced expression of HIF-1α. *Scientific Reports, 7*, 43000. doi:10.1038/srep43000

Brizard, M., Carcenac, C., Bemelmans, A.-P., Feuerstein, C., Mallet, J., & Savasta, M. (2006). Functional reinnervation from remaining DA terminals induced by GDNF lentivirus in a rat model of early Parkinson's disease. *Neurobiology of Disease, 21*(1), 90-101. doi:10.1016/j.nbd.2005.06.015

Brywe, K. G., Mallard, C., Gustavsson, M., Hedtjarn, M., Leverin, A. L., Wang, X., . . . Hagberg, H. (2005). IGF-I neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3beta? *European Journal of Neuroscience, 21*(6), 1489-1502. doi:10.1111/j.1460-9568.2005.03982.x

Burgess, A., Shah, K., Hough, O., & Hynynen, K. (2015). Focused ultrasound-mediated drug delivery through the blood-brain barrier. *Expert Review of Neurotherapeutics, 15*(5), 477-491. doi:10.1586/14737175.2015.1028369

Buser, J. R., Maire, J., Riddle, A., Gong, X., Nguyen, T., Nelson, K., . . . Back, S. A. (2012). Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. *Annals of Neurology, 71*(1), 93-109. doi:10.1002/ana.22627

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A. E., Pluchino, S., . . . Schwartz, M. (2006). Microglia activated by IL-4 or IFN-γ differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Molecular and Cellular Neuroscience 31*(1), 149-160. doi:10.1016/j.mcn.2005.10.006

Butzkueven, H., Emery, B., Cipriani, T., Marriott, M. P., & Kilpatrick, T. J. (2006). Endogenous leukemia inhibitory factor production limits autoimmune demyelination and oligodendrocyte loss. *Glia, 53*(7), 696-703. doi:10.1002/glia.20321

Cai, Z., Fan, L. W., Lin, S., Pang, Y., & 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

Cakmak, H., Schatz, F., Huang, S. T. J., Buchwalder, L., Rahman, M., Arici, A., & Lockwood, C. J. (2005). Progestin suppresses thrombin- and interleukin-1β-induced interleukin-11 production in term decidual cells: Implications for preterm delivery. *Journal of Clinical Endocrinology and Metabolism, 90*(9), 5279-5286. doi:10.1210/jc.2005-0210

Calaora, V., Register, B., Bismuth, K., Murray, K., Brandt, H., Leprince, P., . . . Dubois-Dalcq, M. (2001). Neuregulin signaling regulates neural precursor growth and the generation of oligodendrocytes in vitro. *Journal of Neuroscience, 21*(13), 4740-4751. doi:10.1523/jneurosci.21-13-04740.2001

Cannella, B., & Raine, C. S. (2004). Multiple Sclerosis: Cytokine Receptors on Oligodendrocytes Predict Innate Regulation. *Annals of Neurology, 55*(1), 46-57. doi:10.1002/ana.10764

Canoll, P. D., Kraemer, R., Teng, K. K., Marchionni, M. A., & Salzer, J. L. (1999). GGF/neuregulin induces a phenotypic reversion of oligodendrocytes. *Molecular and Cellular Neurosciences, 13*(2), 79-94. doi:10.1006/mcne.1998.0733

Cao, Y., Gunn, A. J., Bennet, L., Wu, D., George, S., Gluckman, P. D., . . . Guan, J. (2003). Insulin-like growth factor (IGF)-1 suppresses oligodendrocyte caspase-3 activation and increases glial proliferation after ischemia in near-term fetal sheep. *Journal of Cerebral Blood Flow and Metabolism, 23*(6), 739-747. doi:10.1097/01.wcb.0000067720.12805.6f

Cappelletti, M., Della Bella, S., Ferrazzi, E., Mavilio, D., & Divanovic, S. (2016). Inflammation and preterm birth. *Journal of Leukocyte Biology, 99*(1), 67-78. doi:10.1189/jlb.3mr0615-272rr

Carson, M. J., Behringer, R. R., Brinster, R. L., & McMorris, F. A. (1993). Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. *Neuron, 10*(4), 729-740. doi:10.1016/0896-6273(93)90173-o

Casaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T., & Chao, M. V. (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature, 383*, 716-719. doi:10.1038/383716a0

Cekanaviciute, E., Fathali, N., Doyle, K. P., Williams, A. M., Han, J., & Buckwalter, M. S. (2014). Astrocytic transforming growth factor-beta signaling reduces subacute neuroinflammation after stroke in mice. *Glia, 62*(8), 1227-1240. doi:10.1002/glia.22675

Chan, J. R., Watkins, T. A., Cosgaya, J. M., Zhang, C., Chen, L., Reichardt, L. F., . . . Barres, B. A. (2004). NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes. *Neuron, 43*(2), 183-191. doi:10.1016/j.neuron.2004.06.024

Chew, E. Y., Clemons, T. E., Jaffe, G. J., Johnson, C. A., Farsiu, S., Lad, E. M., . . . Friedlander, M. (2019). Effect of Ciliary Neurotrophic Factor on Retinal Neurodegeneration in Patients with Macular Telangiectasia Type 2: A Randomized Clinical Trial. *Ophthalmology, 126*(4), 540-549. doi:10.1016/j.ophtha.2018.09.041

Chew, L. J., Coley, W., Cheng, Y., & Gallo, V. (2010). Mechanisms of regulation of oligodendrocyte development by p38 mitogen-activated protein kinase. *Journal of Neuroscience, 30*(33), 11011-11027. doi:10.1523/JNEUROSCI.2546-10.2010

Chung, S. H., Biswas, S., Selvaraj, V., Liu, X. B., Sohn, J., Jiang, P., . . . Deng, W. (2015). The p38α mitogen-activated protein kinase is a key regulator of myelination and remyelination in the CNS. *Cell Death & Disease, 6*(5), 1-10. doi:10.1038/cddis.2015.119

Cohen, R. I., Marmur, R., Norton, W. T., Mehler, M. F., & Kessler, J. A. (1996). Nerve growth factor and neurotrophin-3 differentially regulate the proliferation and survival of developing rat brain oligodendrocytes. *Journal of Neuroscience, 16*(20), 6433-6442. doi:10.1523/jneurosci.16-20-06433.1996

Compston, A., & Coles, A. (2002). Multiple sclerosis. *Lancet, 359*(9313), 1221-1231. doi:10.1016/s0140-6736(02)08220-x

Covey, M. V., & Levison, S. W. (2007). Leukemia inhibitory factor participates in the expansion of neural stem/progenitors after perinatal hypoxia/ischemia. *Neuroscience, 148*(2), 501-509. doi:10.1016/j.neuroscience.2007.06.015

Cragnolini, A. B., Huang, Y., Gokina, P., & Friedman, W. J. (2009). Nerve growth factor attenuates proliferation of astrocytes via the p75 neurotrophin receptor. *Glia, 57*(13), 1386-1392. doi:10.1002/glia.20857

D'Ercole, A. J., & Ye, P. (2008). Expanding the mind: Insulin-like growth factor I and brain development. *Endocrinology, 149*(12), 5958-5962. doi:10.1210/en.2008-0920

D'Souza, S. D., Alinauskas, K. A., & Antel, J. P. (1996). Ciliary neurotrophic factor selectively protects human oligodendrocytes from tumor necrosis factor-mediated injury. *Journal of Neuroscience Research, 43*(3), 289-298. doi:10.1002/(SICI)1097-4547(19960201)43:3<289::AID-JNR4>3.0.CO;2-F

Dai, X., Chen, J., Xu, F., Zhao, J., Cai, W., Sun, Z., . . . Hu, X. (2019). TGFα preserves oligodendrocyte lineage cells and improves white matter integrity after cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism, 40*(3), 639-655. doi:10.1177/0271678X19830791

Dammann, O., Bueter, W., Leviton, A., Gressens, P., & Dammann, C. E. L. (2008). Neuregulin-1: A potential endogenous protector in perinatal brain white matter damage. *Neonatology, 93*(3), 182-187. doi:10.1159/000111119

Davis, S. M., & Pennypacker, K. R. (2018). The role of the leukemia inhibitory factor receptor in neuroprotective signaling. *Pharmacology and Therapeutics, 183*(August 2017), 50-57. doi:10.1016/j.pharmthera.2017.08.008

Dehghan, S., Javan, M., Pourabdolhossein, F., Mirnajafi-Zadeh, J., & Baharvand, H. (2012). Basic fibroblast growth factor potentiates myelin repair following induction of experimental demyelination in adult mouse optic chiasm and nerves. *Journal of Molecular Neuroscience, 48*(1), 77-85. doi:10.1007/s12031-012-9777-6

Del Bigio, M. R., & Becker, L. E. (1994). Microglial aggregation in the dentate gyrus: a marker of mild hypoxic-ischaemic brain insult in human infants. *Neuropathology and Applied Neurobiology, 20*(2), 144-151. doi:10.1111/j.1365-2990.1994.tb01173.x

Deng, W. (2010). Neurobiology of injury to the developing brain. *Nature Reviews: Neurology, 6*(1759-4766 (Electronic)), 328-336. doi:10.1038/nrneurol.2010.53

Dhobale, M. (2014). Neurotrophins: Role in adverse pregnancy outcome. *International Journal of Developmental Neuroscience, 37*, 8-14. doi:10.1016/j.ijdevneu.2014.06.005

Dizon, M. L., Maa, T., & Kessler, J. A. (2011). The bone morphogenetic protein antagonist noggin protects white matter after perinatal hypoxia-ischemia. *Neurobiology of Disease, 42*(3), 318-326. doi:10.1016/j.nbd.2011.01.023

Dobolyi, A., Vincze, C., Pál, G., & Lovas, G. (2012). The neuroprotective functions of transforming growth factor beta proteins. *International Journal of Molecular Sciences, 13*(7), 8219-8258. doi:10.3390/ijms13078219

Dodge, J. C., Haidet, A. M., Yang, W., Passini, M. A., Hester, M., Clarke, J., . . . Kaspar, B. K. (2008). Delivery of AAV-IGF-1 to the CNS extends survival in ALS mice through modification of aberrant glial cell activity. *Molecular Therapy, 16*(6), 1056-1064. doi:10.1038/mt.2008.60

Dördelmann, M., Kerk, J., Dressler, F., Brinkhaus, M. J., Bartels, D. B., Dammann, C. E. L., . . . Dammann, O. (2006). Interleukin-10 high producer allele and ultrasound-defined periventricular white matter abnormalities in preterm infants: A preliminary study. *Neuropediatrics, 37*(3), 130-136. doi:10.1055/s-2006-924554

Douglas-Escobar, M., & Weiss, M. D. (2012). Biomarkers of brain injury in the premature infant. *Frontiers in Neurology, 3*, 185. doi:10.3389/fneur.2012.00185

Du, L., Zhang, Y., Chen, Y., Zhu, J., Yang, Y., & Zhang, H.-L. (2017a). Role of Microglia in Neurological Disorders and Their Potentials as a Therapeutic Target. *Molecular Neurobiology, 54*(10), 7567-7584. doi:10.1007/s12035-016-0245-0

Du, M., Tan, Y., Liu, G., Liu, L., Cao, F., Liu, J., . . . Xu, Y. (2017b). Effects of the Notch signalling pathway on hyperoxia-induced immature brain damage in newborn mice. *Neuroscience Letters, 653*, 220-227. doi:10.1016/j.neulet.2017.05.065

Duarte Azevedo, M., Sander, S., & Tenenbaum, L. (2020). GDNF, A Neuron-Derived Factor Upregulated in Glial Cells during Disease. *Journal of Clinical Medicine, 9*(2), 456-456. doi:10.3390/jcm9020456

Duarte, E. P., Curcio, M., Canzoniero, L. M., & Duarte, C. B. (2012). Neuroprotection by GDNF in the ischemic brain. *Growth Factors, 30*(4), 242-257. doi:10.3109/08977194.2012.691478

Duchatel, R. J., Harms, L. R., Meehan, C. L., Michie, P. T., Bigland, M. J., Smith, D. W., . . . Tooney, P. A. (2019). Reduced cortical somatostatin gene expression in a rat model of maternal immune activation. *Psychiatry Research, 282*, 112621. doi:10.1016/j.psychres.2019.112621

Dummula, K., Vinukonda, G., Chu, P., Xing, Y., Hu, F., Mailk, S., . . . Ballabh, P. (2011). Bone morphogenetic protein inhibition promotes neurological recovery after intraventricular hemorrhage. *Journal of Neuroscience, 31*(34), 12068-12082. doi:10.1523/jneurosci.0013-11.2011

Dziembowska, M., Tham, T. N., Lau, P., Vitry, S., Lazarini, F., & Dubois-Dalcq, M. (2005). A role for CXCR4 signaling in survival and migration of neural and oligodendrocyte precursors. *Glia, 50*(3), 258-269. doi:10.1002/glia.20170

Eixarch, H., Calvo-Barreiro, L., Montalban, X., & Espejo, C. (2018). Bone morphogenetic proteins in multiple sclerosis: Role in neuroinflammation. *Brain, Behavior, and Immunity, 68*, 1-10. doi:10.1016/j.bbi.2017.02.019

El-Ganzoury, M. M., Awad, H. A., El-Farrash, R. A., El-Gammasy, T. M., Ismail, E. A., Mohamed, H. E., & Suliman, S. M. (2014). Enteral granulocyte-colony stimulating factor and erythropoietin early in life improves feeding tolerance in preterm infants: A randomized controlled trial. *Journal of Pediatrics, 165*(6), 1140-1145.e1141. doi:10.1016/j.jpeds.2014.07.034

Ferrer, I., Alcántara, S., Ballabriga, J., Olivé, M., Blanco, R., Rivera, R., . . . Planas, A. M. (1996). Transforming growth factor-α (TGF-α) and epidermal growth factor-receptor (EGF-R) immunoreactivity in normal and pathologic brain. *Progress in Neurobiology, 49*(2), 99-119. doi:10.1016/0301-0082(96)00009-3

Filipovic, R., Jakovcevski, I., & Zecevic, N. (2003). GRO-alpha and CXCR2 in the human fetal brain and multiple sclerosis lesions. *Developmental Neuroscience, 25*(2-4), 279-290. doi:10.1159/000072275

Filipovic, R., & Zecevic, N. (2008). The Effect of CXCL1 on Human Fetal Oligodendrocyte Progenitor Cells. *Glia, 56*(1), 1-15. doi:10.1002/glia.20582

Fischer, R., Wajant, H., Kontermann, R., Pfizenmaier, K., & Maier, O. (2014). Astrocyte-specific activation of TNFR2 promotes oligodendrocyte maturation by secretion of leukemia inhibitory factor. *Glia, 62*(2), 272-283. doi:10.1002/glia.22605

Fressinaud, C. (2005). Repeated injuries dramatically affect cells of the oligodendrocyte lineage: Effects of PDGF and NT-3 in vitro. *Glia, 49*(4), 555-566. doi:10.1002/glia.20136

Frost, E. E., Nielsen, J. A., Le, T. Q., & Armstrong, R. C. (2003). PDGF and FGF2 regulate oligodendrocyte progenitor responses to demyelination. *Journal of Neurobiology, 54*(3), 457-472. doi:10.1002/neu.10158

Furusho, M., Dupree, J. L., Nave, K. A., & Bansal, R. (2012). Fibroblast growth factor receptor signaling in oligodendrocytes regulates myelin sheath thickness. *Journal of Neuroscience, 32*(19), 6631-6641. doi:10.1523/jneurosci.6005-11.2012

Furusho, M., Roulois, A. J., Franklin, R. J., & Bansal, R. (2015). Fibroblast growth factor signaling in oligodendrocyte-lineage cells facilitates recovery of chronically demyelinated lesions but is redundant in acute lesions. *Glia, 63*(10), 1714-1728. doi:10.1002/glia.22838

Gaesser, J. M., & Fyffe-Maricich, S. L. (2016). Intracellular signaling pathway regulation of myelination and remyelination in the CNS. *Experimental Neurology, 283*, 501-511. doi:10.1016/j.expneurol.2016.03.008

Gao, X., Deng, L., Wang, Y., Yin, L., Yang, C., Du, J., & Yuan, Q. (2016). GDNF Enhances Therapeutic Efficiency of Neural Stem Cells-Based Therapy in Chronic Experimental Allergic Encephalomyelitis in Rat. *Stem Cells International, 2016*, 1431349. doi:10.1155/2016/1431349

Gard, A. L., Burrell, M. R., Pfeiffer, S. E., Rudge, J. S., & Williams, W. C. (1995). Astroglial control of oligodendrocyte survival mediated by PDGF and leukemia inhibitory factor-like protein. *Development, 121*(7), 2187-2197. Retrieved from https://dev.biologists.org/content/121/7/2187.long

Genis, L., Dávila, D., Fernandez, S., Pozo-Rodrigálvarez, A., Martínez-Murillo, R., & Torres-Aleman, I. (2014). Astrocytes require insulin-like growth factor I to protect neurons against oxidative injury. *F1000Res, 3*, 28. doi:10.12688/f1000research.3-28.v2

Gluckman, P., Klempt, N., Guan, J., Mallard, C., Sirimanne, E., Dragunow, M., . . . Nikolics, K. (1992). A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochemical and Biophysical Research Communications, 182*(2), 593-599. doi:10.1016/0006-291x(92)91774-k

Goddard, D. R., Berry, M., Kirvell, S. L., & Butt, A. M. (2001). Fibroblast growth factor-2 inhibits myelin production by oligodendrocytes in vivo. *Molecular and Cellular Neuroscience, 18*(5), 557-569. doi:10.1006/mcne.2001.1025

Goldstein, E. Z., Church, J. S., Hesp, Z. C., Popovich, P. G., & McTigue, D. M. (2016). A silver lining of neuroinflammation: Beneficial effects on myelination. *Experimental Neurology, 283*, 550-559. doi:10.1016/j.expneurol.2016.05.001

Gómez Pinto, L. I., Rodríguez, D., Adamo, A. M., & Mathieu, P. A. (2018). TGF-β pro-oligodendrogenic effects on adult SVZ progenitor cultures and its interaction with the Notch signaling pathway. *Glia, 66*(2), 396-412. doi:10.1002/glia.23253

Gonsalvez, D., Ferner, A. H., Peckham, H., Murray, S. S., & Xiao, J. (2016). The roles of extracellular related-kinases 1 and 2 signaling in CNS myelination. *Neuropharmacology, 110*, 586-593. doi:10.1016/j.neuropharm.2015.04.024

González-Fernández, E., Jeong, H.-K., Fukaya, M., Kim, H., Khawaja, R. R., Srivastava, I. N., . . . Kang, S. H. (2018). PTEN negatively regulates the cell lineage progression from NG2+ glial progenitor to oligodendrocyte via mTOR-independent signaling. *eLife, 2018*(7), 32021. doi:10.7554/eLife.32021

Göttle, P., Kremer, D., Jander, S., Ödemis, V., Engele, J., Hartung, H. P., & Küry, P. (2010). Activation of CXCR7 receptor promotes oligodendroglial cell maturation. *Annals of Neurology, 68*(6), 915-924. doi:10.1002/ana.22214

Grinberg, Y. Y., Dibbern, M. E., Levasseur, V. A., & Kraig, R. P. (2013). Insulin-like growth factor-1 abrogates microglial oxidative stress and TNF-alpha responses to spreading depression. *Journal of Neurochemistry, 126*(5), 662-672. doi:10.1111/jnc.12267

Gródecka-Szwajkiewicz, D., Ulańczyk, Z., Zagrodnik, E., Łuczkowska, K., Rogińska, D., Kawa, M. P., . . . Machaliński, B. (2020). Differential secretion of angiopoietic factors and expression of microRNA in umbilical cord blood from healthy appropriate-for-gestational-age preterm and term newborns—in search of Biomarkers of Angiogenesis-Related Processes in Preterm Birth. *International Journal of Molecular Sciences, 21*(4), 1305. doi:10.3390/ijms21041305

Guan, J., Bennet, L., George, S., Wu, D., Waldvogel, H. J., Gluckman, P. D., . . . Gunn, a. J. (2001). Insulin-like growth factor-1 reduces postischemic white matter injury in fetal sheep. *Journal of Cerebral Blood Flow and Metabolism, 21*(5), 493-502. doi:10.1097/00004647-200105000-00003

Guan, J., Bennet, L., Gluckman, P. D., & Gunn, A. J. (2003). Insulin-like growth factor-1 and post-ischemic brain injury. *Progress in Neurobiology, 70*(6), 443-462. doi:10.1016/j.pneurobio.2003.08.002

Guardia Clausi, M., & Levison, S. W. (2017). Delayed ALK5 inhibition improves functional recovery in neonatal brain injury. *Journal of Cerebral Blood Flow and Metabolism, 37*(3), 787-800. doi:10.1177/0271678X16638669

Gutierrez-Fernandez, M., Rodriguez-Frutos, B., Ramos-Cejudo, J., Otero-Ortega, L., Fuentes, B., & Diez-Tejedor, E. (2013). Stem cells for brain repair and recovery after stroke. *Expert Opinion on Biological Therapy, 13*(11), 1479-1483. doi:10.1517/14712598.2013.824420

Hamaguchi, M., Muramatsu, R., Fujimura, H., Mochizuki, H., Kataoka, H., & Yamashita, T. (2019). Circulating transforming growth factor-β1 facilitates remyelination in the adult central nervous system. *eLife, 8*, 1-20. doi:10.7554/eLife.41869

Hammond, T. R., Robinton, D., & Stevens, B. (2018). Microglia and the Brain: Complementary Partners in Development and Disease. *Annual Review of Cell and Developmental Biology, 34*, 523-544. doi:10.1146/annurev-cellbio-100616-060509

Hansen-Pupp, I., Hellstrom, A., Hamdani, M., Tocoian, A., Kreher, N. C., Ley, D., & Hallberg, B. (2017). Continuous longitudinal infusion of rhIGF-1/rhIGFBP-3 in extremely preterm infants: Evaluation of feasibility in a phase II study. *Growth Hormone and IGF Research, 36*, 44-51. doi:10.1016/j.ghir.2017.08.004

Hansen-Pupp, I., Hovel, H., Lofqvist, C., Hellstrom-Westas, L., Fellman, V., Huppi, P. S., . . . Ley, D. (2013). Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatric Research, 74*(5), 564-569. doi:10.1038/pr.2013.135

Hanson, L. R., & 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 Neuroscience, 9*(3), S5. doi:10.1186/1471-2202-9-S3-S5

Hashimoto, S.-i., Komuro, I., Yamada, M., & Akagawa, K. S. (2001). IL-10 Inhibits Granulocyte-Macrophage Colony-Stimulating Factor-Dependent Human Monocyte Survival at the Early Stage of the Culture and Inhibits the Generation of Macrophages. *Journal of Immunology, 167*(7), 3619-3625. doi:10.4049/jimmunol.167.7.3619

Heldin, C. H., & Moustakas, A. (2016). Signaling receptors for TGF-β family members. *Cold Spring Harbor Perspectives in Biology, 8*(8), 1-34. doi:10.1101/cshperspect.a022053

Hellstrom, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Lofqvist, C., van Marter, L., . . . Smith, L. E. (2016). Insulin-like growth factor 1 has multisystem effects on foetal and preterm infant development. *Acta Paediatrica, 105*(6), 576-586. doi:10.1111/apa.13350

Hill, R. A., Patel, K. D., Medved, J., Reiss, A. M., & Nishiyama, A. (2013). NG2 cells in white matter but not gray matter proliferate in response to PDGF. *Journal of Neuroscience, 33*(36), 14558-14566. doi:10.1523/JNEUROSCI.2001-12.2013

Hoffmann, I., Bueter, W., Zscheppang, K., Brinkhaus, M. J., Liese, A., Riemke, S., . . . Dammann, C. E. L. (2010). Neuregulin-1, the fetal endothelium, and brain damage in preterm newborns. *Brain, Behavior, and Immunity, 24*(5), 784-791. doi:10.1016/j.bbi.2009.08.012

Hortensius, L. M., van Elburg, R. M., Nijboer, C. H., Benders, M. J. N. L., & de Theije, C. G. M. (2019). Postnatal Nutrition to Improve Brain Development in the Preterm Infant: A Systematic Review From Bench to Bedside. *Frontiers in Physiology, 10*(July), 1-18. doi:10.3389/fphys.2019.00961

Hu, J. G., Fu, S. L., Wang, Y. X., Li, Y., Jiang, X. Y., Wang, X. F., . . . Xu, X. M. (2008). Platelet-derived growth factor-AA mediates oligodendrocyte lineage differentiation through activation of extracellular signal-regulated kinase signaling pathway. *Neuroscience, 151*(1), 138-147. doi:10.1016/j.neuroscience.2007.10.050

Huang, E. J., & Reichardt, L. F. (2001). Neurotrophins: Roles in Neuronal Development and Function. *Annual Review of Neuroscience, 24*(1), 677-736. doi:10.1146/annurev.neuro.24.1.677

Huang, J., Zhang, L., Qu, Y., Zhou, Y., Zhu, J., Li, Y., . . . Mu, D. (2018). Histone acetylation of oligodendrocytes protects against white matter injury induced by inflammation and hypoxia-ischemia through activation of BDNF-TrkB signaling pathway in neonatal rats. *Brain Research, 1688*, 33-46. doi:10.1016/j.brainres.2017.11.005

Huang, Y., & Dreyfus, C. F. (2016). The role of growth factors as a therapeutic approach to demyelinating disease. *Experimental Neurology, 283*(Pt B), 531-540. doi:10.1016/j.expneurol.2016.02.023

Ikawa, D., Makinodan, M., Iwata, K., Ohgidani, M., Kato, T. A., Yamashita, Y., . . . Kishimoto, T. (2017). Microglia-derived neuregulin expression in psychiatric disorders. *Brain, Behavior, and Immunity, 61*, 375-385. doi:10.1016/j.bbi.2017.01.003

Ishibashi, T., Lee, P. R., Baba, H., & Fields, R. D. (2009). Leukemia inhibitory factor regulates the timing of oligodendrocyte development and myelination in the postnatal optic nerve. *Journal of Neuroscience Research, 87*(15), 3343-3355. doi:10.1002/jnr.22173

Ishii, A., Furusho, M., Macklin, W., & Bansal, R. (2019). Independent and cooperative roles of the Mek/ERK1/2-MAPK and PI3K/Akt/mTOR pathways during developmental myelination and in adulthood. *Glia, 67*(7), 1277-1295. doi:10.1002/glia.23602

Ishii, A., Fyffe-Maricich, S. L., Furusho, M., Miller, R. H., & Bansal, R. (2012). ERK1/ERK2 MAPK signaling is required to increase myelin thickness independent of oligodendrocyte differentiation and initiation of myelination. *Journal of Neuroscience, 32*(26), 8855-8864. doi:10.1523/JNEUROSCI.0137-12.2012

Jean, I., Lavialle, C., Barthelaix-Pouplard, A., & Fressinaud, C. (2003). Neurotrophin-3 specifically increases mature oligodendrocyte population and enhances remyelination after chemical demyelination of adult rat CNS. *Brain Research, 972*(1-2), 110-118. doi:10.1016/S0006-8993(03)02510-1

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

Jorissen, R. N., Walker, F., Pouliot, N., Garrett, T. P. J., Ward, C. W., & Burgess, A. W. (2003). Epidermal growth factor receptor: Mechanisms of activation and signalling. *Experimental Cell Research, 284*(1), 31-53. doi:10.1016/S0014-4827(02)00098-8

Kadota, R., Koda, M., Kawabe, J., Hashimoto, M., Nishio, Y., Mannoji, C., . . . Yamazaki, M. (2012). Granulocyte colony-stimulating factor (G-CSF) protects oligodendrocyte and promotes hindlimb functional recovery after spinal cord injury in rats. *PloS One, 7*(11), e50391. doi:10.1371/journal.pone.0050391

Kastin, A. J., Akerstrom, V., & Pan, W. (2003). Glial cell line-derived neurotrophic factor does not enter normal mouse brain. *Neuroscience Letters, 340*(3), 239-241. doi:10.1016/s0304-3940(03)00007-7

Kaur, C., Sivakumar, V., Yip, G. W., & Ling, E. A. (2009). Expression of syndecan-2 in the amoeboid microglial cells and its involvement in inflammation in the hypoxic developing brain. *Glia, 57*(3), 336-349. doi:10.1002/glia.20764

Keelan, J. A., Yang, J., Romero, R. J., Chaiworapongsa, T., Marvin, K. W., Sato, T. A., & Mitchell, M. D. (2004). Epithelial Cell-Derived Neutrophil-Activating Peptide-78 Is Present in Fetal Membranes and Amniotic Fluid at Increased Concentrations with Intra-amniotic Infection and Preterm Delivery1. *Biology of Reproduction, 70*(1), 253-259. doi:10.1095/biolreprod.103.016204

Keller, M., Simbruner, G., Gorna, A., Urbanek, M., Tinhofer, I., Griesmaier, E., . . . Gressens, P. (2006). Systemic application of granulocyte-colony stimulating factor and stem cell factor exacerbates excitotoxic brain injury in newborn mice. *Pediatric Research, 59*(4 Pt 1), 549-553. doi:10.1203/01.pdr.0000205152.38692.81

Keunen, K., Van Elburg, R. M., Van Bel, F., & Benders, M. J. N. L. (2015). Impact of nutrition on brain development and its neuroprotective implications following preterm birth. *Pediatric Research, 77*(1), 148-155. doi:10.1038/pr.2014.171

Kim, B. H., Guardia Clausi, M., Frondelli, M., Nnah, I. C., Saqcena, C., Dobrowolski, R., & Levison, S. W. (2017). Age-Dependent Effects of ALK5 Inhibition and Mechanism of Neuroprotection in Neonatal Hypoxic-Ischemic Brain Injury. *Developmental Neuroscience, 39*(1-4), 338-351. doi:10.1159/000477490

Kim, B. R., Shim, J. W., Sung, D. K., Kim, S. S., Jeon, G. W., Kim, M. J., . . . Choi, E. S. (2008). Granulocyte stimulating factor attenuates hypoxic-ischemic brain injury by inhibiting apoptosis in neonatal rats. *Yonsei Medical Journal, 49*(5), 836-842. doi:10.3349/ymj.2008.49.5.836

Kim, H., Xu, R., Padmashri, R., Dunaevsky, A., Liu, Y., Dreyfus, C. F., & Jiang, P. (2019). Pluripotent Stem Cell-Derived Cerebral Organoids Reveal Human Oligodendrogenesis with Dorsal and Ventral Origins. *Stem Cell Reports, 12*(5), 890-905. doi:10.1016/j.stemcr.2019.04.011

Koh, H., Rah, W. J., Kim, Y. J., Moon, J. H., Kim, M. J., & Lee, Y. H. (2018). Serial changes of cytokines in children with cerebral palsy who received intravenous granulocyte-colony stimulating factor followed by autologous mobilized peripheral blood mononuclear cells. *Journal of Korean Medical Science, 33*(21), 1-9. doi:10.3346/jkms.2018.33.e102

Konofagou, E. E., Tung, Y. S., Choi, J., Deffieux, T., Baseri, B., & Vlachos, F. (2012). Ultrasound-induced blood-brain barrier opening. *Current Pharmaceutical Biotechnology, 13*(7), 1332-1345. doi:10.2174/138920112800624364

Kornblum, H. I., Hussain, R. J., Bronstein, J. M., Gall, C. M., Lee, D. C., & Seroogy, K. B. (1997). Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. *Journal of Comparative Neurology, 380*(2), 243-261. doi:10.1002/(SICI)1096-9861(19970407)380:2<243::AID-CNE7>3.0.CO;2-3

Kremer, D., Cui, Q. L., Gottle, P., Kuhlmann, T., Hartung, H. P., Antel, J., & Kury, P. (2016). CXCR7 Is Involved in Human Oligodendroglial Precursor Cell Maturation. *PloS One, 11*(1), e0146503. doi:10.1371/journal.pone.0146503

Kuban, K. C. K., Heeren, T., O'Shea, T. M., Joseph, R. M., Fichorova, R. N., Douglass, L., . . . Investigators, E. S. (2018). Among Children Born Extremely Preterm a Higher Level of Circulating Neurotrophins Is Associated with Lower Risk of Cognitive Impairment at School Age. *Journal of Pediatrics, 201*, 40-48. doi:10.1016/j.physbeh.2017.03.040

Kuhn, H. G., Winkler, J., Kempermann, G., Thal, L. J., & Gage, F. H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *Journal of Neuroscience, 17*(15), 5820-5829. doi:10.1523/jneurosci.17-15-05820.1997

Kwilasz, A. J., Grace, P. M., Serbedzija, P., Maier, S. F., & Watkins, L. R. (2015). The therapeutic potential of interleukin-10 in neuroimmune diseases. *Neuropharmacology, 96*(PA), 55-69. doi:10.1016/j.neuropharm.2014.10.020

Lacaille, H., Vacher, C. M., Bakalar, D., O'Reilly, J. J., Salzbank, J., & Penn, A. A. (2019). Impaired Interneuron Development in a Novel Model of Neonatal Brain Injury. *eNeuro, 6*(1), ENEURO..0300-0318.2019. doi:10.1523/ENEURO.0300-18.2019

Ladiwala, U., Lachance, C., Simoneau, S. J. J., Bhakar, A., Barker, P. A., & Antel, J. P. (1998). p75 neurotrophin receptor expression on adult human oligodendrocytes: Signaling without cell death in response to NGF. *Journal of Neuroscience, 18*(4), 1297-1304. doi:10.1523/jneurosci.18-04-01297.1998

Lai, C., & Feng, L. (2004). Implication of γ-secretase in neuregulin-induced maturation of oligodendrocytes. *Biochemical and Biophysical Research Communications, 314*(2), 535-542. doi:10.1016/j.bbrc.2003.12.131

Lai, S. W., Chen, J. H., Lin, H. Y., Liu, Y. S., Tsai, C. F., Chang, P. C., . . . Lin, C. (2018). Regulatory Effects of Neuroinflammatory Responses Through Brain-Derived Neurotrophic Factor Signaling in Microglial Cells. *Molecular Neurobiology, 55*(9), 7487-7499. doi:10.1007/s12035-018-0933-z

Larroque, B., Ancel, P. Y., Marret, S., Marchand, L., Andre, M., Arnaud, C., . . . Kaminski, M. (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*(9615), 813-820. doi:10.1016/s0140-6736(08)60380-3

Lee, W. H., Clemens, J. A., & Bondy, C. A. (1992). Insulin-like growth factors in the response to cerebral ischemia. *Molecular and Cellular Neuroscience, 3*(1), 36-43. doi:10.1016/1044-7431(92)90006-n

Lee, W. H., Wang, G. M., Seaman, L. B., & Vannucci, S. J. (1996). Coordinate IGF-I and IGFBP5 Gene Expression in Perinatal Rat Brain after Hypoxia-Ischemia. *Journal of Cerebral Blood Flow and Metabolism, 16*(2), 227-236. doi:10.1097/00004647-199603000-00007

Lee, Y. A. (2017). White Matter Injury of Prematurity: Its Mechanisms and Clinical Features. *Journal of Pathology and Translational Medicine, 51*(5), 449-455. doi:10.4132/jptm.2017.07.25

Leviton, A., Allred, E. N., Yamamoto, H., Fichorova, R. N., Kuban, K., O'Shea, T. M., & Dammann, O. (2017). Antecedents and correlates of blood concentrations of neurotrophic growth factors in very preterm newborns. *Cytokine, 94*(October 2016), 21-28. doi:10.1016/j.cyto.2017.03.012

Ley, D., Hansen-Pupp, I., Niklasson, A., Domellof, M., Friberg, L. E., Borg, J., . . . Hellstrom, A. (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. *Pediatric Research, 73*(1), 68-74. doi:10.1038/pr.2012.146

Li, B., Concepcion, K., Meng, X., & Zhang, L. (2017). Brain-immune interactions in perinatal hypoxic-ischemic brain injury. *Progress in Neurobiology, 159*, 50-68. doi:10.1016/j.pneurobio.2017.10.006

Li, W. J., Mao, F. X., Chen, H. J., Qian, L. H., & Buzby, J. S. (2015a). Treatment with UDP-glucose, GDNF, and memantine promotes SVZ and white matter self-repair by endogenous glial progenitor cells in neonatal rats with ischemic PVL. *Neuroscience, 284*, 444-458. doi:10.1016/j.neuroscience.2014.10.012

Li, Y., Tang, G., Liu, Y., He, X., Huang, J., Lin, X., . . . Wang, Y. (2015b). CXCL12 Gene Therapy Ameliorates Ischemia-Induced White Matter Injury in Mouse Brain. *Stem Cells Translational Medicine 4*(10), 1122-1130. doi:10.5966/sctm.2015-0074

Liddelow, S. A., & Barres, B. A. (2017). Reactive Astrocytes: Production, Function, and Therapeutic Potential. *Immunity, 46*(6), 957-967. doi:10.1016/j.immuni.2017.06.006

Lim, S. T., Airavaara, M., & Harvey, B. K. (2010). Viral vectors for neurotrophic factor delivery: a gene therapy approach for neurodegenerative diseases of the CNS. *Pharmacological Research, 61*(1), 14-26. doi:10.1016/j.phrs.2009.10.002

Lima Giacobbo, B., Doorduin, J., Klein, H. C., Dierckx, R. A. J. O., Bromberg, E., & de Vries, E. F. J. (2019). Brain-Derived Neurotrophic Factor in Brain Disorders: Focus on Neuroinflammation. *Molecular Neurobiology, 56*(5), 3295-3312. doi:10.1007/s12035-018-1283-6

Lin, H. W., Jain, M., Li, H., & Levison, S. W. (2009a). Ciliary neurotrophic factor (CNTF) plus soluble CNTF receptor α increases cyclooxygenase-2 expression, PGE2 release and interferon-γ-induced CD40 in murine microglia. *Journal of Neuroinflammation, 6*, 1-13. doi:10.1186/1742-2094-6-7

Lin, S., Fan, L. W., Rhodes, P. G., & Cai, Z. (2009b). Intranasal administration of IGF-1 attenuates hypoxic-ischemic brain injury in neonatal rats. *Experimental Neurology, 217*(2), 361-370. doi:10.1016/j.expneurol.2009.03.021

Linker, R. A., Maurer, M., Gaupp, S., Martini, R., Holtmann, B., Giess, R., . . . Gold, R. (2002). CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. *Nature Medicine, 8*(6), 620-624. doi:10.1038/nm0602-620

Linsell, L., Johnson, S., Wolke, D., O'Reilly, H., Morris, J. K., Kurinczuk, J. J., & Marlow, N. (2018). Cognitive trajectories from infancy to early adulthood following birth before 26 weeks of gestation: a prospective, population-based cohort study. *Archives of Disease in Childhood, 103*(4), 363-370. doi:10.1136/archdischild-2017-313414

Liu, X. F., Fawcett, J. R., Thorne, R. G., & Frey, W. H., 2nd. (2001). Non-invasive intranasal insulin-like growth factor-I reduces infarct volume and improves neurologic function in rats following middle cerebral artery occlusion. *Neuroscience Letters, 308*(2), 91-94. Retrieved from https://www.sciencedirect.com/science/article/pii/S0304394001019826?via%3Dihub

Liu, Y., Gibson, S. A., Benveniste, E. N., & Qin, H. (2015). Opportunities for Translation from the Bench: Therapeutic Intervention of the JAK/STAT Pathway in Neuroinflammatory Diseases. *Critical Reviews in Immunology, 35*(6), 505-527. doi:10.1615/CritRevImmunol.2016015517

Lochhead, J. J., & Thorne, R. G. (2012). Intranasal delivery of biologics to the central nervous system. *Advanced Drug Delivery Reviews, 64*(7), 614-628. doi:10.1016/j.addr.2011.11.002

Lofqvist, C., Niklasson, A., Engstrom, E., Friberg, L. E., Camacho-Hubner, C., Ley, D., . . . Hellstrom, A. (2009). A pharmacokinetic and dosing study of intravenous insulin-like growth factor-I and IGF-binding protein-3 complex to preterm infants. *Pediatric Research, 65*(5), 574-579. doi:10.1203/PDR.0b013e31819d9e8c

Lu, H., Huang, W., Chen, X., Wang, Q., Zhang, Q., & Chang, M. (2018). Relationship between premature brain injury and multiple biomarkers in cord blood and amniotic fluid. *Journal of Maternal-Fetal and Neonatal Medicine, 31*(21), 2898-2904. doi:10.1080/14767058.2017.1359532

Lu, H. y., Zhang, Q., Wang, Q. x., & Lu, J. y. (2016). Contribution of Histologic Chorioamnionitis and Fetal Inflammatory Response Syndrome to Increased Risk of Brain Injury in Infants With Preterm Premature Rupture of Membranes. *Pediatric Neurology, 61*, 94-98.e91. doi:10.1016/j.pediatrneurol.2016.05.001

Lundgaard, I., Luzhynskaya, A., Stockley, J. H., Wang, Z., Evans, K. A., Swire, M., . . . Káradóttir, R. T. (2013). Neuregulin and BDNF Induce a Switch to NMDA Receptor-Dependent Myelination by Oligodendrocytes. *PLoS Biology, 11*(12), e1001743. doi:10.1371/journal.pbio.1001743

Luo, Y., Yang, H., Zhou, Y. F., & Hu, B. (2020). Dual and multi-targeted nanoparticles for site-specific brain drug delivery. *Journal of Controlled Release, 317*, 195-215. doi:10.1016/j.jconrel.2019.11.037

MacKay, D. F., Smith, G. C., Dobbie, R., & Pell, J. P. (2010). Gestational age at delivery and special educational need: retrospective cohort study of 407,503 schoolchildren. *PLoS Medicine, 7*(6), e1000289. doi:10.1371/journal.pmed.1000289

Madhavan, M., Nevin, Z. S., Shick, H. E., Garrison, E., Clarkson-Paredes, C., Karl, M., . . . Tesar, P. J. (2018). Induction of myelinating oligodendrocytes in human cortical spheroids. *Nature Methods, 15*(9), 700-706. doi:10.1038/s41592-018-0081-4

Maheshwari, A., Janssens, K., Bogie, J., Van Den Haute, C., Struys, T., Lambrichts, I., . . . Hellings, N. (2013). Local overexpression of interleukin-11 in the central nervous system limits demyelination and enhances remyelination. *Mediators of Inflammation, 2013*, 685317. doi:10.1155/2013/685317

Malamitsi-Puchner, A., Vrachnis, N., Samoli, E., Baka, S., Iliodromiti, Z., Puchner, K. P., . . . Hassiakos, D. (2006). Possible early prediction of preterm birth by determination of novel proinflammatory factors in midtrimester amniotic fluid. *Annals of the New York Academy of Sciences, 1092*, 440-449. doi:10.1196/annals.1365.043

Marks, W. J., Baumann, T. L., & Bartus, R. T. (2016). Long-Term Safety of Patients with Parkinson's Disease Receiving rAAV2-Neurturin (CERE-120) Gene Transfer. *Human Gene Therapy, 27*(7), 522-527. doi:10.1089/hum.2015.134

Martín, R., Cordova, C., & Nieto, M. L. (2012). Secreted phospholipase A 2-IIA-induced a phenotype of activated microglia in BV-2 cells requires epidermal growth factor receptor transactivation and proHB-EGF shedding. *Journal of Neuroinflammation, 9*, 1-20. doi:10.1186/1742-2094-9-154

Masters, B. A., Werner, H., Roberts, C. T., Jr., LeRoith, D., & Raizada, M. K. (1991). Insulin-like growth factor I (IGF-I) receptors and IGF-I action in oligodendrocytes from rat brains. *Regulatory Peptides, 33*(2), 117-131. doi:10.1016/0167-0115(91)90207-w

Mayer, M., Bhakoo, K., & Noble, M. (1994). Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro. *Development, 120*(1), 143-153. Retrieved from https://dev.biologists.org/content/120/1/143.long

Maysami, S., Nguyen, D., Zobel, F., Pitz, C., Heine, S., Hopfner, M., & Stangel, M. (2006). Modulation of rat oligodendrocyte precursor cells by the chemokine CXCL12. *Neuroreport, 17*(11), 1187-1190. doi:10.1097/01.wnr.0000227985.92551.9a

McCloy, M. P., Roberts, I. A. G., Howarth, L. J., Watts, T. L., & Murray, N. A. (2002). Interleukin-11 levels in healthy and thrombocytopenic neonates. *Pediatric Research, 51*(6), 756-760. doi:10.1203/00006450-200206000-00016

McKinnon, R. D., Piras, G., Ida, J. A., Jr., & Dubois-Dalcq, M. (1993). A role for TGF-beta in oligodendrocyte differentiation. *Journal of Cell Biology, 121*(6), 1397-1407. doi:10.1083/jcb.121.6.1397

McMillan, J., Batrakova, E., & Gendelman, H. E. (2011). Cell delivery of therapeutic nanoparticles. *Progress in Molecular Biology and Translational Science, 104*, 563-601. doi:10.1016/B978-0-12-416020-0.00014-0

McMorris, F. A., Smith, T. M., DeSalvo, S., & Furlanetto, R. W. (1986). Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development. *Proceedings of the National Academy of Sciences of the United States of America, 83*(3), 822-826. doi:10.1073/pnas.83.3.822

Mei, L., & Nave, K. A. (2014). Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases. *Neuron, 83*(1), 27-49. doi:10.1016/j.neuron.2014.06.007

Mekhail, M., Almazan, G., & Tabrizian, M. (2015). Purine-crosslinked injectable chitosan sponges promote oligodendrocyte progenitor cells' attachment and differentiation. *Biomaterials Science, 3*(2), 279-287. doi:10.1039/c4bm00215f

Mela, A., & Goldman, J. E. (2009). The tetraspanin KAI1/CD82 is expressed by late-lineage oligodendrocyte precursors and may function to restrict precursor migration and promote oligodendrocyte differentiation and myelination. *Journal of Neuroscience, 29*(36), 11172-11181. doi:10.1523/JNEUROSCI.3075-09.2009

Mela, A., & Goldman, J. E. (2013). CD82 blocks cMet activation and overcomes hepatocyte growth factor effects on oligodendrocyte precursor differentiation. *Journal of Neuroscience, 33*(18), 7952-7960. doi:10.1523/JNEUROSCI.5836-12.2013

Mesples, B., Plaisant, F., & Gressens, P. (2003). Effects of interleukin-10 on neonatal excitotoxic brain lesions in mice. *Developmental Brain Research, 141*(1-2), 25-32. doi:10.1016/S0165-3806(02)00636-3

Mi, P., Cabral, H., & Kataoka, K. (2020). Ligand-Installed Nanocarriers toward Precision Therapy. *Advanced Materials, 32*(13), 1-29. doi:10.1002/adma.201902604

Mifsud, G., Zammit, C., Muscat, R., Di Giovanni, G., & Valentino, M. (2014). Oligodendrocyte Pathophysiology and Treatment Strategies in Cerebral Ischemia. *CNS Neuroscience & Therapeutics, 20*(7), 603-612. doi:10.1111/cns.12263

Miljkovic, D., Stanojevic, Z., Momcilovic, M., Odoardi, F., Flugel, A., & Mostarica-Stojkovic, M. (2011). CXCL12 expression within the CNS contributes to the resistance against experimental autoimmune encephalomyelitis in Albino Oxford rats. *Immunobiology, 216*(9), 979-987. doi:10.1016/j.imbio.2011.03.013

Mirabelli-Badenier, M., Braunersreuther, V., Viviani, G. L., Dallegri, F., Quercioli, A., Veneselli, E., . . . Montecucco, F. (2011). CC and CXC chemokines are pivotal mediators of cerebral injury in ischaemic stroke. *Thrombosis and Haemostasis, 105*(3), 409-420. doi:10.1160/TH10-10-0662

Mitew, S., Hay, C. M., Peckham, H., Xiao, J., Koenning, M., & 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

Miyamoto, N., Maki, T., Shindo, A., Liang, A. C., Maeda, M., Egawa, N., . . . Arai, K. (2015). Astrocytes Promote Oligodendrogenesis after White Matter Damage via Brain-Derived Neurotrophic Factor. *Journal of Neuroscience, 35*(41), 14002-14008. doi:10.1523/jneurosci.1592-15.2015

Molina-Holgado, E., J.M., V., Arévalo-Martín, A., & Guaza, C. (2001). LPS/IFN-gamma cytotoxicity in oligodendroglial cells: Role of nitric oxide and protection by the anti-inflammatory cytokine IL-10. *European Journal of Neuroscience, 13*(3), 493-502. doi:10.1046/j.0953-816x.2000.01412.x

Morell, M., Tsan, Y.-c., & O'Shea, K. S. (2015). Inducible expression of noggin selectively expands neural progenitors in the adult SVZ. *Stem Cell Research, 14*(1), 79-94. doi:10.1016/j.scr.2014.11.001

Moretti, R., Pansiot, J., Bettati, D., Strazielle, N., Ghersi-Egea, J. F., Damante, G., . . . Gressens, P. (2015). Blood-brain barrier dysfunction in disorders of the developing brain. *Frontiers in Neuroscience, 17*, 9-40. doi:10.3389/fnins.2015.00040

Moster, D., Lie, R. T., & Markestad, T. (2008). Long-term medical and social consequences of preterm birth. *New England Journal of Medicine, 359*(3), 262-273. doi:10.1056/NEJMoa0706475

Mozell, R. L., & McMorris, F. A. (1991). Insulin-like growth factor I stimulates oligodendrocyte development and myelination in rat brain aggregate cultures. *Journal of Neuroscience Research, 30*(2), 382-390. doi:10.1002/jnr.490300214

Mukhopadhyay, A., McGuire, T., Peng, C. Y., & Kessler, J. A. (2009). Differential effects of BMP signaling on parvalbumin and somatostatin interneuron differentiation. *Development, 136*(15), 2633-2642. doi:10.1242/dev.034439

Murtie, J. C., Zhou, Y. X., Le, T. Q., Vana, A. C., & Armstrong, R. C. (2005). PDGF and FGF2 pathways regulate distinct oligodendrocyte lineage responses in experimental demyelination with spontaneous remyelination. *Neurobiology of Disease, 19*(1-2), 171-182. doi:10.1016/j.nbd.2004.12.006

Musah, A. S., Brown, T. L., Jeffries, M. A., Shang, Q., Hashimoto, H., Evangelou, A. V., . . . Wood, T. L. (2020). Mechanistic target of rapamycin regulates the oligodendrocyte cytoskeleton during myelination. *Journal of Neuroscience, 40*(15), 2993-3007. doi:10.1523/JNEUROSCI.1434-18.2020

Nag, O. K., & Delehanty, J. B. (2019). Active Cellular and Subcellular Targeting of Nanoparticles for Drug Delivery. *Pharmaceutics, 11*(10), 543. doi:10.3390/pharmaceutics11100543

Ness, J. K., Mitchell, N. E., & Wood, T. L. (2002). IGF-I and NT-3 signaling pathways in developing oligodendrocytes: Differential regulation and activation of receptors and the downstream effector Akt. *Developmental Neuroscience, 24*(5), 437-445. doi:10.1159/000069050

Ness, J. K., Scaduto, R. C., Jr., & Wood, T. L. (2004). IGF-I prevents glutamate-mediated bax translocation and cytochrome C release in O4+ oligodendrocyte progenitors. *Glia, 46*(2), 183-194. doi:10.1002/glia.10360

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

Neumann, H., Misgeld, T., Matsumuro, K., & Wekerle, H. (1998). Neurotrophins inhibit major histocompatibility class II inducibility of microglia: Involvement of the p75 neurotrophin receptor. *Proceedings of the National Academy of Sciences of the United States of America, 95*(10), 5779-5784. doi:10.1073/pnas.95.10.5779

Nicholson, M., Wood, R. J., Fletcher, J. L., van den Buuse, M., Murray, S. S., & Xiao, J. (2018). BDNF haploinsufficiency exerts a transient and regionally different influence upon oligodendroglial lineage cells during postnatal development. *Molecular and Cellular Neuroscience, 90*(November 2017), 12-21. doi:10.1016/j.mcn.2018.05.005

O'Kusky, J., & Ye, P. (2012). Neurodevelopmental effects of insulin-like growth factor signaling. *Frontiers in Neuroendocrinology, 33*(3), 230-251. doi:10.1016/j.yfrne.2012.06.002

Ohja, K., Gozal, E., Fahnestock, M., Cai, L., Cai, J., Freedman, J. H., . . . Barnes, G. N. (2018). Neuroimmunologic and Neurotrophic Interactions in Autism Spectrum Disorders: Relationship to Neuroinflammation. *Neuromolecular Medicine, 20*(2), 161-173. doi:10.1007/s12017-018-8488-8

Ohno, M., Hiraoka, Y., Matsuoka, T., Tomimoto, H., Takao, K., Miyakawa, T., . . . Nishi, E. (2009). Nardilysin regulates axonal maturation and myelination in the central and peripheral nervous system. *Nature Neuroscience, 12*(12), 1506-1513. doi:10.1038/nn.2438

Ohya, W., Funakoshi, H., Kurosawa, T., & Nakamura, T. (2007). Hepatocyte growth factor (HGF) promotes oligodendrocyte progenitor cell proliferation and inhibits its differentiation during postnatal development in the rat. *Brain Research, 1147*, 51-65. doi:10.1016/j.brainres.2007.02.045

Olivier, P., Baud, O., Evrard, P., Gressens, P., & Verney, C. (2005). Prenatal ischemia and white matter damage in rats. *Journal of Neuropathology and Experimental Neurology, 64*(11), 998-1006. doi:10.1097/01.jnen.0000187052.81889.57

Ormel, P. R., Vieira de Sá, R., van Bodegraven, E. J., Karst, H., Harschnitz, O., Sneeboer, M. A. M., . . . Pasterkamp, R. J. (2018). Microglia innately develop within cerebral organoids. *Nature Communications, 9*(1), 4167. doi:10.1038/s41467-018-06684-2

Ornelas, I. M., Khandker, L., Wahl, S. E., Hashimoto, H., Macklin, W. B., & Wood, T. L. (2020). The mechanistic target of rapamycin pathway downregulates bone morphogenetic protein signaling to promote oligodendrocyte differentiation. *Glia, 68*(6), 1274-1290. doi:10.1002/glia.23776

Oyagi, A., & Hara, H. (2012). Essential roles of heparin-binding epidermal growth factor-like growth factor in the brain. *CNS Neuroscience and Therapeutics, 18*(10), 803-810. doi:10.1111/j.1755-5949.2012.00371.x

Padovani-Claudio, D. A., Liu, L., Ransohoff, R. M., & Miller, R. H. (2006). Alterations in the oligodendrocyte lineage, myelin, and white matter in adult mice lacking the chemokine receptor CXCR2. *Glia, 54*(5), 471-483. doi:10.1002/glia.20383

Paintlia, A. S., Paintlia, M. K., Singh, I., & Singh, A. K. (2006). IL-4-Induced Peroxisome Proliferator-Activated Receptor γ Activation Inhibits NF-κB Trans Activation in Central Nervous System (CNS) Glial Cells and Protects Oligodendrocyte Progenitors under Neuroinflammatory Disease Conditions: Implication for CNS-Demy. *The Journal of Immunology, 176*(7), 4385-4398. doi:10.4049/jimmunol.176.7.4385

Palacios, N., Sanchez-Franco, F., Fernandez, M., Sanchez, I., & Cacicedo, L. (2005). Intracellular events mediating insulin-like growth factor I-induced oligodendrocyte development: modulation by cyclic AMP. *Journal of Neurochemistry, 95*(4), 1091-1107. doi:10.1111/j.1471-4159.2005.03419.x

Palazuelos, J., Klingener, M., & Aguirre, A. (2014). TGFβ signaling regulates the timing of CNS myelination by modulating oligodendrocyte progenitor cell cycle exit through SMAD3/4/FoxO1/Sp1. *Journal of Neuroscience, 34*(23), 7917-7930. doi:10.1523/JNEUROSCI.0363-14.2014

Pan, W., & Kastin, A. J. (2000). Interactions of IGF-1 with the blood-brain barrier in vivo and in situ. *Neuroendocrinology, 72*(3), 171-178. doi:10.1159/000054584

Panda, S., Dohare, P., Jain, S., Parikh, N., Singla, P., Mehdizadeh, R., . . . Ballabh, P. (2018). Estrogen Treatment Reverses Prematurity-Induced Disruption in Cortical Interneuron Population. *Journal of Neuroscience, 38*(34), 7378-7391. doi:10.1523/jneurosci.0478-18.2018

Pang, Y., Rodts-Palenik, S., Cai, Z., Bennett, W. A., & Rhodes, P. G. (2005). Suppression of glial activation is involved in the protection of IL-10 on maternal E. coli induced neonatal white matter injury. *Developmental Brain Research, 157*(2), 141-149. doi:10.1016/j.devbrainres.2005.03.015

Pang, Y., Zheng, B., Campbell, L. R., Fan, L. W., Cai, Z., & Rhodes, P. G. (2010). IGF-1 can either protect against or increase LPS-induced damage in the developing rat brain. *Pediatric Research, 67*(6), 579-584. doi:10.1203/PDR.0b013e3181dc240f

Pang, Y., Zheng, B., Fan, L. W., Rhodes, P. G., & Cai, Z. (2007). IGF-1 protects oligodendrocyte progenitors against TNFalpha-induced damage by activation of PI3K/Akt and interruption of the mitochondrial apoptotic pathway. *Glia, 55*(11), 1099-1107. doi:10.1002/glia.20530

Pardridge, W. (2003). Neurotrophins, neuroprotection and the blood-brain barrier. *Current opinion in investigational drugs (London, England : 2000), 3*, 1753-1757.

Parthasarathy, G., & Philipp, M. T. (2017). Receptor tyrosine kinases play a significant role in human oligodendrocyte inflammation and cell death associated with the Lyme disease bacterium Borrelia burgdorferi. *Journal of Neuroinflammation, 14*(1), 1-11. doi:10.1186/s12974-017-0883-9

Patel, J. R., McCandless, E. E., Dorsey, D., & Klein, R. S. (2010). CXCR4 promotes differentiation of oligodendrocyte progenitors and remyelination. *Proceedings of the National Academy of Sciences of the United States of America, 107*(24), 11062-11067. doi:10.1073/pnas.1006301107

Patra, J. K., Das, G., Fraceto, L. F., Campos, E. V. R., Rodriguez-Torres, M. d. P., Acosta-Torres, L. S., . . . Shin, H.-S. (2018). Nano based drug delivery systems: recent developments and future prospects. *Journal of Nanobiotechnology, 16*(1), 71. doi:10.1186/s12951-018-0392-8

Peng, W. (2017). G-CSF treatment promotes apoptosis of autoreactive T cells to restrict the inflammatory cascade and accelerate recovery in experimental allergic encephalomyelitis. *Experimental Neurology, 289*, 73-84. doi:10.1016/j.expneurol.2016.10.008

Popken, G. J., Hodge, R. D., Ye, P., Zhang, J., Ng, W., O'Kusky, J. R., & D'Ercole, A. J. (2004). In vivo effects of insulin-like growth factor-I (IGF-I) on prenatal and early postnatal development of the central nervous system. *European Journal of Neuroscience, 19*(8), 2056-2068. doi:10.1111/j.0953-816X.2004.03320.x

Psachoulia, K., Chamberlain, K. A., Heo, D., Davis, S. E., Paskus, J. D., Nanescu, S. E., . . . Huang, J. K. (2016). IL4I1 augments CNS remyelination and axonal protection by modulating T cell driven inflammation. *Brain, 139*(12), 3121-3136. doi:10.1093/brain/aww254

Pusic, K. M., Pusic, A. D., Kemme, J., & Kraig, R. P. (2014). Spreading depression requires microglia and is decreased by their M2a polarization from environmental enrichment. *Glia, 62*(7), 1176-1194. doi:10.1002/glia.22672

Qu, X., Guo, R., Zhang, Z., Ma, L., Wu, X., Luo, M., . . . Yao, R. (2015). bFGF Protects Pre-oligodendrocytes from Oxygen/Glucose Deprivation Injury to Ameliorate Demyelination. *Cellular and Molecular Neurobiology, 35*(7), 913-920. doi:10.1007/s10571-015-0186-6

Raabe, T. D., Suy, S., Welcher, A., & DeVries, G. H. (1997). Effect of neu differentiation factor isoforms of neonatal oligodendrocyte function. *Journal of Neuroscience Research, 50*(5), 755-768. doi:10.1002/(SICI)1097-4547(19971201)50:5<755::AID-JNR12>3.0.CO;2-0

Rabchevsky, A. G., Weinitz, J. M., Coulpier, M., Fages, C., Tinel, M., & Junier, M. P. (1998). A role for transforming growth factor α as an inducer of astrogliosis. *Journal of Neuroscience, 18*(24), 10541-10552. doi:10.1523/jneurosci.18-24-10541.1998

Ravanelli, A. M., Kearns, C. A., Powers, R. K., Wang, Y., Hines, J. H., Donaldson, M. J., & Appel, B. (2018). Sequential specification of oligodendrocyte lineage cells by distinct levels of Hedgehog and Notch signaling. *Developmental Biology, 444*(2), 93-106. doi:10.1016/j.ydbio.2018.10.004

Razavi, S., Nazem, G., Mardani, M., Esfandiari, E., Esfahani, S., & Salehi, H. (2015). Neurotrophic factors and their effects in the treatment of multiple sclerosis. *Advanced Biomedical Research, 4*(1), 53-53. doi:10.4103/2277-9175.151570

Reid, M. V., Murray, K. A., Marsh, E. D., Golden, J. A., Simmons, R. A., & Grinspan, J. B. (2012). Delayed myelination in an intrauterine growth retardation model is mediated by oxidative stress upregulating bone morphogenetic protein 4. *Journal of Neuropathology and Experimental Neurology, 71*(7), 640-653. doi:10.1097/NEN.0b013e31825cfa81

Reinhardt, R. R., & Bondy, C. A. (1994). Insulin-like growth factors cross the blood-brain barrier. *Endocrinology, 135*(5), 1753-1761. doi:10.1210/endo.135.5.7525251

Rittchen, S., Boyd, A., Burns, A., Park, J., Fahmy, T. M., Metcalfe, S., & Williams, A. (2015). Myelin repair in vivo is increased by targeting oligodendrocyte precursor cells with nanoparticles encapsulating leukaemia inhibitory factor (LIF). *Biomaterials, 56*, 78-85. doi:10.1016/j.biomaterials.2015.03.044

Rizzi, C., Tiberi, A., Giustizieri, M., Marrone, M. C., Gobbo, F., Carucci, N. M., . . . Cattaneo, A. (2018). NGF steers microglia toward a neuroprotective phenotype. *Glia, 66*(7), 1395-1416. doi:10.1002/glia.23312

Robinson, S., Tani, M., Strieter, R. M., Ransohoff, R. M., & Miller, R. H. (1998). The chemokine growth-regulated oncogene-alpha promotes spinal cord oligodendrocyte precursor proliferation. *Journal of Neuroscience, 18*(24), 10457-10463. doi:10.1523/JNEUROSCI.18-24-10457.1998

Rolan, P. E., O'Neill, G., Versage, E., Rana, J., Tang, Y., Galluppi, G., & Aycardi, E. (2015). First-in-human, double-blind, placebo- controlled, randomized, dose-escalation study of BG00010, a glial cell line-derived neurotrophic factor family member, in subjects with unilateral sciatica. *PloS One, 10*(5), 1-16. doi:10.1371/journal.pone.0125034

Roth, G. A., Spada, V., Hamill, K., & Bornstein, M. B. (1995). Insulin-like growth factor I increases myelination and inhibits demyelination in cultured organotypic nerve tissue. *Developmental Brain Research, 88*(1), 102-108. doi:10.1016/0165-3806(95)00088-u

Rothaug, M., Becker-Pauly, C., & Rose-John, S. (2016). The role of interleukin-6 signaling in nervous tissue. *Biochimica et Biophysica Acta - Molecular Cell Research, 1863*(6), 1218-1227. doi:10.1016/j.bbamcr.2016.03.018

Rowe, D. D., Collier, L. A., Seifert, H. A., Chapman, C. B., Leonardo, C. C., Willing, A. E., & Pennypacker, K. R. (2014). Leukemia inhibitor factor promotes functional recovery and oligodendrocyte survival in rat models of focal ischemia. *European Journal of Neuroscience, 40*(7), 3111-3119. doi:10.1111/ejn.12675

Roy, K., Murtie, J. C., El-Khodor, B. F., Edgar, N., Sardi, S. P., Hooks, B. M., . . . Corfas, G. (2007). Loss of erbB signaling in oligodendrocytes alters myelin and dopaminergic function, a potential mechanism for neuropsychiatric disorders. *Proceedings of the National Academy of Sciences of the United States of America, 104*(19), 8131-8136. doi:10.1073/pnas.0702157104

Saini, H. S., Coelho, R. P., Goparaju, S. K., Jolly, P. S., Maceyka, M., Spiegel, S., & Sato-Bigbee, C. (2005). Novel role of sphingosine kinase 1 as a mediator of neurotrophin-3 action in oligodendrocyte progenitors. *Journal of Neurochemistry, 95*(5), 1298-1310. doi:10.1111/j.1471-4159.2005.03451.x

Saraiva, C., Praça, C., Ferreira, R., Santos, T., Ferreira, L., & Bernardino, L. (2016). Nanoparticle-mediated brain drug delivery: Overcoming blood–brain barrier to treat neurodegenerative diseases. *Journal of Controlled Release, 235*, 34-47. doi:10.1016/j.jconrel.2016.05.044

Scafidi, J., Hammond, T. R., Scafidi, S., Ritter, J., Jablonska, B., Roncal, M., . . . Gallo, V. (2014). Intranasal epidermal growth factor treatment rescues neonatal brain injury. *Nature, 506*(7487), 230-234. doi:10.1038/nature12880

Schenk, G. J., Dijkstra, S., van het Hof, A. J., van der Pol, S. M. A., Drexhage, J. A. R., van der Valk, P., . . . de Vries, H. E. (2013). Roles for HB-EGF and CD9 in multiple sclerosis. *Glia, 61*(11), 1890-1905. doi:10.1002/glia.22565

Scheuer, T., Brockmoller, V., Blanco Knowlton, M., Weitkamp, J., Ruhwedel, T., Mueller, S., . . . Schmitz, T. (2015). Oligodendroglial maldevelopment in the cerebellum after postnatal hyperoxia and its prevention by minocycline. *Glia, 63*(10), 1825-1839. doi:10.1016/j.physbeh.2017.03.040

Schlager, G. W., Griesmaier, E., Wegleiter, K., Neubauer, V., Urbanek, M., Kiechl-Kohlendorfer, U., . . . Keller, M. (2011). Systemic G-CSF treatment does not improve long-term outcomes after neonatal hypoxic-ischaemic brain injury. *Experimental Neurology, 230*(1), 67-74. doi:10.1016/j.expneurol.2010.11.021

Schneider, J., & Miller, S. P. (2019). Preterm brain Injury: White matter injury. *Handbook of Clinical Neurology, 162*, 155-172. doi:10.1016/b978-0-444-64029-1.00007-2

See, J., Zhang, X., Eraydin, N., Mun, S. B., Mamontov, P., Golden, J. A., & Grinspan, J. B. (2004). Oligodendrocyte maturation is inhibited by bone morphogenetic protein. *Molecular and Cellular Neuroscience, 26*(4), 481-492. doi:10.1016/j.mcn.2004.04.004

Shijo, T., Warita, H., Suzuki, N., Ikeda, K., Mitsuzawa, S., Akiyama, T., . . . Aoki, M. (2018). Antagonizing bone morphogenetic protein 4 attenuates disease progression in a rat model of amyotrophic lateral sclerosis. *Experimental Neurology, 307*, 164-179. doi:10.1016/j.expneurol.2018.06.009

Shin, J. A., Kim, Y. A., Kim, H. W., Kim, H. S., Lee, K. E., Kang, J. L., & Park, E. M. (2018). Iron released from reactive microglia by noggin improves myelin repair in the ischemic brain. *Neuropharmacology, 133*, 202-215. doi:10.1016/j.neuropharm.2018.01.038

Shiow, L. R., Favrais, G., Schirmer, L., Schang, A. L., Cipriani, S., Andres, C., . . . Rowitch, D. H. (2017). Reactive astrocyte COX2-PGE2 production inhibits oligodendrocyte maturation in neonatal white matter injury. *Glia, 65*(12), 2024-2037. doi:10.1002/glia.23212

Sil, S., Periyasamy, P., Thangaraj, A., Chivero, E. T., & Buch, S. (2018). PDGF/PDGFR axis in the neural systems. *Molecular Aspects of Medicine, 62*, 63-74. doi:10.1016/j.mam.2018.01.006

Silberstein, F. C., De Simone, R., Levi, G., & Aloisi, F. (1996). Cytokine-Regulated Expression of Platelet-Derived Growth Factor Gene and Protein in Cultured Human Astrocytes. *Journal of Neurochemistry, 66*(4), 1409-1417. doi:10.1046/j.1471-4159.1996.66041409.x

Silwedel, C., Speer, C. P., Haarmann, A., Fehrholz, M., Claus, H., Buttmann, M., & Glaser, K. (2018). Novel insights into neuroinflammation: Bacterial lipopolysaccharide, tumor necrosis factor α, and Ureaplasma species differentially modulate atypical chemokine receptor 3 responses in human brain microvascular endothelial cells. *Journal of Neuroinflammation, 15*(1), 1-11. doi:10.1186/s12974-018-1170-0

Sivakumar, V., Ling, E. A., Lu, J., & Kaur, C. (2010). Role of glutamate and its receptors and insulin-like growth factors in hypoxia induced periventricular white matter injury. *Glia, 58*(5), 507-523. doi:10.1002/glia.20940

Song, P., Xia, X., Han, T., Fang, H., Wang, Y., Dong, F., . . . Shen, C. (2018). BMSCs promote the differentiation of NSCs into oligodendrocytes via mediating Id2 and Olig expression through BMP/Smad signaling pathway. *Bioscience Reports, 38*(5), 1-13. doi:10.1042/BSR20180303

Song, Y. S., Joe, J. H., Joo, H. W., Park, I. H., Shen, G. Y., Kim, K. J., . . . Kim, K. S. (2016). The Effects of Granulocyte-Colony Stimulating Factor on Regeneration in Nerve Crush Injuries in Rats. *Neurochemical Research, 41*(7), 1645-1650. doi:10.1007/s11064-016-1879-z

Steelman, A. J., Zhou, Y., Koito, H., Kim, S. J., Payne, H. R., Lu, Q. R., & Li, J. (2016). Activation of oligodendroglial Stat3 is required for efficient remyelination. *Neurobiology of Disease, 91*, 336-346. doi:10.1016/j.nbd.2016.03.023

Stoll, G., Schroeter, M., Jander, S., Siebert, H., Wollrath, A., Kleinschnitz, C., & Brück, W. (2004). Lesion-associated Expression of Transforming Growth Factor-Beta-2 in the Rat Nervous System: Evidence for Down-regulating the Phagocytic Activity of Microglia and Macrophages. *Brain Pathology, 14*(1), 51-58. doi:10.1111/j.1750-3639.2004.tb00497.x

Stolp, H. B., Fleiss, B., Arai, Y., Supramaniam, V., Vontell, R., Birtles, S., . . . Gressens, P. (2019). Interneuron Development Is Disrupted in Preterm Brains With Diffuse White Matter Injury: Observations in Mouse and Human. *Frontiers in Physiology, 10*, 955. doi:10.3389/fphys.2019.00955

Strelau, J., & Unsicker, K. (1999). GDNF family members and their receptors: Expression and functions in two oligodendroglial cell lines representing distinct stages of oligodendroglial development. *Glia, 26*(4), 291-301. doi:10.1002/(SICI)1098-1136(199906)26:4<291::AID-GLIA3>3.0.CO;2-P

Sun, J., Zhou, W., Sha, B., & Yang, Y. (2010). Ischemia induced neural stem cell proliferation and differentiation in neonatal rat involved vascular endothelial growth factor and transforming growth factor-beta pathways. *Brain and Development, 32*(3), 191-200. doi:10.1016/j.braindev.2009.01.004

Suo, N., Guo, Y. e., He, B., Gu, H., & Xie, X. (2019). Inhibition of MAPK/ERK pathway promotes oligodendrocytes generation and recovery of demyelinating diseases. *Glia, 67*(7), 1320-1332. doi:10.1002/glia.23606

Sussman, C. R., Vartanian, T., & Miller, R. H. (2005). The ErbB4 neuregulin receptor mediates suppression of oligodendrocyte maturation. *Journal of Neuroscience, 25*(24), 5757-5762. doi:10.1523/JNEUROSCI.4748-04.2005

Taveggia, C., Thaker, P., Petrylak, A., Caporaso, G. L., Toews, A., Falls, D. L., . . . Salzer, J. L. (2008). Type III Neuregulin-1 Promotes Oligodendrocyte Myelination. *Glia, 56*, 284-293. doi:10.1002/glia.20612

Teleanu, D. M., Negut, I., Grumezescu, V., Grumezescu, A. M., & Teleanu, R. I. (2019). Nanomaterials for Drug Delivery to the Central Nervous System. *Nanomaterials (Basel), 9*(3), 371. doi:10.3390/nano9030371

Thomas, A. M., Seidlits, S. K., Goodman, A. G., Kukushliev, T. V., Hassani, D. M., Cummings, B. J., . . . Shea, L. D. (2014). Sonic hedgehog and neurotrophin-3 increase oligodendrocyte numbers and myelination after spinal cord injury. *Integrative Biology, 6*(7), 694-705. doi:10.1039/c4ib00009a

Thorne, R. G., Pronk, G. J., Padmanabhan, V., & Frey, W. H., 2nd. (2004). Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. *Neuroscience, 127*(2), 481-496. doi:10.1016/j.neuroscience.2004.05.029

Tian, Y., Yin, H., Deng, X., Tang, B., Ren, X., & Jiang, T. (2018). CXCL12 induces migration of oligodendrocyte precursor cells through the CXCR4-activated MEK/ERK and PI3K/AKT pathways. *Molecular Medicine Reports, 18*(5), 4374-4380. doi:10.3892/mmr.2018.9444

Tokita, Y., Keino, H., Matsui, F., Aono, S., Ishiguro, H., Higashiyama, S., & Oohira, A. (2001). Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. *Journal of Neuroscience, 21*(4), 1257-1264. doi:10.1523/jneurosci.21-04-01257.2001

Tong, L., Aleph Prieto, G., Kramár, E. A., Smith, E. D., Cribbs, D. H., Lynch, G., & Cotman, C. W. (2012). Brain-derived neurotrophic factor-dependent synaptic plasticity is suppressed by interleukin-1β via p38 mitogen-activated protein kinase. *Journal of Neuroscience, 32*(49), 17714-17724. doi:10.1523/JNEUROSCI.1253-12.2012

Traiffort, E., Kassoussi, A., Zahaf, A., & Laouarem, Y. (2020). Astrocytes and Microglia as Major Players of Myelin Production in Normal and Pathological Conditions. *Frontiers in Cellular Neuroscience, 14*(April), 1-21. doi:10.3389/fncel.2020.00079

Tsai, S. T., Chu, S. C., Liu, S. H., Pang, C. Y., Hou, T. W., Lin, S. Z., & Chen, S. Y. (2017). Neuroprotection of granulocyte colony-stimulating factor for early stage Parkinson's disease. *Cell Transplantation, 26*(3), 409-416. doi:10.3727/096368916X694247

Vaes, J. E. G., Vink, M. A., de Theije, C. G. M., Hoebeek, F. E., Benders, M., & Nijboer, C. H. A. (2019). The Potential of Stem Cell Therapy to Repair White Matter Injury in Preterm Infants: Lessons Learned From Experimental Models. *Frontiers in Physiology, 10*, 540. doi:10.3389/fphys.2019.00540

Valerio, A., Ferrario, M., Dreano, M., Garotta, G., Spano, P., & Pizzi, M. (2002). Soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro differentiation of purified rat oligodendroglial lineage cells. *Molecular and Cellular Neuroscience, 21*(4), 602-615. doi:10.1006/mcne.2002.1208

Van Heyningen, P., Calver, A. R., & Richardson, W. D. (2001). Control of progenitor cell number by mitogen supply and demand. *Current Biology, 11*(4), 232-241. doi:10.1016/S0960-9822(01)00075-6

van Tilborg, E., Amarouchi, K., Groenendaal, F., Heijnen, C., Benders, M. J., & Nijboer, C. H. *JNK inhibitor D-JNKi promotes myelination in a rat model of diffuse white matter injury*.

van Tilborg, E., de Theije, C. G. M., van Hal, M., Wagenaar, N., de Vries, L. S., Benders, M. J., . . . Nijboer, C. H. (2018). Origin and dynamics of oligodendrocytes in the developing brain: Implications for perinatal white matter injury. *Glia, 66*(2), 221-238. doi:10.1002/glia.23256

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., & Nijboer, C. H. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Progress in Neurobiology, 136*, 28-49. doi:10.1016/j.pneurobio.2015.11.002

Van Velthoven, C. T. J., Braccioli, L., Willemen, H. L. D. M., Kavelaars, A., & Heijnen, C. J. (2014). Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage. *Molecular Therapy, 22*(3), 645-654. doi:10.1038/mt.2013.260

Van Velthoven, C. T. J., Sheldon, R. A., Kavelaars, A., Derugin, N., Vexler, Z. S., Willemen, H. L. D. M., . . . Ferriero, D. M. (2013). Mesenchymal stem cell transplantation attenuates brain injury after neonatal stroke. *Stroke, 44*(5), 1426-1432. doi:10.1161/STROKEAHA.111.000326

Verney, C., Pogledic, I., Biran, V., Adle-Biassette, H., Fallet-Bianco, C., & Gressens, P. (2012). Microglial reaction in axonal crossroads is a hallmark of noncystic periventricular white matter injury in very preterm infants. *Journal of Neuropathology and Experimental Neurology, 71*(3), 251-264. doi:10.1097/NEN.0b013e3182496429

Vinukonda, G., Hu, F., Mehdizadeh, R., Dohare, P., Kidwai, A., Juneja, A., . . . Ballabh, P. (2016). Epidermal growth factor preserves myelin and promotes astrogliosis after intraventricular hemorrhage. *Glia, 64*(11), 1987-2004. doi:10.1002/glia.23037

Volpe, J. J. (2009a). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurology, 8*(1), 110-124. doi:10.1016/s1474-4422(08)70294-1

Volpe, J. J. (2009b). The encephalopathy of prematurity--brain injury and impaired brain development inextricably intertwined. *Seminars in Pediatric Neurology, 16*(4), 167-178. doi:10.1016/j.spen.2009.09.005

Volpe, J. J. (2017). Confusions in Nomenclature: "Periventricular Leukomalacia" and "White Matter Injury"-Identical, Distinct, or Overlapping? *Pediatric Neurology, 73*, 3-6. doi:10.1016/j.pediatrneurol.2017.05.013

Volpe, J. J. (2019). Dysmaturation of Premature Brain: Importance, Cellular Mechanisms, and Potential Interventions. *Pediatric Neurology, 95*, 42-66. doi:10.1016/j.pediatrneurol.2019.02.016

Volpe, J. J., Kinney, H. C., Jensen, F. E., & Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *International Journal of Developmental Neuroscience, 29*(4), 423-440. doi:10.1016/j.ijdevneu.2011.02.012

Vondran, M. W., Clinton-Luke, P., Honeywell, J. Z., & Dreyfus, C. F. (2010). BDNF+/- mice exhibit deficits in oligodendrocyte lineage cells of the basal forebrain. *Glia, 58*(7), 848-856. doi:10.1002/glia.20969

Wang, L., Yang, H., Zang, C., Dong, Y., Shang, J., Chen, J., . . . Zhang, D. (2020a). CXCR2 antagonism promotes oligodendrocyte precursor cell differentiation and enhances remyelination in a mouse model of multiple sclerosis. *Neurobiology of Disease, 134*(May 2019), 104630-104630. doi:10.1016/j.nbd.2019.104630

Wang, L. Y., Tu, Y. F., Lin, Y. C., & 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. *Journal of Neuroinflammation, 13*, 6. doi:10.1186/s12974-015-0474-6

Wang, S., Sdrulla, A. D., DiSibio, G., Bush, G., Nofziger, D., Hicks, C., . . . Barres, B. A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron, 21*(1), 63-75. doi:10.1016/S0896-6273(00)80515-2

Wang, X., Wang, Y., Wang, L., Shi, S., Yang, C., Jiang, W., . . . Yao, R. (2020b). Oligogenesis in the "oligovascular unit" involves PI3K/AKT/mTOR signaling in hypoxic-ischemic neonatal mice. *Brain Research Bulletin, 155*(October 2019), 81-91. doi:10.1016/j.brainresbull.2019.11.013

Watanabe, D., Yoshimura, R., Khalil, M., Yoshida, K., Kishimoto, T., Taga, T., & Kiyama, H. (1996). Characteristic localization of gp130 (the signal-transducing receptor component used in common for IL-6/IL-11/CNTF/LIF/OSM) in the rat brain. *European Journal of Neuroscience, 8*(8), 1630-1640. doi:10.1111/j.1460-9568.1996.tb01307.x

Weickert, C. S., & Blum, M. (1995). Striatal TGF-α: postnatal developmental expression and evidence for a role in the proliferation of subependymal cells. *Developmental Brain Research, 86*(1-2), 203-216. doi:10.1016/0165-3806(95)00026-A

Weiss, A., & Attisano, L. (2013). The TGFbeta superfamily signaling pathway. *Wiley Interdisciplinary Reviews: Developmental Biology, 2*(1), 47-63. doi:10.1002/wdev.86

Whone, A., Luz, M., Boca, M., Woolley, M., Mooney, L., Dharia, S., . . . Gill, S. S. (2019). Randomized trial of intermittent intraputamenal glial cell line-derived neurotrophic factor in Parkinson's disease. *Brain, 142*(3), 512-525. doi:10.1093/brain/awz023

Wilson, H. C., Onischke, C., & Raine, C. S. (2003). Human oligodendrocyte precursor cells in vitro: phenotypic analysis and differential response to growth factors. *Glia, 44*(2), 153-165. doi:10.1002/glia.10280

Winship, A., & Dimitriadis, E. (2017). Interleukin-11 induces preterm birth and modulates decidual inflammasome gene expression in mice. *Placenta, 50*, 99-101. doi:10.1016/j.placenta.2017.01.006

Wong, A. W., Willingham, M., Xiao, J., Kilpatrick, T. J., & Murray, S. S. (2008). Neurotrophin receptor homolog-2 regulates nerve growth factor signaling. *Journal of Neurochemistry, 106*(4), 1964-1976. doi:10.1111/j.1471-4159.2008.05539.x

Wong, A. W., Xiao, J., Kemper, D., Kilpatrick, T. J., & Murray, S. S. (2013). Oligodendroglial expression of TrkB independently regulates myelination and progenitor cell proliferation. *Journal of Neuroscience, 33*(11), 4947-4957. doi:10.1523/JNEUROSCI.3990-12.2013

Wood, T. L., Loladze, V., Altieri, S., Gangoli, N., Levison, S. W., Brywe, K. G., . . . Hagberg, H. (2007). Delayed IGF-1 administration rescues oligodendrocyte progenitors from glutamate-induced cell death and hypoxic-ischemic brain damage. *Developmental Neuroscience, 29*(4-5), 302-310. doi:10.1159/000105471

Wrigley, S., Arafa, D., & Tropea, D. (2017). Insulin-Like Growth Factor 1: At the Crossroads of Brain Development and Aging. *Frontiers in Cellular Neuroscience, 11*, 14. doi:10.3389/fncel.2017.00014

Xiao, B. G., Bai, X. F., Zhang, G. X., & Link, H. (1997). Transforming growth factor-β1 induces apoptosis of rat microglia without relation to bcl-2 oncoprotein expression. *Neuroscience Letters, 226*(2), 71-74. doi:10.1016/S0304-3940(97)00234-6

Xiao, J., Wong, A. W., Willingham, M. M., van den Buuse, M., Kilpatrick, T. J., & Murray, S. S. (2010). Brain-derived neurotrophic factor promotes central nervous system myelination via a direct effect upon oligodendrocytes. *Neuro-Signals, 18*(3), 186-202. doi:10.1159/000323170

Xu, J., Hu, C., Chen, S., Shen, H., Jiang, Q., Huang, P., & Zhao, W. (2017). Neuregulin-1 protects mouse cerebellum against oxidative stress and neuroinflammation. *Brain Research, 1670*, 32-43. doi:10.1016/j.brainres.2017.06.012

Yan, H., & Rivkees, S. A. (2002). Hepatocyte growth factor stimulates the proliferation and migration of oligodendrocyte precursor cells. *Journal of Neuroscience Research, 69*(5), 597-606. doi:10.1002/jnr.10323

Yan, H., & Wood, P. M. (2000). NT-3 weakly stimulates proliferation of adult rat O1- O4+ oligodendrocyte-lineage cells and increases oligodendrocyte myelination in vitro. *Journal of Neuroscience Research, 62*(3), 329-335. doi:10.1002/1097-4547(20001101)62:3<329::AID-JNR2>3.0.CO;2-C

Yang, J., Cheng, X., Qi, J., Xie, B., Zhao, X., Zheng, K., . . . Qiu, M. (2017). EGF Enhances Oligodendrogenesis from Glial Progenitor Cells. *Frontiers in Molecular Neuroscience, 10*, 106. doi:10.3389/fnmol.2017.00106

Yang, J., Cheng, X., Shen, J., Xie, B., Zhao, X., Zhang, Z., . . . Qiu, M. (2016). A novel approach for amplification and purification of mouse oligodendrocyte progenitor cells. *Frontiers in Cellular Neuroscience, 10*(AUG), 1-10. doi:10.3389/fncel.2016.00203

Yang, J., Jiang, Z., Fitzgerald, D. C., Ma, C., Yu, S., Li, H., . . . Zhang, G. X. (2009). Adult neural stem cells expressing IL-10 confer potent immunomodulation and remyelination in experimental autoimmune encephalitis. *Journal of Clinical Investigation, 119*(12), 3678-3691. doi:10.1172/JCI37914

Yap, V., & Perlman, J. M. (2020). Mechanisms of brain injury in newborn infants associated with the fetal inflammatory response syndrome. *Seminars in Fetal and Neonatal Medicine, 25*(4), 101110. doi:10.1016/j.siny.2020.101110

Yata, K., Matchett, G. A., Tsubokawa, T., Tang, J., Kanamaru, K., & Zhang, J. H. (2007). Granulocyte-colony stimulating factor inhibits apoptotic neuron loss after neonatal hypoxia-ischemia in rats. *Brain Research, 1145*(1), 227-238. doi:10.1016/j.brainres.2007.01.144

Ye, P., Carson, J., & D'Ercole, A. J. (1995). In vivo actions of insulin-like growth factor-I (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice. *Journal of Neuroscience, 15*(11), 7344-7356. doi:10.1523/JNEUROSCI.15-11-07344.1995

Ye, P., & D'Ercole, A. J. (1999). Insulin-like growth factor I protects oligodendrocytes from tumor necrosis factor-alpha-induced injury. *Endocrinology, 140*(7), 3063-3072. doi:10.1210/endo.140.7.6754

Ye, P., Li, L., Richards, R. G., DiAugustine, R. P., & D'Ercole, A. J. (2002). Myelination is altered in insulin-like growth factor-I null mutant mice. *Journal of Neuroscience, 22*(14), 6041-6051. doi:10.1523/JNEUROSCI.22-14-06041.2002

Yellowhair, T. R., Newville, J. C., Noor, S., Maxwell, J. R., Milligan, E. D., Robinson, S., & Jantzie, L. L. (2019). CXCR2 blockade mitigates neural cell injury following preclinical chorioamnionitis. *Frontiers in Physiology, 10*(APR), 1-11. doi:10.3389/fphys.2019.00324

Ying, Y. q., Yan, X. q., Jin, S. j., Liang, Y., Hou, L., Niu, W. t., & Luo, X. p. (2018). Inhibitory Effect of LPS on the Proliferation of Oligodendrocyte Precursor Cells through the Notch Signaling Pathway in Intrauterine Infection-induced Rats. *Current Medical Science, 38*(5), 840-846. doi:10.1007/s11596-018-1951-9

Yuan, T. M., & Yu, H. M. (2010). Notch signaling: Key role in intrauterine infection/inflammation, embryonic development, and white matter damage? *Journal of Neuroscience Research, 88*(3), 461-468. doi:10.1002/jnr.22229

Zanno, A. E., Romer, M. A., Fox, L., Golden, T., Jaeckle-Santos, L., Simmons, R. A., & Grinspan, J. B. (2019). Reducing Th2 inflammation through neutralizing IL-4 antibody rescues myelination in IUGR rat brain. *Journal of Neurodevelopmental Disorders, 11*(1), 1-13. doi:10.1186/s11689-019-9297-6

Zeger, M., Popken, G., Zhang, J., Xuan, S., Lu, Q. R., Schwab, M. H., . . . Ye, P. (2007). Insulin-like growth factor type 1 receptor signaling in the cells of oligodendrocyte lineage is required for normal in vivo oligodendrocyte development and myelination. *Glia, 55*(4), 400-411. doi:10.1002/glia.20469

Zhang, J., Zhang, Y., Dutta, D. J., Argaw, A. T., Bonnamain, V., Seto, J., . . . John, G. R. (2011). Proapoptotic and antiapoptotic actions of Stat1 versus Stat3 underlie neuroprotective and immunoregulatory functions of IL-11. *Journal of Immunology, 187*(3), 1129-1141. doi:10.4049/jimmunol.1004066

Zhang, P., Chebath, J., Lonai, P., & Revel, M. (2004). Enhancement of Oligodendrocyte Differentiation from Murine Embryonic Stem Cells by an Activator of gp130 Signaling. *Stem Cells, 22*, 344-354. doi:10.1017/CBO9781107415324.004

Zhang, Q., Zhu, W., Xu, F., Dai, X., Shi, L., Cai, W., . . . Hu, X. (2019). The interleukin-4/PPARγ signaling axis promotes oligodendrocyte differentiation and remyelination after brain injury. *PLoS Biology, 17*(6), e3000330. doi:10.1371/journal. pbio.3000330

Zhang, Y., Taveggia, C., Melendez-Vasquez, C., Einheber, S., Raine, C. S., Salzer, J. L., . . . John, G. R. (2006). Interleukin-11 potentiates oligodendrocyte survival and maturation, and myelin formation. *Journal of Neuroscience, 26*(47), 12174-12185. doi:10.1523/JNEUROSCI.2289-06.2006

Zhang, Y., Zhang, J., Navrazhina, K., Argaw, A. T., Zameer, A., Gurfein, B. T., . . . John, G. R. (2010). TGFβ1 induces Jagged1 expression in astrocytes via ALK5 and Smad3 and regulates the balance between oligodendrocyte progenitor proliferation and differentiation. *Glia, 58*(8), 964-974. doi:10.1002/glia.20978

Zhang, Y. J., Zhang, W., Lin, C. G., Ding, Y., Huang, S. F., Wu, J. L., . . . Zeng, Y. S. (2012). Neurotrophin-3 gene modified mesenchymal stem cells promote remyelination and functional recovery in the demyelinated spinal cord of rats. *Journal of the Neurological Sciences, 313*(1-2), 64-74. doi:10.1016/j.jns.2011.09.027

Zhao, J., Chen, Y., Xu, Y., & Pi, G. (2013). Effect of intrauterine infection on brain development and injury. *International Journal of Developmental Neuroscience, 31*(7), 543-549. doi:10.1016/j.ijdevneu.2013.06.008

Zhou, X., & Spittau, B. (2018). Lipopolysaccharide-Induced Microglia Activation Promotes the Survival of Midbrain Dopaminergic Neurons In Vitro. *Neurotoxicity Research, 33*(4), 856-867. doi:10.1007/s12640-017-9842-6

Zhou, Y., Peng, Z., Seven, E. S., & Leblanc, R. M. (2018). Crossing the blood-brain barrier with nanoparticles. *Journal of Controlled Release, 270*, 290-303. doi:10.1016/j.jconrel.2017.12.015

Zhou, Z., Peng, X., Insolera, R., Fink, D. J., & Mata, M. (2009). Interleukin-10 provides direct trophic support to neurons. *Journal of Neurochemistry, 110*(5), 1617-1627. doi:10.1111/j.1471-4159.2009.06263.x

Zonouzi, M., Scafidi, J., Li, P., McEllin, B., Edwards, J., Dupree, J. L., . . . Gallo, V. (2015). GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury. *Nature Neuroscience, 18*(5), 674-682. doi:10.1038/nn.3990

5

Intranasal mesenchymal stem cell therapy to boost myelination after encephalopathy of prematurity

J.E.G. Vaes, C.M. van Kammen*, C. Trayford*, A. van der Toorn, T. Ruhwedel, M.J.N.L Benders, R.M. Dijkhuizen, W. Möbius, S.H. van Rijt, C.H. Nijboer

*Both authors contributed equally to this manuscript and share the second authorship

Published in GLIA, 2021 Mar;69(3):655-680

ABSTRACT

Encephalopathy of Prematurity (EoP) is a common cause of long-term neurodevelopmental morbidity in extreme preterm infants. Diffuse white matter injury (dWMI) is currently the most commonly observed form of EoP. Impaired maturation of oligodendrocytes (OLs) is the main underlying pathophysiological mechanism. No therapies are currently available to combat dWMI. Intranasal application of mesenchymal stem cells (MSCs) is a promising therapeutic option to boost neuroregeneration after injury. Here, we developed a double-hit dWMI mouse model and investigated the therapeutic potential of intranasal MSC therapy. Postnatal systemic inflammation and hypoxia-ischemia led to transient deficits in cortical myelination and OL maturation, functional deficits and neuroinflammation. Intranasal MSCs migrated dispersedly into the injured brain and potently improved myelination and functional outcome, dampened cerebral inflammation and rescued OL maturation after dWMI. Cocultures of MSCs with primary microglia or OLs show that MSCs secrete factors that directly promote OL maturation and dampen neuroinflammation. We show that MSCs adapt their secretome after ex vivo exposure to dWMI milieu and identified several factors including IGF1, EGF, LIF and IL11 that potently boost OL maturation. Additionally, we showed that MSC-treated dWMI brains express different levels of these beneficial secreted factors. In conclusion, the combination of postnatal systemic inflammation and hypoxia-ischemia leads to a pattern of developmental brain abnormalities that mimics the clinical situation. Intranasal delivery of MSCs, that secrete several beneficial factors *in situ*, is a promising strategy to restore myelination after dWMI and subsequently improve the neurodevelopmental outcome of extreme preterm infants in the future.

INTRODUCTION

Preterm birth is a major cause of life-long neurodevelopmental sequelae in neonates, ranging from motor impairments and cognitive deficits to behavioral problems (Larroque et al., 2008; Linsell et al., 2018; MacKay et al., 2010; Moster et al., 2008). These impairments are believed to be the consequence of Encephalopathy of Prematurity (EoP), a collective term used to delineate a variety of anomalies that result from disturbances in brain development due to an (extreme) premature birth (Volpe, 2009a). Diffuse white matter injury (dWMI), characterized by global hypomyelination in absence of cystic lesions, is a prominent hallmark of EoP (Back, 2017; Volpe, 2009b, 2017). Peri- and postnatal insults associated with preterm birth, such as inflammation and respiratory problems, are thought to negatively impact oligodendrocyte (OL) lineage development, resulting in insufficient myelination and subsequently dWMI (Lee, 2017; van Tilborg et al., 2016; Volpe et al., 2011). Despite the significant morbidity associated with dWMI, at present only supportive treatment is available for preterm infants. Therefore, novel treatment strategies to combat dWMI, tested in clinically relevant animal models of EoP, are urgently needed.

Mesenchymal stem cell (MSC) therapy has been shown to effectively improve functional outcome and aid in endogenous brain repair in numerous experimental models of adult and neonatal brain injury (Paul & Anisimov, 2013; van Velthoven et al., 2010a; Volkman & Offen, 2017; Wagenaar et al., 2018). While these models often do not represent the pattern of dWMI observed in human patients, these studies do provide support of the potential of MSCs to protect and regenerate the white matter (Vaes et al., 2019). MSCs are believed, through paracrine signaling, to directly stimulate OL survival and maturation whilst attenuating neuroinflammation thereby favoring a cerebral milieu permissive for repair and (re)myelination (Cunningham et al., 2018; Vaes et al., 2019). To non-invasively target the brain with MSCs without loss of cells in the periphery, we and others have successfully applied stem cells via the nasal route (Danielyan et al., 2009; Donega et al., 2014b; van Velthoven et al., 2010b).

In the present study we investigated neurodevelopmental outcome in a novel mouse model of dWMI, in which two clinically relevant hits, i.e. postnatal hypoxia/ischemia and inflammation, were combined during a cerebral developmental stage corresponding with human preterm birth. Moreover, we aimed to determine the therapeutic potential of intranasal MSC therapy in this novel dWMI mouse model. To elucidate

the mechanisms underlying the potential regenerative effect of MSCs, *ex vivo* MSC secretome analysis and primary cell cultures were used to assess the cell-specific effects of MSCs on OL maturation and microglia activation.

MATERIALS AND METHODS

MSC culture

GIBCO® mouse (C57BL/6) bone marrow-derived MSCs were purchased (Invitrogen, S1502-100; Carlsbad, California, USA) and cultured according to the manufacturer's instructions. Cells were passaged once (from P2 to P3) prior to *in vivo* administration or in *in vitro* experiments.

In vivo mouse model of dWMI

All animal experiments were carried out according to the Dutch and European guidelines (Directive 86/609, ETS 123, Annex II) and were approved by the Experimental Animal Committee Utrecht (Utrecht University, Utrecht, Netherlands) and the Central Authority for Scientific Procedures on Animals (the Hague, the Netherlands). The animals were kept under standard housing conditions with food and water available *ad libitum*, a 12-hour light/dark cycle and in a temperature-controlled environment. Both sexes were included in all described experiments and randomly assigned to experimental groups with an equal distribution across groups. We used an adaptation of the mouse model described by Shen et al. (2010). Hypoxia-ischemia (HI) was induced at postnatal day 5 (P5) in C57BL/6j mouse pups (in-house breeding) by permanent unilateral occlusion of the right common carotid artery under isoflurane anesthesia (5-10 mins; 5% induction, 3-4% maintenance with flow O_2: air 1:1). After a recovery period of 75 min pups were exposed to 35 minutes of hypoxia (6% O_2) in a temperature-controlled hypoxic incubator (35.8-36.0°C). Directly following hypoxia, pups were i.p. injected with 1mg/kg LPS (List Biological Laboratories, Campbell, CA) dissolved in 0.9% NaCl. Sham-control littermates underwent surgical incision only, without occlusion of the carotid artery, hypoxia or LPS injection. Afterwards all animals were returned to their dams. Prior to MSC administration (on P8 (i.e. D3 after induction of dWMI), P11(i.e. D6) or P15 (i.e. D10)), 2 dosages of 2µl Hyaluronidase (12.5 U/µl in total, Sigma-Aldrich, St. louis, MO) dissolved in H_2O were administered in each nostril (total of 8 µl) and inhaled spontaneously. Hyaluronidase is commonly used to improve permeability of the nasal mucosa and thereby facilitate intranasal cell delivery (Danielyan et al., 2009; van Velthoven et al., 2010b). 30 minutes after

hyaluronidase treatment, mice received intranasally MSCs (different dosages; total of 0.1×10^6,0.5×10^6,1.0×10^6 or 2.0×10^6 MSCs) in dPBS (Thermo-fisher, 14190-169, Waltham, MA) in 2 dosages of 2µl in each nostril (total of 8µl). dPBS was used as vehicle solution. Mice were euthanized at various time points (P8 (i.e. D3 after induction of dWMI), P11 (i.e. D6), P19 (i.e. 2 weeks), P26 (i.e. 3 weeks) and P33 (i.e. 4 weeks)) for different experiments by an i.p. overdose of pentobarbital (250 mg/kg).

MSC tracing

MSC labeling

A detailed description of the synthesis of gold core-mesoporous and lipid coated silica nanoparticles (AuNP-MSN-LIP) can be found in supplemental data S1. Two hours after cell passaging, MSCs were incubated with 25µg/ml AuNP-MSN-LIP in culture medium during 48 hours. Following labeling, 0.5×10^6 MSCs in PBS were administered at P8 as described in the previous paragraph to both dWMI and sham-controls.

Cell tracing in mouse tissue

Twelve hours after MSC administration, mice were sacrificed by overdose pentobarbital followed by decapitation. Brains were collected, cerebrum was divided in ipsilateral and contralateral hemispheres and frozen separately in liquid nitrogen, as well as cerebellum and olfactory bulbs. Spleens, lungs and livers were also collected and frozen in liquid nitrogen. Inductively coupled plasma mass spectrometry (ICP-MS) was performed for quantitative assessment of the biodistribution of MSCs by detection of gold in mouse tissue homogenates. Snap frozen tissue sections were weighed in 2mL plastic eppendorfs. Each sample was lyophilized overnight and re-weighed. Each sample was then transferred to graduated glass vials (VWR). Next, freshly prepared aqua regia (HCl 30% and HNO3 60%, VWR) was added to each tissue section for w/v; 1mg/50µL. The samples were disintegrated overnight at 40°C using an ultrasonic bath (Bransonic®, Thermo Scientific). The samples were further homogenized by microwaving (5×30 seconds, 600W) until the solutions were transparent and free of particulates. Next, freshly prepared 1% HNO3 was functionalized with 20ppb of ruthenium (VWR) to form the matrix solution. All homogenized tissue and cell samples were diluted 1:10 in the as prepared matrix (typically 100µL:900µL). Additionally, a gold standard curve ranging from 1ng L-1 to 100ug L-1 was made by dilution of a gold stock solution (VWR) in as-prepared matrix. ICP-MS was measured

using an iCAP™ RQ ICP-MS (Thermo Scientific). Gold content measured at mass 197 will have no interference from other ions. The detection limit was 1ng/L.

Behavioral assessments

All behavioral paradigms were videotaped and scored by researchers blinded to the experimental conditions. Between all runs all setups were cleaned with soapy water and ethanol to eliminate smells.

Cylinder rearing test

To evaluate motor impairment the cylinder rearing test (CRT) was used at P19, P26 and P33. Animals were individually placed in a transparent cylinder (80mm diameter and 300mm height) and videotaped for a minimum of three minutes. At least 10 full weight-bearing rearings against the cylinder wall were recorded for each animal. Mice that did not perform 10 rearings within the timeframe were retested approximately 30 minutes later. Forepaw usage was scored as left (impaired), right (non-impaired) or both. Preference to use the non-impaired forepaw was calculated as ((non-impaired – impaired)/(non-impaired + impaired + both))x100%.

Delayed spontaneous alternation in T-maze

Executive memory functioning was assessed by measuring correct alternating behavior in a T-maze at P26. Each trial consisted of two runs in a T-shaped maze, a sample run and a choice run. During the sample run, animals were placed in the starting arm of the T-maze. The investigator then waited (max 2 minutes) for the animal to enter one of either goal arms, followed by closing of the chosen arm, forcing the animal to remain in the same spot for 30 seconds. Animals were then returned to the home cage for two minutes, and put back into the starting arm for the choice run. If the animal would first enter the unexplored arm, this was scored as a correct alternation, while exploration of the previously explored arm was scored as an incorrect alternation. Animals performed two trials a day, during three consecutive days (i.e. 6 trials in total). Percentage of correct alternations was calculated as (the total number of choice runs choosing the alternate/unexplored arm of the 6 total trials) x100%.

Open field test

Anxiety-related behavior was assessed using the open field paradigm. Animals were placed in an arena (52,5cm x 35,5cm x 40,5cm) during 30 minutes and locomotion was automatically tracked using Ethovision software (Noldus, Wageningen, The

Netherlands). The arena was divided in two sections, the outer rim and the inner zone. Time spent in both zones, the number of entries into the inner zone and time spent in the inner zone was recorded.

Electron microscopy

After anesthesia mice (n=3, SHAM; n=4 dWMI) were perfused transcardially with HBSS (for flushing) followed by fixative containing 4 % formaldehyde (Sigma, G5882), 2.5 % glutaraldehyde (EM grade, Sigma, 16005) and 0.5% NaCl in phosphate buffer pH 7.4 according to Karlsson and Schultz as described previously (Weil et al., 2019). Brains were dissected and postfixed in the same fixative overnight. Vibratome sections of 300 µm thickness were prepared in sagittal direction and area of interest, the caudal corpus callosum of the ipsilateral hemisphere, were punched out using a hollow biopsy punch. After postfixation in 2% OsO_4 (Science Services, München, Germany) in 0.1 M phosphate buffer pH 7.3 pieces of tissue were embedded in EPON (Serva) after dehydration with acetone. Ultrathin sections were prepared using a Leica UC7 ultramicrotome (Leica, Vienna, Austria) and stained with UranylLess™ (Science Services, Munich, Germany). EM overview images were obtained with a Zeiss LEO912 electron microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at 6300 x magnification with an on-axis 2k CCD camera (TRS, Moorenweis, Germany). Image analysis was performed using ImageJ (Fiji, Version 2.0.0-rc-69/1.52). For g-ratio analysis approx. 150 axons were chosen in a systematic random sampling fashion by overlaying the image with a line grid and selecting axons hit by crossing lines. G-ratios (axonal diameter divided by the fibre diameter including the myelin sheath) were calculated from circular areas equivalent to the measured areas of axons and myelin sheath including the axon.

Post-mortem MRI

Image acquisition

After a lethal dose of pentobarbital animals were transcardially perfused with PBS and 4% PFA in PBS at P26. Prior to scanning, brains with intact skulls fixed in 4% PFA were kept in PBS with sodium azide during a minimum of 7 days. High-resolution structural MRI scans were performed on a 9.4 T horizontal bore MR system (Varian, Palo Alto, CA, USA) equipped with a 6 cm internal diameter (ID) gradient insert with gradients up to 1 T/m. Three perfusion-fixed mouse brains collected with intact skulls and immersed in Galden PFPE (PerFluoroPolyEther) ®D05 were positioned within a custom made solenoid coil with an internal diameter of 2.6 cm. Diffusion tensor

imaging (DTI) was performed using a 3D diffusion-weighted spin-echo sequence with an isotropic spatial resolution of 150 µm, where the read- and phase- encode directions were acquired using 8-shot Echo Planar Imaging (EPI)-encoding, while the second phase direction was linearly phase-encoded (repetition time (TR)/echo time (TE) 500/33 ms, 160×144×140 matrix, field-of-view (FOV) 24×21.6×20 mm3, D/d 8.83/5.5 ms, b 1000,2498,3999 s/mm^2, 30 diffusion-weighted images in non-collinear directions for each diffusion gradient and 6 images without diffusion-weighting (b=0), number of averages per image 1, total number of images 96).

Morphometric analysis of the DTI images

All images were Fourier-transformed and the complete data set was fit using the dtifit routine from the FMRIB's Diffusion Toolbox (Behrens et al., 2007; Behrens et al., 2003; Jbabdi et al., 2012). This resulted in fractional anisotropy (FA) and mean diffusivity (MD) maps and a b0 image of the brains. The brain image on the b0-image was extracted from surrounding skull and tissue signal using the brain extraction tool BET2 of FSL on the b0-image. All FA images were then registered to one of the sham mouse brains using FMRIB's Linear Image Registration Tool (FLIRT) and FMRIB's non-linear registration tool (FNIRT) (Jenkinson et al., 2002; Jenkinson & Smith, 2001). The median of all non-linear registered FA images from control mouse brains was used to obtain a new reference FA image, which was used for non-linear co-registration of the FA images from all mouse brains. The FA and MD maps and the b0 images of the control mouse brains were transformed to the reference FA image, and new median images were obtained for the FA, MD and b0. These were used as DTI reference files.

The Waxholm mouse brain T2* weighted image was downloaded from the Neuro-Imaging Tools and Resources Collaboratory (NITRC) website (https://www.nitrc.org/projects/incfwhsmouse) and converted to a gray/white matter- and CSF-segmented image. This image was non-linearly registered to a similarly segmented b0 reference image. In this manner the regions of interest (hippocampus, lateral ventricle, internal capsule, anterior commissure, cerebellum) drawn on the Waxholm mouse brain were converted to the b0 reference image. Some manual adjustments had to be made to optimize the overlay of the ROIs on the reference image. Not all ROIs were available in the downloaded Waxholm atlas. Additional ROIs (e.g. the secondary motor cortex (M2)) were manually drawn based on the segmentation in the Paxinos and Watson atlas (M2). ROIs overlaid on the reference anatomical image were inversely registered

to the individual mice FA and MD maps, and the ROI volume was determined. In addition, the pixels in the ROI were assigned to CSF, white matter or gray matter based on their FA and MD values. Pixels with FA >0.3 were assigned to be white matter, pixels with an FA < 0.2 and an MD > 0.4×10-3 mm^3/s were assigned to CSF, and remaining pixels were assigned to gray matter.

Immunohistochemistry

Following a lethal dose of pentobarbital animals were transcardially perfused with PBS and 4% PFA in PBS at P19, P26 and P33. Brains were post-fixed during 24 hours in 4% PFA and dehydrated followed by embedment in paraffin. Coronal sections (8µm) were cut at hippocampal level (-1.80mm from bregma in adult mice). To assess hippocampal size, paraffin sections were stained with hematoxylin and eosin (HE). For immunofluorescent stainings sections were deparaffinized in xylene, and subsequently rehydrated in decreasing ethanol concentrations. For antigen retrieval, sections were heated to 95°C in sodium citrate buffer (0.01M, pH 6). After cooling down and PBS (+ 0.05-0.1% Tween20) washings, sections were blocked with 5-10% normal species-specific serum in PBS-Tween20 or 2% bovine serum albumin (BSA)/0.1% saponin in PBS and incubated overnight with primary antibodies (table 1). The following day, sections were washed in PBS and incubated with alexafluor-594 and -488 conjugated secondary antibodies (Life technologies, Carlsbad, CA; 1:200-500) for 1-2 hours at room temperature, followed by DAPI (1:5000) counterstaining and embedment in Fluorsave (Merck Millipore, 345789). For 3,3'-Diaminobenzidine (DAB) staining, sections were deparaffinized in xylene and 100% ethanol. Sections were incubated with 3% H2O2 to block endogenous peroxidase and then hydrated using decreasing ethanol concentrations. For antigen retrieval, sections were heated to 95°C in sodium citrate buffer (0.01M, pH 6). After cooling down and PBS washings, sections were blocked with 5% normal horse serum in PBS, followed by overnight incubation with mouse anti-MAP2 (M4403, Sigma; 1:1000) in 2% normal horse serum in PBS. The following day, sections were washed with PBS and incubated with biotinylated horse-anti-mouse (BA-2000, Vector Laboratories; 1:100), followed by washing in PBS. Biotin was HRP-labeled using a vectastain ABC kit (Vector laboratories) according to manufacturer's protocol, followed by 0.05M Tris-HCl (pH: 7.6) washing. Sections were incubated in 0.5mg/ml DAB (Sigma) in 0.05M Tris-HCl with 0.009% H_2O_2 for staining. Sections were dehydrated in ethanol and embedded.

Table 1. Overview of primary antibodies used in the study

Antigen	Species (host)	Company Productcode	Dilution
Anti-MBP	Rat	Merck Millipore MAB386	1:500
	Mouse	Biolegend SMI-94	1:1000
Anti-NF200	Rabbit	Sigma N-4142	1:400
Anti-Iba1	Rabbit	Wako 019-19741	1:500
Anti-GFAP	Mouse	Origene BM2287	1:200
Anti-Ki67	Rabbit	Abcam 15580	1:300
Anti-Olig2	Rabbit	Merck Millipore AB9610	1:500
	Mouse	Merck Millipore MABN50	1:500
Anti-APC/CC1	Mouse	Calbiochem OP80	1:300
Anti-Cleaved Caspase 3	Rabbit	Cell signal 9664	1:800

Microscopy

Investigators were blinded for experimental conditions during image acquisition. In HE- and MAP2-stained sections a 2,5x magnification was used to visualize both hemispheres using a light microscope (Zeiss, Oberkochen, Germany) with an AxioCam ICc 5 camera (Zeiss). For immunofluorescent stainings, images of both hemispheres were acquired using a Cell Observer microscope with an AxioCam MRm camera (Zeiss, Oberkochen, Germany). For MBP/NF200 stainings 2.5x images were taken to visualize the cortex. In addition, 3 adjacent 40x images were taken at a fixed distance from the external capsule into the cortex (layer III/IV), superjacent to the ipsi- and contralateral hippocampal areas. For exact locations we refer to van Tilborg et al. (2017). For both Iba1 and GFAP analyses, three 40x images were acquired in the corpus callosum. Moreover, one 40x image of the CA1 region of the hippocampus (dorsal from the dentate gyrus) was obtained for GFAP area analyses. For CC1/Olig2, Ki67/Olig2 and Caspase3/Olig2 stainings two 20x micrographs were taken in corpus callosum and the cortex, directly dorsal from the external capsule.

Analyses

All image analyses were performed in a blinded fashion. Hippocampal areas in the contra- and ipsilateral hemispheres were measured on images of HE-stained sections using Zen software (Zeiss, Oberkochen, Germany). Ipsilateral hippocampal area loss was expressed as the ratio of ipsilateral to contralateral hippocampal areas. Microglial morphology was evaluated using the particle analysis function of ImageJ v1.47 (Schneider et al., 2012) as described by Zanier et al. (2015). Microglia were manually selected and morphological description parameters were measured. Mor-

phological parameters of microglia were normalized for sham-control values. GFAP (40x) and NF200 (40x) threshold analyses to calculate the positive area of staining, were carried out using ImageJ software. GFAP$^+$ area measurements were normalized for sham-control values. Following MBP/NF200 staining, the extent of cortical myelination and the microstructural integrity of myelinated axons was assessed as described earlier (van Tilborg et al., 2017). Values of all acquired images were averaged per animal. Cell counts of proliferation (Ki67), microglia (Iba1), apoptotic (cleaved caspase 3) and OL lineage (CC1/Olig2) markers were performed manually using Zeiss software (Axiovision and Zen; Zeiss, Oberkochen, Germany) and corrected for measured area. Cells with a clear DAPI$^+$ nucleus in combination with clear Ki67$^+$/Olig2$^+$, CC1$^+$/Olig2$^+$, cleaved caspase3$^+$/Olig2$^+$ or Iba1$^+$ staining were counted. Images with excessive background staining or large artefacts were excluded.

MSC gene expression profiles

Brain extracts

At P8 (i.e. D3 after induction of dWMI) mice were sacrificed by overdose pentobarbital, decapitated, brains were collected and hemispheres were separately frozen in liquid nitrogen. The ipsilateral hemispheres of sham-operated (n=4) and dWMI mice (n=4) were grinded with a mortar and pestle chilled on liquid nitrogen. The tissue pieces were then weighed and homogenized at 150 mg/ml in knock-out DMEM (Thermo Fisher, 10829018) containing a protease inhibitor cocktail (1:50 dilution; Invitrogen) using a potter tissue homogenizer (10 strokes), followed by centrifugation for 10 minutes at 10,000 x g at 4°C. The supernatant was collected as "brain extract" and protein concentration was measured with a protein assay (Biorad) with BSA as standard. Brain extracts were aliquoted and kept at -80C until use.

PCR arrays

To evaluate the response of MSCs to the milieu encountered in the brain after induction of dWMI, MSCs were cultured and seeded in six-wells plates at 2.0×10^5 cells per well in standard MSC medium. After 24 hours, culture medium was replenished with knock-out DMEM containing either dWMI of sham-operated brain extract at a concentration of 1mg protein/ml. After 72 hours in culture, brain extract-enriched medium was completely removed, wells were washed with ice-cold PBS and total RNA of the MSCs was isolated using the RNeasy minikit (Qiagen). RNA quantity and quality was assessed by spectrophotometry (NanoDrop 2000, Thermo-scientific, Waltham, MS) at 260mm and OD 260/280 ratio was determined to evaluate quality.

cDNA transcription was performed using the RT2 first strand synthesis kit (Qiagen) according to manufacturer's protocol. The cDNA of MSCs exposed to sham-operated or dWMI brain extracts were pooled per condition and expression profiles of 168 growth factor- and cytokine- related genes were assessed by commercially available PCR arrays (Qiagen; PAMM-041Z and PAMM-150Z). PCR array analysis was carried out once following manufacturer's protocol with the RT2 Real-Time SYBR green PCR Master Mix (Qiagen) on the Biorad iQ5. Data were normalized using multiple housekeeping genes provided within the PCR array and analyzed by comparing 2-ΔCt using Qiagen software. Gene expression changes in MSCs exposed to dWMI brain extracts were calculated relative to MSCs exposed to brain extracts from sham-operated mice. A fold regulation threshold of 2.0 was considered as either down- or upregulation.

Quantitative PCR validation

PCR array results were validated by quantitative PCR analysis on individual cDNA samples for selected genes. Real time RT-PCR was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR green supermix (Biorad, 1708887, Hercules, CA). Primer sequences can be found in table 2. Mean expression of GAPDH and ß-actin were used to normalize the data.

Table 2. Overview of primer sequences used in validation qPCR

Gene	Forward (FW) and reverse (REV) primer sequences
Gapdh	FW: TGAAGCAGGCATCTGAGGG REV: CGAAGGTGGAAGAGTGGGAG
ß-actin	FW: AGAGGGAAATCGTGCGTGAC REV: CAATAGTGATGACCTGGCCGT
CXCL12	FW: CGGTGTCCTCTTGCTGTCC REV: CTCTGGCGATGTGGCTCTC
LIF	FW: CTTCTCCCTCTGGTCTCCAA REV: GGGTCAGGATGTTTCAGCAC
IL11	FW: CTGCACAGATGAGAGACAAATTCC REV: GAAGCTGCAAAGATCCCAATG
IL10	FW: GCACCCACTTCCCAGTCG REV: GCATTAAGGAGTCGGTTAGCAG
IGF1	FW: CACATCATGTCGTCTTCACACC REV: GGAAGCAACACTCATCCACAATG
EGF	FW: GTCCGTCTTATCAGGCATCAA REV: TGAGTAGAAGATCCGATCACCAA
CSF3 (GCSF)	FW: AGTGTTCCCAAACTGGGTTCT REV: TTAGGGACTTCGTTCCTGTGA

Cerebral expression of trophic factors following MSC treatment

To investigate the effect of MSC treatment on cerebral mRNA expression changes of trophic factors, dWMI mice that received vehicle or 0.5×10^6 MSCs intranasally at D3 after injury, were sacrificed by overdose pentobarbital followed by decapitation at 12 hours after intranasal treatment. Brains were collected, hemispheres were isolated and divided in a rostral and caudal part and frozen separately in liquid nitrogen. The cerebellum was frozen separately. The ipsilateral brain parts and cerebellum of vehicle-treated (n=3) and MSC-treated mice (n=4) were grinded with a mortar and pestle chilled on liquid nitrogen. All tissue parts were lysated in RLT lysis buffer using a TissueLyser LT Adapter and stainless-steel beads (all Qiagen, Hilden, DE), at 50Hz during 2 minutes following the supplier's protocol. RNA isolation was carried out using the RNeasy minikit (Qiagen), including on-column DNase digestion with the RNase-free DNase set (79254, Qiagen). RNA quantity and quality were assessed by spectrophotometry (NanoDrop 2000, Thermo-scientific, Waltham, MS) at 260mm and OD 260/280 ratio was determined to evaluate quality. cDNA transcription was performed using the iScript reverse transcription supermix (Bio-rad, Hercules, CA). Real time RT-PCR for a selection of genes was carried out using the QuantStudio 3 (Applied Biosystems, Foster City, CA) using SYBR select master mix (4472903, Applied Biosystems). Primer sequences can be found in table 2. Mean expression of GAPDH and b-actin were used to normalize the data.

In vitro models of dWMI

Primary rat cultures

A mixed glial culture was obtained from P0-2 Sprague Dawley rat pup cortices, as described in detail by Chen et al. (2007) with minor adaptations. In short, brains were isolated and cortices were dissected, followed by removal of the meninges. Cortices were pooled, minced, and subsequently dissociated using DNase I (10ug/l, Sigma Aldrich, D5025) and Trypsin solution (0.01%, Sigma Aldrich, T1426) in HBSS. Tissue suspensions were dissociated by pipetting and filtered through a 0.70µm filter twice. Cells were plated in poly-D-lysine-coated (0.1mg/ml, Sigma Aldrich, P6407) T75 culture flasks. For details on the media used, please see the paper. After a minimum of 10 DIV, microglia and oligodendrocyte precursor cells (OPCs) were harvested by mechanical shaking on an orbital shaker. Cultures were kept for a remaining 7 DIV for a second harvest.

Production of MCM

The flasks were shaken for 1 hour at 200rpm at 37°C to collect microglia. Culture medium with detached microglia was centrifuged (10 minutes at 1200rpm at RT) and microglia were counted. Microglia were plated at 0.5×10^6 cells per well on poly- L-ornithine (Sigma Aldrich, P3655)-coated 24 wells plates. Microglia-conditioned-medium (MCM) was produced to induce a maturational arrest in primary OLs. Activated microglia produce pro-inflammatory cytokines, mimicking *in vivo* inflammation, one of the etiological hallmarks of dWMI. To this means, 24 hours after plating, microglia were cultured in Basal Defined Medium (BDM) as described by Chen et al. (2007), with or without 50ng/ml LPS (Sigma, L4616) during 24 hours at 37°C, producing respectively "MCM+LPS" or "MCM-LPS". MCM was collected, filter sterilized (0.20µm) and stored at -80°C until use.

After removal of detached microglia from the culture flasks, flasks were filled with fresh medium and shaken for an additional 20 hours at 200 rpm at 37°C to isolate OPCs. The cell suspension was passed through a 20µm sterile screening pouch (Merck Millipore, NY2004700, Burlington, MS) to remove possible remaining/detached microglia and astrocytes. The OPCs were collected by centrifugation (100g during 10 minutes RT) and plated at 4.0×10^4 cells/well on poly-D,L-ornithine (Sigma Aldrich, P0421)-coated 24-wells plates in OPC medium (BDM with PDGF-aa (Peprotech, 100-13A) and bFGF (Peprotech, 100-18B)). OPCs were cultured for 4 days before starting the experiments. Cells were fed with a complete OPC medium change every other day.

Primary mouse cultures

Primary cultures of cortical microglia were prepared from P1 C57BL/6 mice. In short, after dissection, meninges were removed and cortices were minced and incubated with 0.25% trypsin (Sigma, T4799) in Gey's balanced salt solution (GBSS) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 mM D (+)-glucose during 15 minutes. The dissociated cell suspension was resuspended until homogenous and cultured in poly-L-ornithine-coated flasks (Sigma, P3655) (at a concentration of cortices of 1 animal/flask) in DMEM/HamF10 (1:1) (Gibco, 41965-039 and 31550-023) supplemented with 10% FCS, 2mM glutamine and antibiotics (see above). After 10-12 DIV culture flasks were shaken during 20-22 hours (130-135rpm, 37°C) to detach microglia. The microglia were collected by centrifugation (120 rpm during 10 minutes RT), counted and seeded in poly-L-ornithine-coated 24-wells plates at a density of

1.0-2.0×10^5 cells per well. Microglia were cultured for 1 day before starting the experiments. After shaking, flasks were directly replenished with new culture medium and new microglia were harvested with a second shake after another 10 DIV.

Non-contact MSC-glia cocultures

To study the effect of the MSCs' secretome on glial cells, MSCs were embedded in gel inserts to establish non-contact cocultures between MSCs and primary OPCs or microglia. This culture system enables cells to remain in their own medium, ensuring optimal cell viability. For these experiments MSCs were cultured as described above. One day prior to the start of cocultures MSCs were embedded in Hydromatrix gel (Sigma, A6982) inserts (Merck Millipore, MCHT24H48) according to manufacturer's protocol in different cell densities (2.0-8.0×10^4 cells per insert).

At day 4 after OPC plating, when the majority of OLs displayed an immature pre-OL morphology (i.e. a round cell body with multiple extensions), OPC medium containing PDGF-AA and bFGF was replaced with either MCM+LPS or MCM-LPS containing NAC (Sigma, A8199), CNTF (Peprotech, 450-50) and T3 (Sigma, T2752), leading to either induction of OL maturation (MCM-LPS) or OL maturational arrest (MCM+LPS). Aside from MCM, we used TNFα as an alternative strategy to induce OL maturational arrest, as described previously (Bonora et al., 2014). For these experiments BDM containing TNFα (10ng/ml, Peprotech, rat recombinant 400-19) and the differentiation growth factors (NAC (Sigma, A8199), CNTF (Peprotech, 450-50) and T3 (Sigma, T2752))) was added to wells 4 days after OL plating. Concurrently, transwell inserts containing MSCs or hydromatrix gel without MSCs as a control, were added to the wells. After 72 hours of coculturing, inserts were removed and OLs were fixated.

Microglia were cultured and plated as described above. After 24 hours microglia were stimulated with 50ng/ml LPS (Sigma, L4515). Concurrently, transwell inserts containing MSCs (4.0×10^4 cells per insert) were placed in each well. After 48 hours of coculture, inserts were removed and microglia supernatant was collected, aliquoted and stored at -80C until ELISA.

Growth or immunomodulatory factor administration

At day 4 following OPC plating, when cells displayed a pre-OL morphology, OPC differentiation or maturational arrest was induced using MCM or TNFα (see above). To the OPC cultures, the following selected factors were added: Noggin (murine

recombinant, Peprotech 250-38), IL10 (rat recombinant, Peprotech 400-19), IL11 (murine recombinant, Peprotech 220-11), LIF (murine recombinant, Peprotech 250-02), CXCL12 (murine recombinant, Peprotech 250-20a), GCSF (murine recombinant, Peprotech 250-05), IGF1 (murine recombinant, Peprotech 250-19) and EGF (murine recombinant, Peprotech 250-09). Factors were added simultaneously with MCM+LPS or MCM-LPS or 10ng/ml TNFα to study their potential to boost OPC differentiation. All experiments were terminated after 72 hours. Optimal concentrations of the selected factors (Noggin 250ng/ml, IL10 25ng/ml, IL11 100ng/ml, LIF 100ng/ml, CXCL12 100ng/ml, GCSF 100ng/ml, IGF1 100ng/ml, EGF 20ng/ml) were obtained in dose-response experiments in which MBP expression by matured OLs was the final read-out (Supplemental data; S2).

ELISA

TNFα concentrations in the microglia supernatant were measured using an ELISA kit for murine TNFα (Ucytech, Utrecht, The Netherlands) according to manufacturer's protocol. Because TNFα levels varied slightly between independent experiments, TNFα data were normalized to positive control conditions (50ng/ml LPS; no insert).

Immunocytochemistry

Oligodendrocyte cultures were fixated with 4% PFA in PBS during 10 minutes. Subsequently 2% BSA and 0.1% saponin in PBS was used to block nonspecific binding. Plates were incubated with primary antibodies (rabbit-anti-Olig2, Merck Millipore AB9610; 1:1000 and mouse-anti-MBP, Biolegend SMI-94, 1:1000) overnight at 4°C, washed with PBS, followed by incubation with alexafluor-594 and -488 conjugated secondary antibodies (Life technologies; 1:1000) for 1 hour at room temperature. Cell nuclei were counterstained with Hoechst 33342 (Sigma) and wells were embedded in Fluorsave (Merck Millipore, 345789).

For each well, six adjacent fields were photographed (10x), starting at a fixed distance of the well edges. Olig2- and Hoechst-positive cells were counted automatically using the analyze particles function in ImageJ v1.47. MBP area was determined using manual threshold analyses in ImageJ software v1.47. In order to reliably compare independent experiments, all results were normalized for the positive control (MCM+LPS; insert without MSCs/ 0ng factor).

Statistics

All data are presented as mean ± standard error of the mean (SEM). Statistics were performed with Graphpad Prism 8.3. Comparison of two groups was tested using unpaired t-tests, or in the event of unequal variances, non-parametric Mann-Whitney tests. When comparing >2 groups, one-way ANOVA with Bonferroni posthoc tests was carried out. A non-parametric Kruskal-Wallis test with Dunn's posthoc correction was used for comparison of multiple groups with unequal variances. p-values <0.05 were considered statistically significant. Specific sample sizes are mentioned in the figure captions.

RESULTS

A double-hit mouse model of dWMI

Two postnatal hits cause transient myelination deficits without cortical neuronal/axonal loss

To mimic preterm birth-related hits newborn P5 mouse pups were subjected to two hits: postnatal hypoxia-ischemia plus inflammation. To investigate the effects of these double hits on myelination later in life, brain sections were stained for expression of myelin basic protein (MBP) at P19, P26 and P33 (i.e. 2, 3 and 4 weeks after induction of dWMI, respectively). In comparison to sham-operated control littermates, we observed a significant decrease in cortical myelination, indicated by a reduction in MBP^+ cortical coverage in the ipsilateral hemisphere, at P19 (*p<0.001*) and P26 (*p<0.001*) in animals subjected to dWMI, indicating myelination failure (figure 1A-B). At P33 no significant differences in cortical myelination were observed anymore in dWMI animals compared to sham-operated controls (*p=0.395*). More detailed examination of the myelin microstructure by segmentation analysis showed that dWMI animals display a less complex organization of myelinated axons in the cortical white matter of the ipsilateral hemisphere, indicated by a significant reduction in the number of intersections (P19 *p=0.013;* P26 *p=0.010*) and fiber length (P26 *p=0.013*), up to three weeks after injury induction (figure 1 C-E). Both hits were required to induce deficits in myelin microstructure; only LPS injection in sham-operated animals or induction of HI without LPS injection did not result in significant myelin deficits (data not shown). In line with the cortical myelination data (figure 1A), at P33 no significant differences in microstructural parameters (intersections *p=0.110* and fiber length *p=0.485*) were observed between dWMI and control animals (figure 1C-E). To assess more in depth whether myelination at P33 was endogenously recovered, we measured myelin sheath thickness using electron microscopy. We did not observe

any significant differences in myelin g-ratio, representing the relationship between axon size and myelin thickness, between dWMI and control animals (p=0.759) (figure1 F-G). Taken together, these results indicate that our double-hit model induces a delay in myelination, rather than irreversible long-lasting changes in white matter development. We did not observe any myelin deficits in the contralateral hemisphere of dWMI animals compared to both hemispheres of sham-control littermates (data not shown), thus, from here onwards data will be shown from the ipsilateral hemisphere only.

Besides studying myelination, we also assessed possible effects of our model on cerebral gray matter integrity by using HE, NF200 and MAP2 stainings to look at hippocampal area, axonal integrity and neuronal loss in cortical areas, respectively. Area measurements on HE sections revealed a unilateral reduction in ipsilateral hippocampal area in dWMI animals, stable over time and persistent up to 4 weeks after injury induction (P19 *p=0.004*; P26 *p=0.003*; P33 *p=0.004*) (figure 2A-B). We did not observe any loss of MAP2 staining in the cortex as a measure for acute neuronal damage at P19 (figure 2E). Additionally, we did not detect any axonal damage in cortical regions as determined by NF200 stainings at P19-P33 (figure 2C-D). These findings indicate that gray matter area loss (i.e. reduced hippocampal area) observed in dWMI animals is not the result of acute neuronal loss or axonal damage, but rather due to maldevelopment of the gray matter structure secondary to myelination failure.

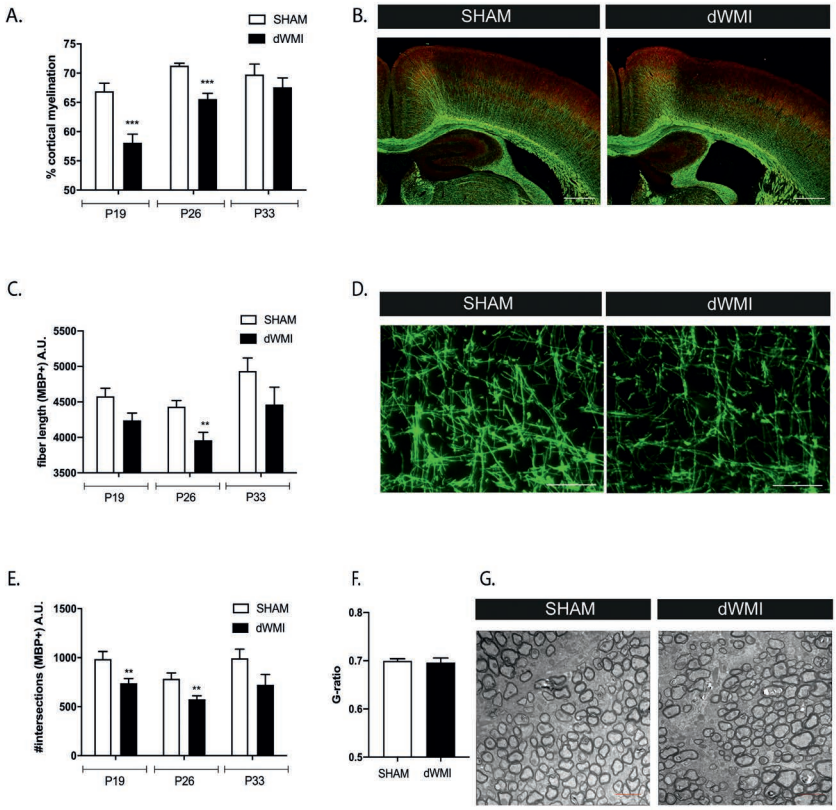


Figure 1. The combination of postnatal hypoxia/ischemia and systemic inflammation at P5 (dWMI model) causes a delay in myelination in neonatal mice. **A.** Mice exposed to the double-hit model displayed a transient reduction in cortical myelination (P19 SHAM n=11 dWMI n=11, P26 n=7 in both groups, P33 SHAM n=4 dWMI n=5) **B.** Representative fluorescent images (2.5x) of the ipsilateral cortex of a sham-operated control mouse (left) and dWMI mouse (right) stained for axonal marker NF200 (red) and myelin marker MBP (green). Scale bars: 500 µm. **C/E.** Microstructural changes in MBP⁺ fibers following dWMI induction were observed at P19 and P26, assessed by measuring fiber length (**C**) and number of intersections (**E**) (P19 SHAM n=11 dWMI n=11, P26 n=8 in both groups, P33 SHAM n=4 dWMI n=5) **D.** Representative fluorescent images (40x) of MBP⁺ axons in the ipsilateral cortex of a sham-operated control mouse (left) and dWMI mouse (right). Scale bars: 100µm **F.** G-ratio analyses (axonal diameter divided by the fiber diameter including the myelin sheath) reveal no changes in myelin enwrapment at P33 in SHAM (n=3) and dWMI (n=4) animals. **G**. Representative electron microscopy images of the caudal corpus callosum in SHAM and dWMI animals at P33. Scale bars: 2µm. **: p<0.01; ***: p<0.001 sham-operated control vs dWMI animals at the specified timepoint.

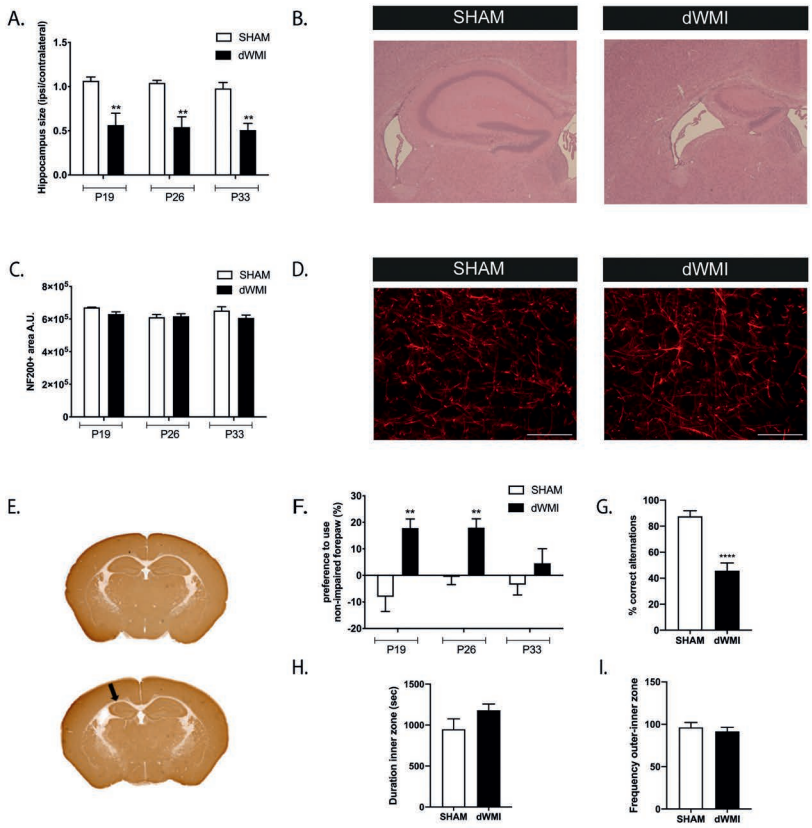


Figure 2. dWMI induction causes functional impairments in absence of cortical axonal deficits or acute neuronal loss. **A.** Mice with dWMI displayed a persistent unilateral reduction in ipsilateral hippocampal size compared to sham-control mice (P19/26 SHAM n=7 dWMI=7, P33 SHAM n=4 dWMI n=5). **B.** Representative images of the ipsilateral HE-stained hippocampus of a sham-operated control mouse (left) and dWMI mouse (right). **C.** No significant changes in NF200⁺ area were observed in the ipsilateral cortex of sham-control (P19 n=4, P26 n=7, P33 n=4) vs. dWMI (P19 n=4, P26 n=7, P33 n=5) animals. **D.** Representative fluorescent images (40x) of NF200⁺ axons in the ipsilateral cortex of a sham-control mouse (left) and dWMI mouse (right). Scale bars: 100µm **E.** Representative whole brain images stained for MAP2 at P19 showing reduced hippocampal area (arrow) but no further indications of overt neuronal loss. **F.** dWMI animals (P19 n=6, P26 n=7, P33 n=7) performed worse compared to sham-controls (P19 n=8, P26 n=8, P33 n=5) animals in the cylinder rearing test up to P26, indicating unilateral motor impairment. **G.** Compared to sham-control animals (n=8), dWMI mice (n=8) made less correct spontaneous alterations in the T-maze at P26. **H/ I.** No changes in time spent in the inner zone (**H**) or frequency of inner zone entry (**I**) between sham-control (n=11) and dWMI (n=11) were observed in the open field test at P26. **: p<0.01;****: p<0.0001 sham-operated control vs dWMI animals at the specified timepoint.

dWMI-induced functional deficits include motor impairment and cognitive deficits

The functional consequences of dWMI were assessed by the cylinder rearing test (CRT) for motor function, open field for anxiety-related behavior and T-maze for executive memory functioning. dWMI animals demonstrated a significant increase in non-impaired forelimb preference in the CRT, indicating unilateral motor impairment corresponding to unilateral myelination deficits, up to P26 (P19/26 *p=0.003*; P33 *p=0.299*) (figure 2F). Sham-operated littermates did not show a significant preference to use either the left or right forepaw in the CRT. Executive memory and anxiety-related behavior were assessed at P26 as the tests were not reliably performable with animals younger than this age and most prominent histological deficits of dWMI were observed up to P26. T-maze performance of sham-operated mice was very accurate with ~90% correct alternations, indicating high executive memory functioning under control conditions. In contrast, dWMI animals displayed executive memory deficits, with significant less correct alternations (even <50%) in the T-maze ($p<0.0001$) (figure 2G). Using the open field task, we did not find any indication for anxiety-related behavior in dWMI animals as we did not detect differences in entries into, or time spent, in the inner zone of the arena between dWMI and control animals (*p=0.133)* (figure 2H-I).

dWMI induction is associated with microglia activation and astrogliosis

To study neuroinflammation, an important etiological hallmark of dWMI, brain sections of dWMI and control mice were stained for the microglia marker Iba1 and astrocyte marker GFAP at P19, P26 and P33. In the corpus callosum of dWMI animals a higher number of Iba1-positive cells were observed both at P19 and P26 after induction of injury (P19 *p=0.037*; P26 *p=0.015*) (figure 3A). At P33 Iba1-positive cell numbers in dWMI animals were reduced to sham-operated control levels. Detailed analyses of numerous microglial morphological aspects (e.g. cell circularity and perimeter), revealed a more amoeboid, active morphology of microglia in dWMI animals compared to controls at P19 and P26 (figure 3B-E). These morphological activation hallmarks of Iba1-positive cells were restored at P33 after dWMI. An increase in $GFAP^+$ area was observed in the corpus callosum and hippocampus of dWMI animals compared to sham-controls at P19, with the most pronounced changes observed at P26 (CC *p=0.048* and HC *p=0.029*). The increase in $GFAP^+$ area in both brain areas was restored to control level at P33 (figure 3 F-I). Collectively, these data indicate that the double-hits applied at P5 in newborn mice induce a potent but transient neuroinflammatory response.

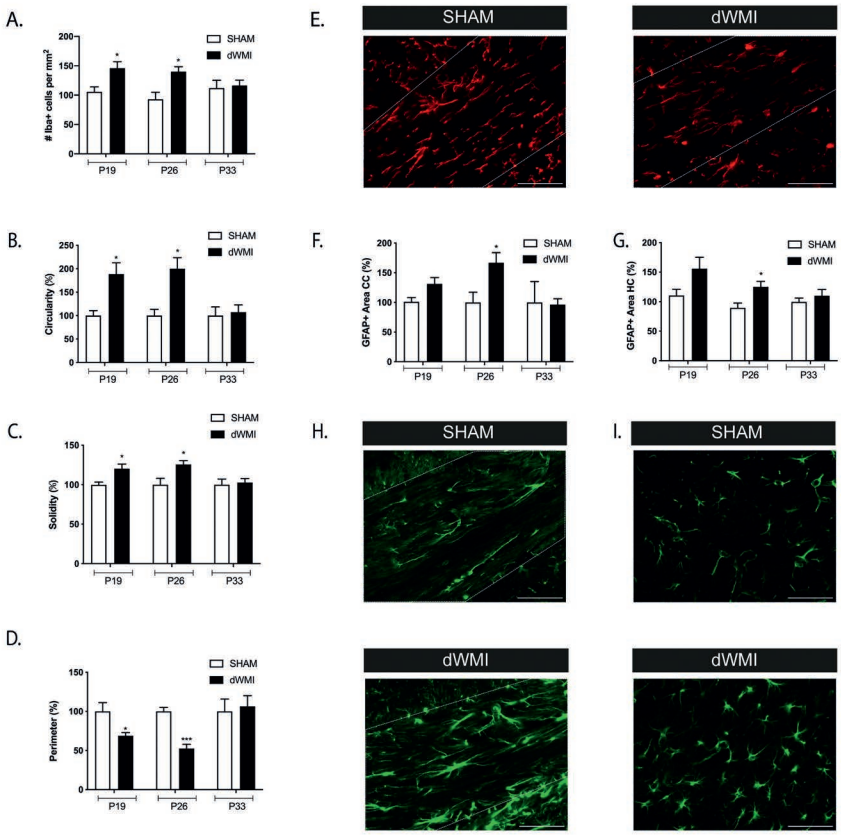


Figure 3. Microglia and astrocyte activity is transiently increased in dWMI animals. **A.** dWMI induction leads to a transient rise in microglia (Iba1⁺) numbers in the corpus callosum (P19 SHAM n=6 dWMI n=9, P26 SHAM n=6 dWMI n=10, P33 SHAM n=7 dWMI n=8). **B-D.** Microglia in the corpus callosum of dWMI animals (P19/26/33 n=8) demonstrate a more pro-inflammatory phenotype with increased cell circularity (**B**)/solidity (**C**) and decrease in cell perimeter (**D**) up to P26 compared to sham-control (P19/26 n=5, P33 n=7, normalized to control values) mice. **E.** Representative fluorescent images (40x) of Iba1⁺ cells in the corpus callosum (white outline) in a sham-control (left) and dWMI animal (right). Scale bars: 100µm **F/G.** Quantification of GFAP⁺ area revealed increased astrocyte reactivity in dWMI animals (P19 n=14, P26 n=8, P33 n=5, normalized to control values) compared to sham-controls (P19 n=10, P26 n=7, P33 n=4) in the corpus callosum and hippocampus at P26. **H/I.** Representative fluorescent images (40x) of GFAP⁺ staining in the corpus callosum (**H**) and hippocampus (**I**) in a sham-control (upper) and dWMI (lower) animal. Scale bars: 100µm. *: $p<0.05$; ***: $p<0.001$ sham-operated control vs dWMI animals at the specified timepoint.

dWMI leads to white matter volume loss measured by postmortem MRI

Over the years, multiple human MR studies have identified long-term region-specific reductions in brain volumes of preterm infants compared to term peers. These volumetric changes have been associated with a poorer functional outcome (Keunen et al., 2016; Lind et al., 2011; Ment et al., 2009; Peterson et al., 2000). Using *postmortem* MRI, we measured the volumes of a range of predefined ROIs in both gray and white matter areas in sham-control and dWMI mice at P26. The double-hit model was associated with a reduction in total cerebral volume (*p=0.001*) and borderline significant reduction in global WM volume (*p=0.052)* compared to sham-control animals (figure 4A). Specific analyses of important WM structures showed reduction in volumes of the corpus callosum (*p=0.001*), internal capsule (trend, *p=0.060*) and anterior commissure (*p=0.006*) in dWMI animals compared to sham (figure 4B-D). Moreover, dWMI animals displayed enlargement of the lateral ventricles (*p=0.046*), a prominent hallmark of dWMI in preterm infants (Keunen et al., 2016; Lind et al., 2011) (figure 4E). In line with findings in human studies (Keunen et al., 2016; Volpe, 2009a), dWMI animals also showed a loss of global gray matter volume (*p=0.008*), along with volumetric deficits of gray matter structures, such as the hippocampus (*p=0.003*) and secondary motor cortex (M2) (*p=0.004*) (figure 4G-I). Additionally, dWMI animals exhibited volumetric deficits of the cerebellum (*p=0.013*) compared to sham-controls (figure 4J).

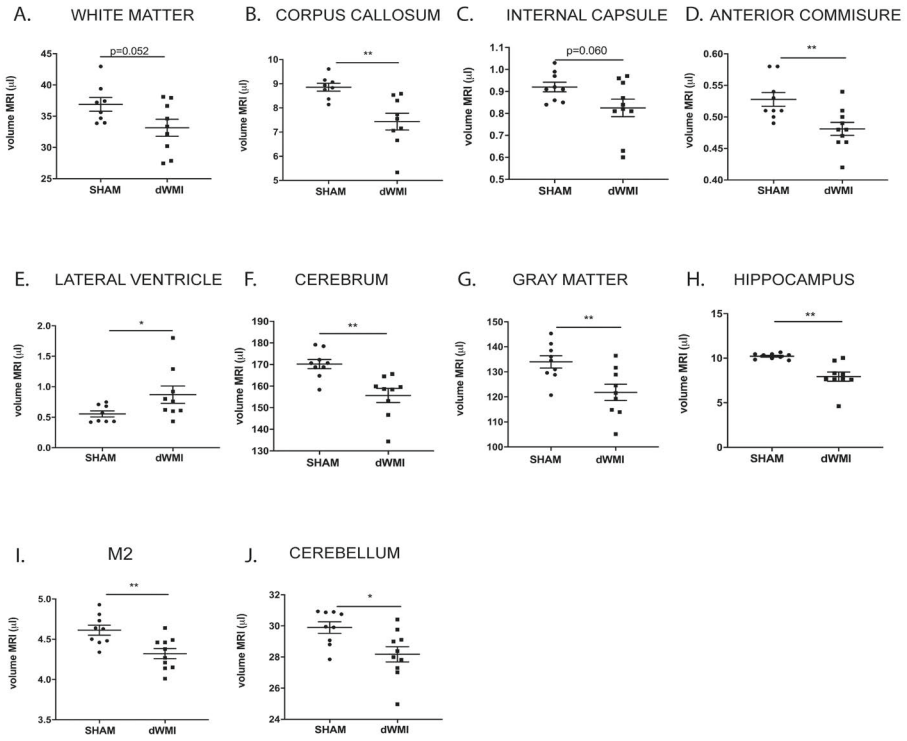


Figure 4. dWMI induction leads to global volumetric deficits on post-mortem MRI. **A-J.** dWMI animals (n=9) displayed a reduction in volume (µL) of multiple white and gray matter structures compared to sham-controls (n=9) at P26. *: p<0.05; **: p<0.01. Nearly significant p values are indicated in A and C.

dWMI is associated with disturbed oligodendrocyte lineage maturation

Multiple studies have suggested that myelination failure in dWMI is the result of arrested maturation of the oligodendrocyte (OL) lineage (Billiards et al., 2008; Buser et al., 2012). Double-stainings using the proliferative marker Ki67 (indicative for immature, proliferative OL precursors) or the mature OL-specific marker CC1, in combination with the nuclear OL marker Olig2 were used to investigate development of the OL lineage in animals exposed to the double-hit model. Interestingly, at P8 (i.e. 3 days after induction of dWMI) an increase in the number of proliferating $Ki67^+/Olig2^+$ cells was observed in the cortex of dWMI animals compared to sham-controls (*p=0.041*) (figure 5A-B). No significant differences in $Ki67^+/Olig2^+$ cell numbers were observed in the corpus callosum at P8 (*p=0.932*) (figure 5C). At P19, increased numbers of $Ki67^+/Olig2^+$ cells were observed in corpus callosum, but not the cortex, of dWMI animals compared to controls (*p=0.008 and p=0.936* respectively) (figure 5D-F). Importantly,

the number of mature CC1$^+$/Olig2$^+$ cells was significantly reduced in cortical areas in dWMI animals compared to sham-controls (*p=0.047*), whereas CC1$^+$/Olig2$^+$ cell numbers in the corpus callosum were unchanged (*p=0.873*) (figure 5G-I). We were unable to detect any differences in mature (or immature) OLs between dWMI animals and sham-controls at P26 (data not shown). CC1 analyses were not performed on P8, as this marker was hardly expressed at this time point.

To assess the contribution of OL cell death to the observed reduced number of mature oligodendrocytes, we performed double-stainings for cleaved caspase 3 and Olig2 on sections of dWMI and control mice at P8. Very low numbers of cleaved caspase3$^+$/Olig2$^+$ cells were observed in both the corpus callosum and cortex at P8 and no significant differences in numbers were observed between sham-control and dWMI groups (cortex *p=0.279*; corpus callosum *p=0.889*) (figure 5J-L). To check for possible cell death of OL progenitors before P8, we also assessed the total number of Olig2$^+$ cells at P8. In line with the observed rise in proliferating cortical OLs, an increase in total Olig2$^+$ cell numbers was detected at P8 in the cortex of dWMI animals compared to sham-controls (*p=0.007*) (figure 5M). No significant differences in total Olig2$^+$ numbers were observed in the corpus callosum at P8 between dWMI and sham-control animals (*p=0.435*) (figure 5N).

In sum, our data show an early and persistent proliferative response of the OL lineage after dWMI induction, with a concurrent reduction of mature cortical OLs, without clear evident OL cell death or loss. These data together imply that in this model myelination failure is likely the result of arrested oligodendrocyte maturation rather than extensive loss of (immature) OLs.

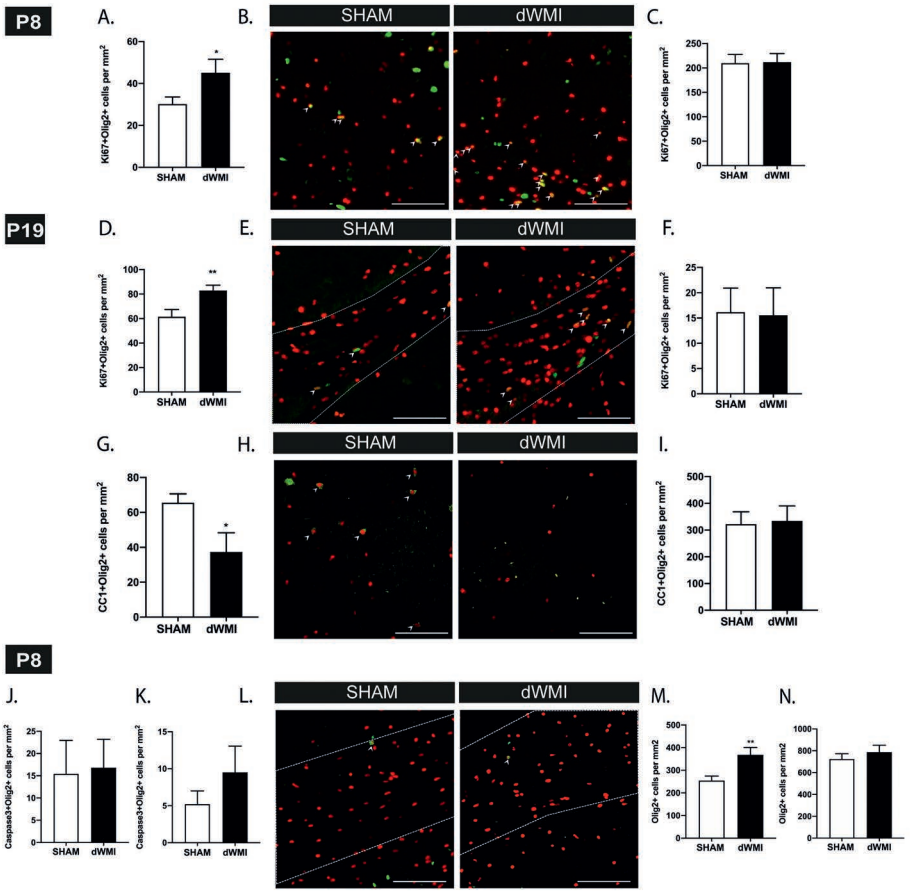


Figure 5. dWMI is associated with an early proliferative response of the OL lineage, followed by maturation arrest. **A/C.** dWMI induction leads to an early increase (P8) of Ki67⁺/Olig2⁺ cells in the cortex (**A**), indicative of a proliferative response of the OL lineage. This proliferative response is absent in the corpus callosum (**C**) (SHAM n=10, dWMI n=7). **B.** Representative fluorescent images of the cortex of a sham-control (left) and dWMI (right) animal at P8, double-stained for Ki67 (green) and Olig2 (red). Double-positive cells are marked with an arrowhead. Scale bars: 100µm. **D/F.** dWMI mice (n=12) showed a higher quantity of Ki67⁺Olig2⁺ cells in the corpus callosum (**D**) compared to sham-controls (n=12) at P19, indicative of increased OL proliferation. No differences in Ki67⁺Olig2⁺ cells were found in the cortex (**F**). **E.** Representative fluorescent images (20x) of the corpus callosum (white outline), double-stained for Ki67 (green) and Olig2 (red), of a sham-control (left) and dWMI (right) mouse at P19. Double-positive cells are marked with an arrowhead. Scale bars: 100µm. **G/I.** At P19, a lower quantity of mature (CC1⁺/Olig⁺) OLs was observed in the cortex (**G**) of dWMI animals (n=5), compared to sham-control animals (n=5). We did not detect differences in the number of mature CC1⁺/Olig⁺ OLs in the corpus callosum (**I**). **H.** Representative fluorescent images of the P19 cortex, double-stained for CC1(green) and Olig2 (red), of a sham-control (left) and dWMI (right) mouse. Double-positive cells are marked with an arrowhead. Scale bars: 100µm. **J/K.** At P8, no significant changes in the

number of cleaved caspase3⁺/Olig2⁺ cells were observed in the corpus callosum (**J**) or cortex (**K**) of dWMI (n=9) vs. sham-control (n=10) mice. **L.** Representative fluorescent images of the corpus callosum (white outline), double-stained for cleaved caspase 3 (green) and Olig2 (red) of a sham-control (left) and dWMI (right) animal at P8. Double-positive cells are marked with an arrowhead. Scale bars: 100µm. **M/N.** dWMI induction is associated with an increase in Olig2⁺ cells, representing the total OL population, in the cortex (**M**), but not in the corpus callosum (**N**) (SHAM n=10, dWMI n=7).*: p<0.05; **: p<0.01 sham-operated control vs dWMI animals.

Intranasal MSC treatment to repair dWMI

MSCs widely distribute throughout the injured brain following intranasal administration

To study MSC distribution, mesoporous silica coated gold nanoparticles (AuNP-MSN) were synthesized (Figure S1A-D) and used to label MSCs. The labeling was most efficient after 48h of incubation and remained stable after cell concentrating (Figure S1E). Nanoparticle labeled MSCs were administered intranasally at D3 after dWMI induction (i.e.at P8) to investigate cell distribution in our model. MSC distribution could be measured by detecting gold signal in tissue homogenates using ICP-MS. Twelve hours after MSC administration, the majority of the measured gold was found in the brain of dWMI animals and a significantly lower amount of gold in peripheral organs (i.e. lungs, liver and spleen) *(p=0.0005; p=0.002; p=0.0006 respectively)* (figure 6A). In contrast, in sham-controls the highest mass of gold was observed in the lungs and liver after intranasal MSC administration (figure 6B). To investigate cerebral distribution of MSCs, the brain was divided in ipsi- and contralateral rostral and caudal cerebrum, cerebellum and bulbi. An even distribution of the total amount of gold was observed in both ipsi- and contralateral cerebrum and cerebellum of dWMI animals (figure 6C) indicating a dispersed distribution throughout the diffusely affected brain.

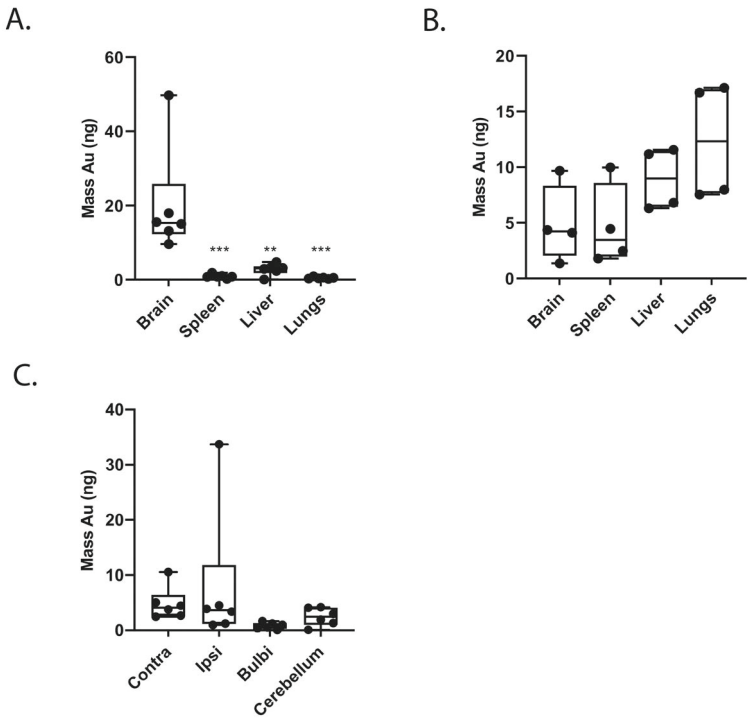


Figure 6. Intranasally administered silica coated gold nanoparticle-labeled MSCs evenly distribute throughout the brain after dWMI induction. **A/B**. After intranasal administration of nanoparticle-labeled MSCs at D3 (i.e. P8) after injury induction or sham operation, the highest mass of gold was observed in the brain for dWMI animals (n=6) (**A**) versus peripheral organs in sham-control animals (n=6) (**B**). **C**. Gold nanoparticles, used to label MSCs, were evenly distributed throughout the brain parts after dWMI **: p<0.01; ***: p<0.001 peripheral organs vs. brain.

Intranasal administration of MSCs improves myelination and functional deficits

To investigate the potential of intranasal MSC therapy on myelin deficits, dWMI animals were intranasally treated with different dosages of MSCs at 3 days after induction of brain injury. At P19, vehicle-treated dWMI animals displayed a significant reduction in cortical myelination compared to sham-control animals (*p=0.0002*) which could be rescued by intranasal treatment with 0.5×10^6, 1.0×10^6 or 2.0×10^6 MSCs, as indicated by increased cortical MBP^+ coverage (*p=0.001, p=0.0008 and p=0.0316* dWMI-veh vs dWMI-MSC dosis respectively) (figure 7A). A lower dose of 0.1×10^6 MSCs did not significantly enhance cortical myelination after dWMI and was therefore not assessed further in this study (data not shown). Microstructural MBP analyses showed similar results: complexity of myelin fibers was negatively affected by dWMI (fiber length

p=0.048, intersections *p=0.0026*), however an intranasal dose of 0.5×10^6 and 1.0×10^6 MSCs completely restored the number of intersections (*p=0.0003* and *p=0.0039 vs veh* respectively) and fiber length (*p=0.0048* and *p=0.0171 vs veh* respectively) to control level. The highest dose of 2.0×10^6 MSCs provided partial recovery of myelin complexity after dWMI: increasing the number of intersections (*p=0.0349*) without significantly boosting fiber length (*p=0.421*) (figure 7B-C). Intranasal treatment with MSCs did not significantly restore developmental gray matter deficits after dWMI, with persistent hippocampal area loss in HE-stained sections after MSC treatment (figure 7D).

To examine the effect of MSC treatment on motor outcome after dWMI, animals were subjected to the CRT at P19. Intranasal treatment with 0.5×10^6, 1.0×10^6 and 2.0×10^6 MSCs at 3 days after dWMI potently improved motor outcome by reducing forepaw preference (*p=0.003, p<0.0001* and *p<0.0001* respectively). In line with our histological findings on myelination, motor outcome did not improve after treatment with 0.1×10^6 MSCs (data not shown). Based on the histological and functional outcome parameters as shown in Figure 7A-E, we determined 0.5×10^6 MSCs to be the lowest effective dose ensuring optimal recovery after dWMI. The effect of 0.5×10^6 MSCs on executive memory at P26 was assessed next. dWMI animals treated with vehicle demonstrated impairments in executive memory determined by a reduction in correct alternation in the T-maze compared to sham-controls. Intranasal MSC treatment showed a potent favorable effect on executive functioning by restoring levels of correct alternations up to sham-control levels (*p=0.029*) (figure 7F).

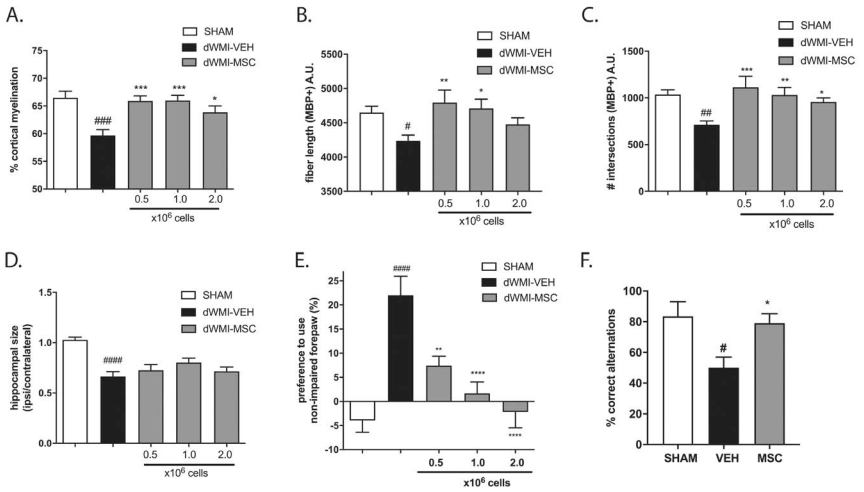


Figure 7. Intranasal MSC treatment boosts myelination and rescues motor and cognitive impairments in dWMI mice. **A.** Intranasal administration of 0.5×10^6 (n=11), 1.0×10^6 (n=11) and 2.0×10^6 (n=14) MSCs restores MBP$^+$ coverage of the cortex up to sham-control levels (n=13), when compared to vehicle treatment (n=12). **B/C.** An intranasal dose of 0.5×10^6 and 1.0×10^6 MSCs completely restored myelin complexity assessed by fiber length (**B**) and the number of intersections (**C**), to sham-control level (SHAM n=13, VEH n=16, 0.5×10^6 MSCs n=12, 1.0×10^6 MSCs n=13 and 2.0×10^6 MSCs n=13). **D.** Intranasal MSC treatment does not restore hippocampal size (SHAM n=14, VEH n=14 ,0.5×10^6 MSCs n=18, 1.0×10^6 MSCs n=18 and 2.0×10^6 MSCs n=16). **E.** Motor performance measured with the cylinder rearing test improved after MSC treatment (SHAM n=14, VEH n=14, 0.5×10^6 MSCs n=12, 1.0×10^6 MSCs n=15 and 2.0×10^6 MSCs n=11). **F.** Intranasal treatment with 0.5×10^6 MSCs restores the percentage of correct alternations in the T-maze up to sham-control levels in dWMI mice (SHAM n=4, VEH n=4 and 0.5×10^6 MSCs n=8). # p<0.05; ##: p<0.01; ###: p<0.001; ####: p<0.0001 vehicle-treated dWMI animals vs sham-controls; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 MSC-treated dWMI animals vs vehicle-treated dWMI animals.

MSC treatment attenuates microglia and astrocyte activation following dWMI

In line with our previous findings, an increase in the number of Iba1$^+$ cells was observed in the corpus callosum of vehicle-treated animals compared to sham-control animals at P19 (*p=0.031*). Intranasal MSC treatment at 3 days after induction of injury successfully reduced the number of Iba1$^+$ cells to levels observed in sham-control animals (*p=0.045*) (figure 8A). More detailed analyses of microglial morphology revealed an amoeboid (activated) microglia morphology in vehicle-treated dWMI mice, while MSC treatment significantly reduced the activation state of Iba1$^+$ cells (figure 8B-D). In addition, we assessed astrocyte reactivity in the corpus callosum and hippocampus. In line with the microglia data, intranasal MSC treatment strongly decreased GFAP$^+$ area compared to vehicle-treatment in both brain regions (CC *p=0.026* and HC *p=0.001*) (figure 8E-F).

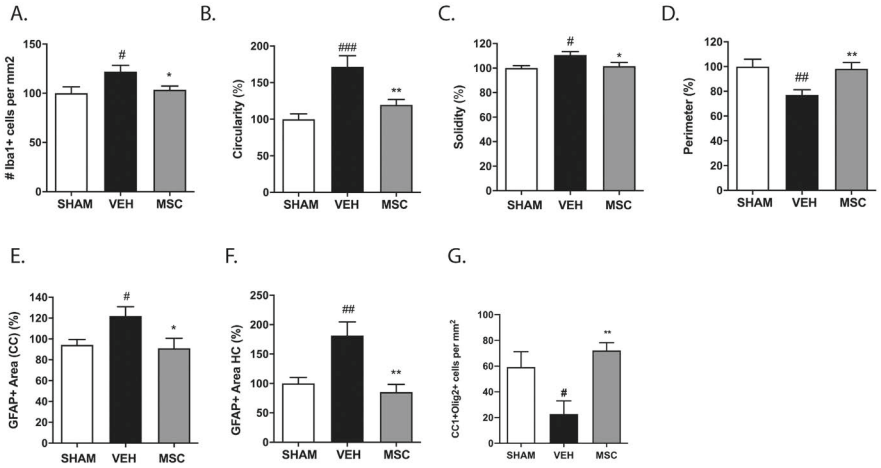


Figure 8. Intranasal MSC treatment dampens the neuro-inflammatory response and restores OL maturation following dWMI **A.** Quantification of microglia density in the corpus callosum revealed a reduction of Iba1⁺ cells after MSC treatment compared to vehicle treatment (SHAM n=9, VEH n=10, MSC n=14)**. B-D.** Microglia morphology analyses assessed by cell circularity (**B**), solidity (**C**) and perimeter (**D**), showed a less pro-inflammatory phenotype following MSC treatment compared to vehicle treatment (SHAM n=9, VEH n=11, MSC n=12, normalized to control values). **E-F.** A reduction in GFAP⁺ area in the corpus callosum (**E**) and hippocampus (**F**) was observed following intranasal MSC treatment compared to vehicle-treatment (SHAM n=8, VEH n=8, MSC n=8, normalized to control values). **G**. Intranasal administration of 0.5×10^6 MSCs restored CC1⁺/Olig2⁺ cells numbers up to sham-control levels in dWMI animals, indicating a boost in OL lineage maturation (SHAM n=6, VEH n=6, MSC n=13). # p<0.05; ##: p<0.01; ###: p<0.001 vehicle-treated dWMI animals vs sham-controls; *: p< 0.05; **: p<0.01 MSC-treated dWMI vs vehicle-treated dWMI animals.

Treatment with intranasal MSCs boosts OL maturation

To determine if the observed recovery of myelination after MSC treatment corresponds with an increase in mature OLs, double-stainings for CC1 and Olig2 were performed on brain sections of vehicle- and MSC-treated animals at P19. dWMI induced a reduction in cortical CC1⁺/Olig2⁺ (*p=0.032*), which was rescued by intranasal treatment with 0.5×10^6 MSCs at P8 (i.e. 3 days after injury induction) (*p=0.001)*, indicating recovery of mature OL numbers by MSCs after dWMI (figure 8G).

Intranasal MSC treatment for dWMI: starting earlier is better

To gain insight in the treatment window of intranasal MSC therapy, we delayed the timing of MSC administration from 3 days to 6 or 10 days after induction of dWMI. The lowest effective dose of MSCs (0.5×10^6 cells) was administered and complexity

of cortical myelination was assessed using segmentation analyses at P26. Figure 9A shows that the dWMI-induced reduction in fiber length (*p=0.0076 compared to sham)* was restored in dWMI animals that received intranasal MSC treatment at 3 days after injury induction (*p=0.039 vs veh*). Delay in MSC treatment to 6 or 10 days after induction of brain injury led to a strong reduction in treatment efficacy (*D6/10 p>0.999 vs veh*) (figure 9A).

Assessment of motor outcome supported these histological findings. The beneficial effect of D3 MSC treatment (*p=0.046*) was partially lost when treatment was postponed to D6 or D10 after dWMI (figure 9B). Collectively, these results indicate a relative limited time-window for intranasal MSC treatment in our double-hit mouse model of dWMI.

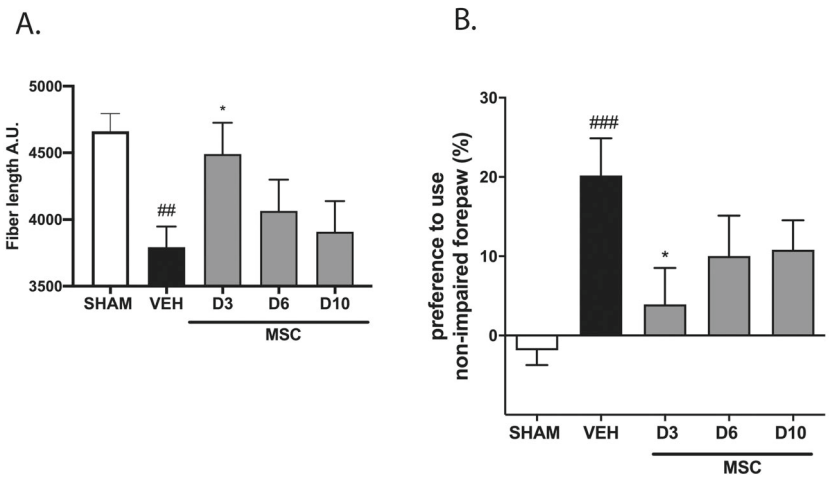


Figure 9. Delayed intranasal administration reduces the regenerative potential of MSCs. **A.** Fiber length, a microstructural myelin parameter, was restored in dWMI animals that received MSC treatment at 3 days after injury induction. Delay in MSC treatment to 6 or 10 days led to a reduction in treatment efficacy (SHAM n=13, VEH n=13, MSC-day 3 n=14, MSC-day 6 n=9, MSC-day 10 n=9). **B.** The beneficial effect of MSC treatment on motor performance, measured with the cylinder rearing test, was partially lost when MSC treatment was postponed to day 6 or day 10 after dWMI induction (SHAM n=18, VEH n=14, MSC-day 3 n=7, MSC-day 6 n=11, MSC-day 10 n=9). ##: p<0.01; ###: p<0.001 vehicle-treated dWMI animals vs sham-controls; *: p< 0.05; MSC-day 3 treated dWMI vs vehicle-treated dWMI animals.

MSCs boost OL maturation and attenuate microglia activation in non-contact cocultures

Previous studies have shown that the regenerative potential of MSC treatment is primarily mediated by paracrine signaling, without engraftment of stem cells (Cunningham et al., 2018; Vaes et al., 2019; van Velthoven et al., 2010a). To investigate whether paracrine signaling by MSCs can act on OLs directly, we subjected primary cultured pre-OLs to medium of LPS-stimulated microglia (MCM+LPS), in a non-contact coculture assay with MSCs, and measured MBP^+ area as maturational readout. Pre-OLs exposed to MCM+LPS demonstrated a strong reduction in MBP^+ area (*p=0.002),* indicating impaired maturation, compared to pre-OLs cultured in medium of non-stimulated microglia (MCM-LPS). Subsequent coculture with 2, 4, 8×10^4 embedded MSCs led to a significant increase in MBP^+ area in the OL cultures exposed to MCM+LPS ((borderline) *p=0.084, p=0.001* and *p<0.0001* respectively) (figure 10A-B).

MCM+LPS is a stringent stimulus to mimic dWMI in vitro, leading to both reduced OL maturation and reduced OL survival, and varies slightly per batch of LPS-stimulated microglia. To be in line with our *in vivo* findings of OL maturation arrest in *absence* of pronounced OL apoptosis, we repeated the experiments with a low dose of TNFα as stimulus. Exposure of pre-OLs to TNFα reduced MBP^+ area (*p<0.0001*), without affecting $Olig2^+$ cell numbers (*p=0.758*), implying a true OL maturational arrest (Supplemental data; S2). Pre-OLs cocultured in the presence of 4×10^4 embedded MSCs displayed a significant increase in MBP^+ area compared to cells cocultured without MSCs (p=0.0002) (figure 10C-D).

To study the paracrine effects of MSCs directly on microglia, we exposed primary cultured microglia to 50ng/ml LPS and cocultured with 4×10^4 MSCs. LPS stimulation strongly increased the production of TNFα by microglia in the supernatant compared to non-stimulated cells (*p<0.0001*). Coculture with MSCs led to a significant decrease in TNFα secretion, indicating attenuation of microglial activation (*p=0.0093*) (figure 10E). Taken together, these results indicate that MSCs can act on both oligodendrocytes and microglia directly through paracrine signaling.

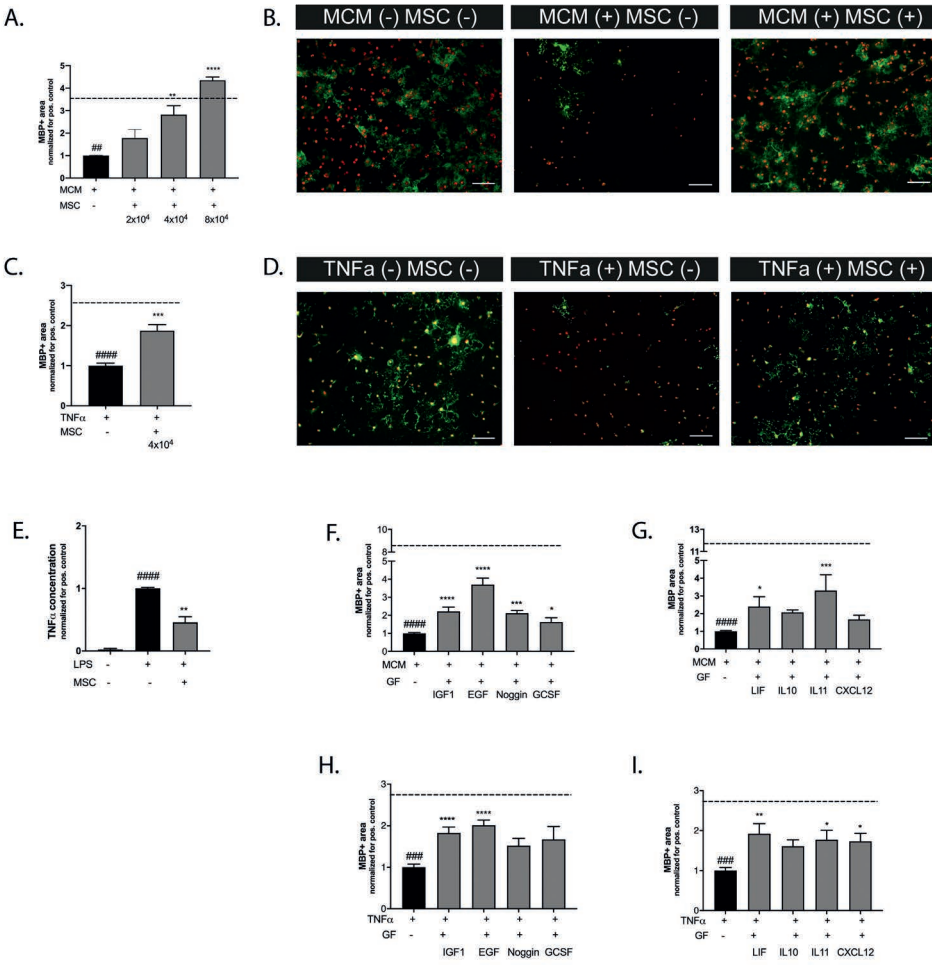


Figure 10. The MSCs secretome boosts OL maturation and attenuates microglia activation in vitro. **A.** MCM+LPS causes a reduction in MBP⁺ area (dashed line represents MBP⁺ area in MCM-LPS control condition), MSC treatment with 4×10⁴ or 8×10⁴ MSCs in a noncontact co-culture significantly improves OL maturation (n=3 independent experiments, 3-4 observations per experiment normalized for the positive control, e.g. cells exposed to MCM+LPS). **B.** Representative fluorescent images (10x) of primary cultured oligodendrocytes stained for oligodendrocyte marker Olig2 (red) and myelin component MBP (green). Cells were exposed to MCM-LPS (MCM-) or MCM+LPS (MCM+) and 4×10⁴ MSCs (MSC+) in a noncontact gel-insert. Scale bars: 100µm. **C.** Exposure to 10 ng/ml TNFα leads to a reduction in MBP production (dashed line represents MBP⁺ area in medium without TNFα), MSC treatment with 4×10⁴ MSCs in a noncontact co-culture significantly boosts OL maturation (n=2 independent experiments, 3-4 observations per experiment, normalized for the positive control, e.g. cells exposed to TNFα). **D.** Representative fluorescent images (10x) of primary cultured oligodendrocytes stained for oligodendrocyte marker Olig2 (red) and myelin component MBP (green). Cells were exposed to medium with (+) or without (-) 10ng/ml TNFα and 4×10⁴ MSCs (MSC+) in noncontact gel-in-

sert. Scale bars: 100µm. **E.** Treatment with 4×10^4 MSCs in a noncontact gel-insert attenuates microglial TNF-α production (n=2 independent experiments, 2 observations per experiment, normalized for the positive control, e.g. cells exposed to LPS). **F-G.** Addition of IGF1, EGF, Noggin, GCSF, LIF and IL11 but not IL10 and CXCL12 significantly improves MBP^+ area by primary cultured oligodendrocytes following MCM+LPS exposure (dashed line represents MBP^+ area in MCM-LPS control condition) (n=2 independent experiments, 3-4 observations per experiment normalized for the positive control, e.g. cells exposed to MCM+LPS). **H-I.** Addition of IGF1, EGF, LIF, IL11 and CXCL12 but not Noggin, G-CSF and IL10 boosts OL maturation after TNFα-induced OL maturational arrest (dashed line represents MBP^+ area in medium without TNFα) (n=2 independent experiments, 3-4 observations per experiment, normalized for the positive control, e.g. cells exposed to TNFα). ##: p<0.01; ###: p<0.001; ####: p<0.0001 MCM+ or TNF+ condition (black bars) vs MCM- or TNFα- control (dashed line) respectively; *: p< 0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 factor-exposed MCM+ or TNF+ condition (gray bars) vs MCM+ or TNF+ control condition (black bars)

Changes in MSC gene expression profile in dWMI environment

To identify candidate factors in the secretome of MSCs that could underlie the regenerative capacity after encountering the dWMI brain milieu, we cultured MSCs in presence of brain protein extracts from either dWMI or sham-control animals obtained at 3 days after injury (i.e. the timepoint of MSC treatment in vivo). Gene expression changes in MSCs were examined using PCR arrays focused on growth factors and cyto/chemokines. Using a cut-off of 2-fold change, 56 differentially regulated genes were identified (Supplemental data; S3). To investigate the potency of individual MSC-secreted factors to boost OL maturation and subsequent myelination, we selected candidate factors from the PCR arrays that have been associated with white matter development or repair in literature (i.e. IGF1, EGF, GCSF(CSF3), IL10, IL11, LIF and CXCL12). IGF1 and CXCL12 expression by MSCs seemed negatively impacted by the dWMI milieu, however, both these factors have been shown to play a role in OL lineage development (Masters et al., 1991; Maysami et al., 2006; Patel et al., 2010; Wilson et al., 2003). Moreover, Noggin, an inhibitor of factors strongly associated with OL maturation arrest (i.e. the BMP family), was added to the panel of factors to mimic BMP4 downregulation by MSCs. PCR array results for these 7 factors were confirmed by real-time RT-PCR on the individual samples, with a downregulation of CXCL12 and IGF1 and an upregulation of LIF, IL11 and GCSF (CSF3). IL10 and EGF were both upregulated in MSCs exposed to dWMI extracts, but did not reach the two-fold cut-off (Supplemental data; S3). Interestingly, EGF expression seemed to be exclusively induced by the dWMI milieu, as MSCs cultured with sham-control brain extracts did not express any EGF.

MSC treatment regulates expression of trophic factors after dWMI

To investigate whether MSC treatment changes the cerebral gene expression of the selected trophic factors (i.e. IGF1, EGF, GCSF(CSF3), IL10, IL11, LIF and CXCL12) in dWMI animals *in vivo*, we compared mRNA expression levels of the factors between vehicle- and MSC-treated dWMI animals at 12 hours after intranasal treatment. MSC-treated dWMI animals displayed an upregulation of EGF, GCSF and LIF mRNA in the ipsilateral hemisphere compared to vehicle-treated dWMI animals, though mean expression levels varied between tissue parts (table 3). Expression of IL11 showed a modest upregulation following MSC administration, however restricted to the caudal part of the ipsilateral hemisphere. IGF1 and CXCL12 expression was (slightly) downregulated in dWMI animals that received MSC treatment (table 3). IL10 expression was not detected in any of the tissue parts.

Table 3. Trophic gene expression changes in dWMI animals after MSC treatment versus vehicle treatment

Gene symbol	**Fold regulation 2^-(ΔΔCt)** dWMI-MSC vs. dWMI-VEH
IGF1	
Rostral	1.16
Caudal	0.99
Cerebellum	0.85
EGF	
Rostral	4.40
Caudal	0.56
Cerebellum	0.69
IL11	
Rostral	0.70
Caudal	1.63
Cerebellum	0.67
LIF	
Rostral	1.96
Caudal	2.05
Cerebellum	1.34
GCSF	
Rostral	3.69
Caudal	2.48
Cerebellum	1.32
CXCL12	
Rostral	1.00
Caudal	0.95
Cerebellum	0.66

MSC-secreted factors boost myelin production in vitro

The selected factors were tested for their potential to boost maturation in primary OL cultures challenged with inflammatory stimuli. MCM+LPS as a stimulus led to a strong decrease in MBP⁺ area compared to pre-OLS cultured under non-inflammatory (MCM-LPS) conditions (*p<0.0001)* as observed earlier (figure 10F-G). Optimal concentrations of all selected factors on OL maturation were determined in dose-response experiments (Supplemental data; S2). Addition of IGF1, EGF, Noggin, GCSF, LIF or IL11 significantly increased maturation in the OL cultures determined by MBP⁺ area (*p<0.0001, p<0.0001, p= 0.0005, p=0.0176, p=0.048, p=0.0002* respectively) (figure 10F-G). Addition of CXCL12 and IL10 failed to significantly boost OL differentiation in this assay (*p=0.934* and *p=0.629* respectively). Moreover, to mimic OL maturation arrest without significant loss of cells, pre-OLs were exposed to TNFα. Addition of IGF1, EGF, LIF, IL11 and CXCL12 significantly increased MBP⁺ area in OLs challenged with 10ng/ml TNFα, indicating that these factors help OLs to overcome their maturational arrest (*p<0.0001, p<0.0001, p=0.002, p=0.011, p=0.023* respectively) (figure 10H-I). Moreover, GCSF therapy was associated with a borderline significant increase in MBP⁺ area (*p=0.056).* Addition of IL10 and Noggin did not significantly rescue maturation of pre-OLs after TNFa exposure (figure 10H-I).

DISCUSSION

Here we investigated a novel double-hit model of dWMI in mouse pups using behavioral paradigms, postmortem MRI, electron microscopy and immunostainings. We show that combined postnatal inflammation and hypoxia/ischemia in P5 mice induces transient myelination deficits, neuroinflammation, a maturational arrest in OLs, behavioral impairments and global volumetric deficits of white and gray matter structures. Moreover, we explored the regenerative potential of intranasal MSC treatment after dWMI. Intranasally administered MSCs were dispersedly distributed throughout the brain in dWMI animals compared to sham-controls. We report that intranasal administration of MSCs restores OL maturation and myelination, dampens the neuroinflammatory response and improves functional outcome. Furthermore, we demonstrate that MSCs modify their secretome dependent on the cerebral environment. Exposure to dWMI milieu leads to an increased expression of beneficial growth factors and/or anti-inflammatory cytokines, valuable in boosting OL differentiation. Intranasal treatment with MSCs modulates availability of beneficial factors *in vivo*, promoting a cerebral milieu more permissive for repair. Moreover, we show *in vitro* that MSCs

can act on both oligodendrocytes and microglia directly, boosting OL maturation under pro-inflammatory conditions, and attenuating microglia activation. Additionally, MSC-produced factors, identified in our PCR array and associated with white matter repair in literature, were able to rescue maturation of OLs individually *in vitro*. Collectively, these results imply that intranasal MSC therapy is a potent treatment strategy to restore myelination in dWMI, and that the neuroregenerative properties of MSCs are likely mediated by their secretome.

Clinically relevant animal models of EoP are essential for translation of potential novel treatment options and further elucidation of underlying pathophysiological mechanisms. Key translational aspects for relevant animal models for EoP are (multiple) etiological factors relevant for the target patient, induction at a developmental brain stage comparable to the (extreme) preterm infant and a diffuse pattern of WMI that resembles EoP in the human patient. Our mouse model incorporates two postnatal insults, both unequivocally linked to EoP pathophysiology. Both hypoxia/ischemia and inflammation have been linked to OL maturation arrest and insufficient myelination in EoP, and are believed to work synergistically (Rezaie & Dean, 2002; Zhao et al., 2013). Inflammation in the preterm brain is believed to be triggered by maternal inflammation/infection and postnatal infections, such as neonatal sepsis (Back & Miller, 2014; Bennet et al., 2018). Moreover, disturbances in cerebral oxygenation (either hypoxia or hyperoxia) as a result of an underdeveloped respiratory system and mechanical ventilation, are often encountered by the human preterm infant (Brown & DiBlasi, 2011; Stoll et al., 2015). Unlike a number of previously proposed single-hit models (reviewed in (van Tilborg et al., 2016)), our model incorporated two postnatal hits, reflecting more closely the multi-factorial etiology of EoP (Deng, 2010; Khwaja & Volpe, 2008). In our model, mice were subjected to two hits at P5, a developmental time-window roughly corresponding to white matter development at 24-28 weeks of human gestation, with a peak in immature non-myelinating OLs (Salmaso et al., 2014; Semple et al., 2013). We did not observe myelination deficits following a single hit of hypoxia-ischemia or systemic inflammation. Although the method of hypoxia/ischemia is also used in older postnatal models to induce acute gray matter loss often accompanied by cystic lesions spreading into the white matter tracts, we did not observe (cortical) neuronal loss. Instead, our double-hit model at P5 induced brain injury closely resembling human post-mortem and imaging data in preterm dWMI, including a reduction in cortical myelination and myelin complexity in absence of (macroscopic) cystic lesions (Back, 2017; Back & Miller, 2014; Volpe, 2017). These

differences in brain injury patterns can mainly be explained by the developmental stage of the rodent brain at P5 versus e.g. P9 and the depth and duration of the systemic hypoxia (Semple et al., 2013).

As proposed in human studies (Billiards et al., 2008; Buser et al., 2012; Verney et al., 2012), hampered OL maturation is likely to underlie the observed insufficient myelination, indicated by the early and persistent rise in immature OLs, with reduction of mature OL numbers and without evident OL cell death. In line with these human studies, in our mouse model we observed an early proliferative response of OPCs in the cortex at P8. Subsequently, at P19, we detected a proliferative response in the corpus callosum, but not the cortex. The increase in proliferation was accompanied by a deficit in mature OLs in the cortex, while the number of mature OLs in the corpus callosum seemed unaffected. Importantly, at P8 we did not find any evidence of extensive OL loss, measured by the number of cleaved caspase3^{+} OLs. Overt OL cell death prior to P8 also seems unlikely, as dWMI animals did not show Olig2^{+} cell loss in the corpus callosum, and even an increase in cortical Olig2^{+} cells, possibly reflecting the early surge in proliferation. These results are also in line with previous observations in our rat double-hit model of fetal inflammation and postnatal hypoxia (van Tilborg et al., 2018a). The spatial differences in immature and mature OLs in the cortex and corpus callosum could be the result of temporal differences in OL migration and myelination. Previous studies have shown that myelination of the corpus callosum precedes that of the somatosensory cortex in rodents (Downes & Mullins, 2013; Vincze et al., 2008). Therefore, it is possible that injury induction at P5 mainly targets a population of vulnerable immature cortical OLs, leading to increased proliferation at P8, followed by a deficit in cortical mature OLs and myelination at P19. The observed proliferative response in the corpus callosum at P19 could indicate an endogenous (regenerative) response aimed at restoring cortical myelination deficits of local quiescent OPCs in the corpus callosum, or cells derived from other progenitor pools, such as the subventricular zone (Bonfanti et al., 2017; Hughes et al., 2013; van Tilborg et al., 2018b; Viganò et al., 2016; Xing et al., 2014). This suggestion is supported by observations in a multiple sclerosis model, with temporal differences in OPC repopulation and maturation between the cortex and corpus callosum (Baxi et al., 2017). Interestingly, at P26 we were unable to detect any differences in mature (or immature) OLs between dWMI animals and sham-controls, followed by endogenous restoration of cortical myelination at P33. White matter deficits were confirmed using post-mortem MRI, with volumetric changes in important white matter

structures and enlargement of the lateral ventricles, similar to imaging observations in human extreme preterm infants (Keunen et al., 2016; Lind et al., 2011). In line with the hypothesis of EoP as a global dysmaturation disorder, affecting all developing cell types, including interneurons, and structures in the immature brain, we also observed a reduction in gray matter volumes on MRI and histological hippocampal size, without overt neuronal loss (Keunen et al., 2016; Volpe, 2009a). In depth investigation of potential interneuron disturbances in our model should be considered in the future. Moreover, in line with human post-mortem studies, we observed a potent neuro-inflammatory response after induction of dWMI (Billiards et al., 2008; Buser et al., 2012; Haynes et al., 2003; Kadhim et al., 2001; Verney et al., 2012) illustrated by increased microglia numbers (likely the consequence of increased proliferation or migration (Umekawa et al., 2015)), with an activated, pro-inflammatory morphology (Davis et al., 1994). Microglia activation after dWMI was accompanied by an increase in astrocyte reactivity, characterized by hypertrophy of the soma (Khakh & Sofroniew, 2015; Schmidt-Kastner et al., 1993). On a behavioral level, dWMI animals displayed impaired motor performance and cognitive impairments. Several clinical studies have found an association between preterm birth and (mild) cognitive disability (Johnson et al., 2009; Linsell et al., 2018; Nosarti et al., 2007). Aside from motor or cognitive impairments, clinical follow-up studies often report a higher prevalence in psychiatric disorders, including anxiety disorders and autism spectrum disorder (ASD), in preterm-born children and adolescents (Johnson & Marlow, 2011). In our mouse model we did not find an indication for anxiety-like behavior in the open field test, in contrast to (mild) indications of anxiety previously described in our rat double-hit model (van Tilborg et al., 2018a). In addition, van Tilborg et al. (2018a) identified ASD-like behavioral deficits following dWMI induction. To study whether these deficits are also applicable to our mouse model of postnatal inflammation and hypoxia/ischemia more behavioral tasks should be performed in the future. Taken together, the double-hit model of postnatal inflammation and hypoxia/ischemia leads to a clinically relevant pattern of developmental brain injury in neonatal mice, with regard to anatomical, microstructural and functional outcome.

In our model, the majority of observed deficits were most pronounced up to 3 weeks following injury induction, with evidence of (endogenous) recovery of myelination, neuroinflammation and motor behavior at 4 weeks (i.e. P33) after dWMI. EM analyses confirmed that at 4 weeks after dWMI myelination differences were absent, illustrated by a comparable thickness of the myelin sheath between dWMI and sham animals

at P33. This could imply that our double-hit model induces a developmental delay in the immature rodent brain rather than irreversible long-lasting changes. Similarly, endogenous recovery of histological myelin deficits has been reported in other rodent models of dWMI (van Tilborg et al., 2018a; van Tilborg et al., 2016) . Such findings are in contrast to data from clinical imaging studies of preterm infants, in whom changes on structural and diffusion-weighted MRI persist into adulthood (Allin et al., 2011; Eikenes et al., 2011; Ment et al., 2009; Nagy et al., 2009; Nosarti et al., 2002; Nosarti et al., 2008). This discrepancy could be the result of a relatively higher regenerative capacity of the rodent brain compared to humans, limiting the possibility for long-term follow-up in rodent models of dWMI (Kaplan et al., 2015; van Tilborg et al., 2016). Interestingly, despite apparent histological recovery of myelination, some follow-up studies do report long-lasting alterations in myelin microstructure measured with DTI or behavioral functioning (Chahboune et al., 2009; Favrais et al., 2011; Scafidi et al., 2014; Schmitz et al., 2011; van Tilborg et al., 2018a). When evaluating our model, some considerations should be taken into account. First, we cannot exclude spatial differences in the dWMI brains, in other words the possibility that myelination in other brain regions does not completely restore up to sham-control levels. Additionally, a delay in white matter development could influence maturation of brain connectivity and other linked brain regions/cell types, including interneurons, still leading to long-lasting functional impairments (Benamer et al., 2020; Volpe, 2009a; Zonouzi et al., 2015). As example we did not observe an endogenous recovery of loss of hippocampal area in our model.

Previously, we showed that intranasal MSC treatment was able to improve functional outcome and reduce gray- and white matter deficits in a rodent model of term hypoxia-ischemia, by promoting endogenous repair mechanisms and dampening cerebral inflammation (Donega et al., 2014a; Donega et al., 2013). Additionally, we showed that MSC treatment is safe, as MSCs do not engraft (Donega et al., 2015; van Velthoven et al., 2011). Moreover, a profound body of evidence supports the regenerative potential of MSC therapy in a multitude of white matter pathologies, and was recently summarized by our group (Vaes et al., 2019). In this study we showed via gold nanoparticle cell labelling and ICP-MS that intranasally applied MSCs dispersedly migrate throughout the brain after dWMI induction, with minimal loss of gold signal (i.e. labeled MSCs) to peripheral organs, in clear contrast to what was observed in sham-control animals. Though these data imply that MSCs favor migration toward the injured brain after intranasal administration, the potential beneficial role of MSCs in

the periphery remains unclear in this model (Jellema et al., 2013). The widespread cerebral distribution of MSCs might be the result of systemic inflammation and hypoxia leading to diffuse patterns of myelin deficits, though additional studies are needed to determine the timing of migration and final location of MSCs after intranasal treatment in our model. In the field of EoP, several studies have reported beneficial effects of MSC therapy on OL lineage maturation and survival, subsequent myelination and modulation of the inflammatory response in the CNS and periphery (Vaes et al., 2019). In line with these studies, we report here increased numbers of mature OLs, recovery of cortical myelination and myelin microstructure, dampening of neuroinflammation and improved functional outcome after intranasal MSC administration in our dWMI model. Even though large differences in pathophysiological hallmarks (i.e. prominent cell death of pre-OLs in other models) and methodology (i.e. MSC source and/or route/timing of administration) exist between other studies and ours, these concurrent findings strengthen the promising role of MSCs to restore dWMI in the preterm brain. In contrast to the beneficial effects of MSC treatment on myelination and neuroinflammation, MSCs failed to restore hippocampal area loss after dWMI. We suggest that the loss of hippocampal area in this model possibly is the result of interference with normal development and construction of certain gray matter tracts, for example due to changes in synaptic input, leading to long-lasting hippocampal volume loss. Interestingly, MSC treatment did improve cognitive functioning in the spontaneous alternation T-maze. Though this spatial memory task does (in part) rely on proper hippocampal functioning, other brain regions, such as the prefrontal cortex, thalamus and cerebellum are also required for optimal spatial memory performance (Lalonde, 2002). Thus, the observed improvement in T-maze performance after MSC therapy is likely the result of restoration of myelination in other brain areas involved in executive functioning or improved functioning of the remaining hippocampal tissue.

One of the challenges for future clinical application of MSCs is determination of the optimal treatment protocol. Here, we propose a lowest effective dose of 0.5×10^6 cells BM-MSCs applied intranasally based on histological and functional outcome parameters. A similar dosage has shown to be effective in a P2-3 rat model of EoP using intranasal delivery of cells (Oppliger et al., 2016). Intracranial administration in a P3-4 mouse model of EoP, an invasive but direct route with minimal loss of cells during migration, warranted a lower effective dose of 0.25×10^6 cells (Mueller et al., 2017).

Apart from the dosage, the optimal window of MSC treatment in EoP is still unclear. Here, we observed a relative limited treatment window, measured by myelination parameters and functional outcome when intranasal treatment was postponed until D6 or D10 after dWMI compared to treatment at D3. A similar reduction in MSC treatment efficacy over time has been observed in other preclinical studies in the field of adult ischemic stroke, severe intraventricular hemorrhage and neonatal stroke (Kim et al., 2012; Park et al., 2016; Wang et al., 2014). In contrast, earlier work from our group showed a treatment window of at least 10 days in a mouse model of term hypoxic-ischemic brain injury (van Velthoven et al. (2010a).The reduction in MSC treatment efficacy could be due to reduced homing of cells following intranasal administration when damage patterns are less severe or more diffuse in nature, or could be the result of a limited regenerative capacity of MSCs in later stages of EoP pathophysiology. In the latter option, optimization of MSC therapy, using preconditioning or genetic modification strategies, could be explored to prolong the treatment window. A limited treatment window of MSCs would underline the need for early identification (i.e. biomarkers) of preterm infants at risk for EoP to ensure timely treatment.

MSCs are believed to exert their regenerative properties by adaptation of their secretome, stimulating endogenous repair mechanisms through paracrine signaling (Kassis et al., 2011; Liang et al., 2014; Paton et al., 2017; van Velthoven et al., 2010a). However, the exact mechanisms that underlie the regenerative effect of MSCs on white matter damage *in vivo* remain unclear. On the one hand, the MSCs' secretome, containing growth factors and cytokines, as well as microvesicles and exosomes, could act directly on OL precursors, thereby supporting OL maturation and subsequent myelination. However, the effect of MSC-secreted factors could also be mediated by attenuation of neuroinflammation (i.e. microglia activation), leading indirectly to a more favorable milieu for OL maturation. Here, we demonstrate *in vitro* that the MSCs' secretome can directly promote maturation of OPCs under pro-inflammatory conditions, as well as attenuate microglia activation. Moreover, MSC treatment induced mRNA expression changes of the selected beneficial factors in brains of dWMI mice, when compared to vehicle-treated dWMI brains. Though these in vivo changes closely resembled the observed patterns of up- and downregulation in the secretome when MSCs were exposed to the dWMI milieu ex vivo, the current data do not indisputably prove which cells are responsible for the expression changes. Aside from a direct effect of the MSCs' secretome in situ, the observed effects on beneficial factor expression levels could also reflect an indirect effect of MSCs on

factor production by for example microglia and/or astrocytes, indirectly contributing to a more repair-promoting milieu in the brain. This theory likely applies to EGF, as expression of this trophic factor was upregulated in MSC-treated dWMI brains in vivo, whereas we did not observe any expression changes in the dWMI-exposed MSCs ex vivo. Taken together, these results indicate that the observed potent regenerative effects of MSCs *in vivo* on OL maturation and dampening of neuroinflammation are likely mediated through direct paracrine signaling on *multiple* glial cell types.

To get more insight in which individual MSC-secreted factors are potent to boost OL maturation *in vitro*, we selected promising growth factors and cytokines that are differentially expressed in MSCs encountering dWMI milieu ex vivo. Addition of IGF1, EGF, GCSF, LIF and IL11 restored MBP^+ area of primary OLs up to control levels following inflammation *in vitro*. Interestingly, Noggin, a BMP4 inhibitor, failed to significantly restore OL differentiation in the TNFα maturation arrest assay, suggesting that Noggin predominately plays a role in OL survival and to a lesser extent in OL maturation. The contrary could be proposed for CXCL12. These findings imply a potentially promising future role for trophic factor (mono)therapy or combination therapy with MSCs, in the treatment of EoP. However, additional *in vivo* studies, including back-to-back comparison of efficacy of MSCs, that can deliver a more constant mixture of trophic factors, are needed to substantiate these statements.

In sum this study shows that intranasal MSC treatment is a potent new strategy to support myelination and functional outcome in a mouse model of EoP. Our results indicate that MSCs potently modify their secretome *in situ* to support OL maturation by upregulating a mixture of beneficial factors valuable in underpinning proper OL maturation and subsequent myelination of the developing brain at risk.

Acknowledgements

This work was funded by Brain Foundation the Netherlands. We would like to thank Rik van Vliet, Michiel van der Lelie and Angela Vandersteen for their technical assistance.

REFERENCES

Allin, M. P., Kontis, D., Walshe, M., Wyatt, J., Barker, G. J., Kanaan, R. A., . . . Nosarti, C. (2011). White matter and cognition in adults who were born preterm. *PLoS One, 6*(10), e24525. doi:10.1371/journal.pone.0024525

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathologica, 134*(3), 331-349. doi:10.1007/s00401-017-1718-6

Back, S. A., & Miller, S. P. (2014). Brain injury in premature neonates: A primary cerebral dysmaturation disorder? *Annals of Neurology, 75*(4), 469-486. doi:10.1002/ana.24132

Baxi, E. G., DeBruin, J., Jin, J., Strasburger, H. J., Smith, M. D., Orthmann-Murphy, J. L., . . . Calabresi, P. A. (2017). Lineage tracing reveals dynamic changes in oligodendrocyte precursor cells following cuprizone-induced demyelination. *Glia, 65*(12), 2087-2098. doi:10.1002/glia.23229

Behrens, T. E., Berg, H. J., Jbabdi, S., Rushworth, M. F., & Woolrich, M. W. (2007). Probabilistic diffusion tractography with multiple fibre orientations: What can we gain? *NeuroImage, 34*(1), 144-155. doi:10.1016/j.neuroimage.2006.09.018

Behrens, T. E., Woolrich, M. W., Jenkinson, M., Johansen-Berg, H., Nunes, R. G., Clare, S., . . . Smith, S. M. (2003). Characterization and propagation of uncertainty in diffusion-weighted MR imaging. *Magnetic Resonance in Medicine, 50*(5), 1077-1088. doi:10.1002/mrm.10609

Benamer, N., Vidal, M., & Angulo, M. C. (2020). The cerebral cortex is a substrate of multiple interactions between GABAergic interneurons and oligodendrocyte lineage cells. *Neurosci Lett, 715*, 134615. doi:10.1016/j.neulet.2019.134615

Bennet, L., Dhillon, S., Lear, C. A., van den Heuij, L., King, V., Dean, J. M., . . . Gunn, A. J. (2018). Chronic inflammation and impaired development of the preterm brain. *Journal of Reproductive Immunology, 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., . . . Kinney, H. C. (2008). Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia. *Brain Pathology, 18*(2), 153-163. doi:10.1111/j.1750-3639.2007.00107.x

Bonfanti, E., Gelosa, P., Fumagalli, M., Dimou, L., Viganò, F., Tremoli, E., . . . Abbracchio, M. P. (2017). The role of oligodendrocyte precursor cells expressing the GPR17 receptor in brain remodeling after stroke. *Cell Death Dis, 8*(6), e2871-e2871. doi:10.1038/cddis.2017.256

Bonora, M., De Marchi, E., Patergnani, S., Suski, J. M., Celsi, F., Bononi, A., . . . Pinton, P. (2014). Tumor necrosis factor-alpha impairs oligodendroglial differentiation through a mitochondria-dependent process. *Cell Death & Differentiation, 21*(8), 1198-1208. doi:10.1038/cdd.2014.35

Brown, M. K., & DiBlasi, R. M. (2011). Mechanical Ventilation of the Premature Neonate. *Respiratory Care, 56*(9), 1298-1313. doi:10.4187/respcare.01429

Buser, J. R., Maire, J., Riddle, A., Gong, X., Nguyen, T., Nelson, K., . . . Back, S. A. (2012). Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. *Annals of Neurology, 71*(1), 93-109. doi:10.1002/ana.22627

Chahboune, H., Ment, L. R., Stewart, W. B., Rothman, D. L., Vaccarino, F. M., Hyder, F., & Schwartz, M. L. (2009). Hypoxic injury during neonatal development in murine brain: correlation between in vivo DTI findings and behavioral assessment. *Cerebral Cortex, 19*(12), 2891-2901. doi:10.1093/cercor/bhp068

Chen, Y., Balasubramaniyan, V., Peng, J., Hurlock, E. C., Tallquist, M., Li, J., & Lu, Q. R. (2007). Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc, 2*(5), 1044-1051. doi:10.1038/nprot.2007.149

Cunningham, C. J., Redondo-Castro, E., & Allan, S. M. (2018). The therapeutic potential of the mesenchymal stem cell secretome in ischaemic stroke. *Journal of Cerebral Blood Flow and Metabolism, 38*(8), 1276-1292. doi:10.1177/0271678×18776802

Danielyan, L., Schafer, R., von Ameln-Mayerhofer, A., Buadze, M., Geisler, J., Klopfer, T., . . . Frey, W. H., 2nd. (2009). Intranasal delivery of cells to the brain. *European Journal of Cell Biology, 88*(6), 315-324. doi:10.1016/j.ejcb.2009.02.001

Davis, E. J., Foster, T. D., & Thomas, W. E. (1994). Cellular forms and functions of brain microglia. *Brain Research Bulletin, 34*(1), 73-78. doi:10.1016/0361-9230(94)90189-9

Deng, W. (2010). Neurobiology of injury to the developing brain. *Nature Reviews Neurology, 6*(1759-4766 (Electronic)), 328-336. doi:10.1038/nrneurol.2010.53

Donega, V., Nijboer, C. H., Braccioli, L., Slaper-Cortenbach, I., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2014a). Intranasal administration of human MSC for ischemic brain injury in the mouse: in vitro and in vivo neuroregenerative functions. *PLoS One, 9*(11), e112339-e112339. doi:10.1371/journal.pone.0112339

Donega, V., Nijboer, C. H., van Tilborg, G., Dijkhuizen, R. M., Kavelaars, A., & Heijnen, C. J. (2014b). Intranasally administered mesenchymal stem cells promote a regenerative niche for repair of neonatal ischemic brain injury. *Experimental Neurology, 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., . . . Heijnen, C. J. (2015). Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse. *Pediatric Research, 78*(5), 520-526. doi:10.1038/pr.2015.145

Donega, V., van Velthoven, C. T., Nijboer, C. H., van Bel, F., Kas, M. J., Kavelaars, A., & Heijnen, C. J. (2013). Intranasal mesenchymal stem cell treatment for neonatal brain damage: long-term cognitive and sensorimotor improvement. *PLoS One, 8*(1), e51253-e51253. doi:10.1371/journal.pone.0051253

Downes, N., & Mullins, P. (2013). The Development of Myelin in the Brain of the Juvenile Rat. *Toxicologic Pathology, 42*(5), 913-922. doi:10.1177/0192623313503518

Eikenes, L., Løhaugen, G. C., Brubakk, A. M., Skranes, J., & Håberg, A. K. (2011). Young adults born preterm with very low birth weight demonstrate widespread white matter alterations on brain DTI. *NeuroImage, 54*(3), 1774-1785. doi:10.1016/j.neuroimage.2010.10.037

Favrais, G., van de Looij, Y., Fleiss, B., Ramanantsoa, N., Bonnin, P., Stoltenburg-Didinger, G., . . . Gressens, P. (2011). Systemic inflammation disrupts the developmental program of white matter. *Annals of Neurology, 70*(4), 550-565. doi:10.1002/ana.22489

Haynes, R. L., Folkerth, R. D., Keefe, R. J., Sung, I., Swzeda, L. I., Rosenberg, P. A., . . . Kinney, H. C. (2003). Nitrosative and Oxidative Injury to Premyelinating Oligodendrocytes in Periventricular Leukomalacia. *Journal of Neuropathology & Experimental Neurology, 62*(5), 441-450. doi:10.1093/jnen/62.5.441

Hughes, E. G., Kang, S. H., Fukaya, M., & Bergles, D. E. (2013). Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain. *Nature Neuroscience, 16*(6), 668-676. doi:10.1038/nn.3390

Jbabdi, S., Sotiropoulos, S. N., Savio, A. M., Grana, M., & Behrens, T. E. (2012). Model-based analysis of multishell diffusion MR data for tractography: how to get over fitting problems. *Magnetic Resonance in Medicine, 68*(6), 1846-1855. doi:10.1002/mrm.24204

Jellema, R. K., Wolfs, T. G., Lima Passos, V., Zwanenburg, A., Ophelders, D. R., Kuypers, E., . . . Kramer, B. W. (2013). Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia. *PLoS One, 8*(8), e73031. doi:10.1371/journal.pone.0073031

Jenkinson, M., Bannister, P., Brady, M., & Smith, S. (2002). Improved optimization for the robust and accurate linear registration and motion correction of brain images. *NeuroImage, 17*(2), 825-841. doi:10.1016/s1053-8119(02)91132-8

Jenkinson, M., & Smith, S. (2001). A global optimisation method for robust affine registration of brain images. *Medical Image Analysis, 5*(2), 143-156. doi:10.1016/s1361-8415(01)00036-6

Johnson, S., Fawke, J., Hennessy, E., Rowell, V., Thomas, S., Wolke, D., & Marlow, N. (2009). Neurodevelopmental disability through 11 years of age in children born before 26 weeks of gestation. *Pediatrics, 124*(2), e249-257. doi:10.1542/peds.2008-3743

Johnson, S., & Marlow, N. (2011). Preterm Birth and Childhood Psychiatric Disorders. *Pediatric Research, 69*(8), 11-18. doi:10.1203/PDR.0b013e318212faa0

Kadhim, H., Tabarki, B., Verellen, G., De Prez, C., Rona, A. M., & Sébire, G. (2001). Inflammatory cytokines in the pathogenesis of periventricular leukomalacia. *Neurology, 56*(10), 1278-1284. doi:10.1212/wnl.56.10.1278

Kaplan, H. M., Mishra, P., & Kohn, J. (2015). The overwhelming use of rat models in nerve regeneration research may compromise designs of nerve guidance conduits for humans. *Journal of Materials Science: Materials in Medicine, 26*(8), 226. doi:10.1007/s10856-015-5558-4

Kassis, I., Vaknin-Dembinsky, A., & Karussis, D. (2011). Bone marrow mesenchymal stem cells: agents of immunomodulation and neuroprotection. *Curr Stem Cell Res Ther, 6*(1), 63-68. doi:10.2174/157488811794480762

Keunen, K., Išgum, I., van Kooij, B. J. M., Anbeek, P., van Haastert, I. C., Koopman-Esseboom, C., . . . Benders, M. J. N. L. (2016). Brain Volumes at Term-Equivalent Age in Preterm Infants: Imaging Biomarkers for Neurodevelopmental Outcome through Early School Age. *J Pediatr, 172*, 88-95. doi:10.1016/j.jpeds.2015.12.023

Khakh, B. S., & Sofroniew, M. V. (2015). Diversity of astrocyte functions and phenotypes in neural circuits. *Nature Neuroscience, 18*(7), 942-952. doi:10.1038/nn.4043

Khwaja, O., & Volpe, J. J. (2008). Pathogenesis of cerebral white matter injury of prematurity. *Archives of Disease in Childhood: Fetal & Neonatal Edition, 93*(2), F153-161. doi:10.1136/adc.2006.108837

Kim, E. S., Ahn, S. Y., Im, G. H., Sung, D. K., Park, Y. R., Choi, S. H., . . . Park, W. S. (2012). Human umbilical cord blood–derived mesenchymal stem cell transplantation attenuates severe brain injury by permanent middle cerebral artery occlusion in newborn rats. *Pediatric Research, 72*, 277. doi:10.1038/pr.2012.71

Lalonde, R. (2002). The neurobiological basis of spontaneous alternation. *Neuroscience and Biobehavioral Reviews, 26*(1), 91-104. doi:10.1016/s0149-7634(01)00041-0

Larroque, B., Ancel, P. Y., Marret, S., Marchand, L., Andre, M., Arnaud, C., . . . Kaminski, M. (2008). Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study. *The Lancet, 371*(9615), 813-820. doi:10.1016/s0140-6736(08)60380-3

Lee, Y. A. (2017). White Matter Injury of Prematurity: Its Mechanisms and Clinical Features. *J Pathol Transl Med, 51*(5), 449-455. doi:10.4132/jptm.2017.07.25

Liang, X., Ding, Y., Zhang, Y., Tse, H. F., & Lian, Q. (2014). Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant, 23*(9), 1045-1059. doi:10.3727/096368913×667709

Lind, A., Parkkola, R., Lehtonen, L., Munck, P., Maunu, J., Lapinleimu, H., . . . Group, P. S. (2011). Associations between regional brain volumes at term-equivalent age and development at 2 years of age in preterm children. *Pediatric Radiology, 41*(8), 953-961. doi:10.1007/s00247-011-2071-x

Linsell, L., Johnson, S., Wolke, D., O'Reilly, H., Morris, J. K., Kurinczuk, J. J., & Marlow, N. (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*(4), 363-370. doi:10.1136/archdischild-2017-313414

MacKay, D. F., Smith, G. C., Dobbie, R., & Pell, J. P. (2010). Gestational age at delivery and special educational need: retrospective cohort study of 407,503 schoolchildren. *PLoS Med, 7*(6), e1000289. doi:10.1371/journal.pmed.1000289

Masters, B. A., Werner, H., Roberts, C. T., Jr., LeRoith, D., & Raizada, M. K. (1991). Insulin-like growth factor I (IGF-I) receptors and IGF-I action in oligodendrocytes from rat brains. *Regul Pept, 33*(2), 117-131. doi:10.1016/0167-0115(91)90207-W

Maysami, S., Nguyen, D., Zobel, F., Pitz, C., Heine, S., Hopfner, M., & Stangel, M. (2006). Modulation of rat oligodendrocyte precursor cells by the chemokine CXCL12. *Neuroreport, 17*(11), 1187-1190. doi:10.1097/01.wnr.0000227985.92551.9a

Ment, L. R., Kesler, S., Vohr, B., Katz, K. H., Baumgartner, H., Schneider, K. C., . . . Reiss, A. L. (2009). Longitudinal Brain Volume Changes in Preterm and Term Control Subjects During Late Childhood and Adolescence. *Pediatrics, 123*, 503-511. doi:10.1542%2Fpeds.2008-0025

Moster, D., Lie, R. T., & Markestad, T. (2008). Long-term medical and social consequences of preterm birth. *New England Journal of Medicine, 359*(3), 262-273. doi:10.1056/NEJMoa0706475

Mueller, M., Oppliger, B., Joerger-Messerli, M., Reinhart, U., Barnea, E., Paidas, M., . . . Schoeberlein, A. (2017). Wharton's Jelly Mesenchymal Stem Cells Protect the Immature Brain in Rats and Modulate Cell Fate. *Stem Cells Dev, 26*(4), 239-248. doi:10.1089/scd.2016.0108

Nagy, Z., Ashburner, J., Andersson, J., Jbabdi, S., Draganski, B., Skare, S., . . . Lagercrantz, H. (2009). Structural correlates of preterm birth in the adolescent brain. *Pediatrics, 124*(5), e964-972. doi:10.1542/peds.2008-3801

Nosarti, C., Al-Asady, M. H., Frangou, S., Stewart, A. L., Rifkin, L., & Murray, R. M. (2002). Adolescents who were born very preterm have decreased brain volumes. *Brain, 125*(Pt 7), 1616-1623. doi:10.1093/brain/awf157

Nosarti, C., Giouroukou, E., Healy, E., Rifkin, L., Walshe, M., Reichenberg, A., . . . Murray, R. M. (2008). Grey and white matter distribution in very preterm adolescents mediates neurodevelopmental outcome. *Brain, 131*(Pt 1), 205-217. doi:10.1093/brain/awm282

Nosarti, C., Giouroukou, E., Micali, N., Rifkin, L., Morris, R. G., & Murray, R. M. (2007). Impaired executive functioning in young adults born very preterm. *Journal of the International Neuropsychological Society, 13*(4), 571-581. doi:10.1017/S1355617707070725

Oppliger, B., Joerger-Messerli, M., Mueller, M., Reinhart, U., Schneider, P., Surbek, D. V., & Schoeberlein, A. (2016). Intranasal Delivery of Umbilical Cord-Derived Mesenchymal Stem Cells Preserves Myelination in Perinatal Brain Damage. *Stem Cells and Development, 25*(16), 1234-1242. doi:10.1089/scd.2016.0027

Park, W. S., Sung, S. I., Ahn, S. Y., Sung, D. K., Im, G. H., Yoo, H. S., . . . Chang, Y. S. (2016). Optimal Timing of Mesenchymal Stem Cell Therapy for Neonatal Intraventricular Hemorrhage. *Cell Transplant, 25*(6), 1131-1144. doi:10.3727/096368915×689640

Patel, J. R., McCandless, E. E., Dorsey, D., & Klein, R. S. (2010). CXCR4 promotes differentiation of oligodendrocyte progenitors and remyelination. *PNAS, 107*(24), 11062-11067. doi:10.1073/pnas.1006301107

Paton, M. C. B., McDonald, C. A., Allison, B. J., Fahey, M. C., Jenkin, G., & Miller, S. L. (2017). Perinatal Brain Injury As a Consequence of Preterm Birth and Intrauterine Inflammation: Designing Targeted Stem Cell Therapies. *Frontiers in Neuroscience, 11*, 200. doi:10.3389/fnins.2017.00200

Paul, G., & Anisimov, S. V. (2013). The secretome of mesenchymal stem cells: potential implications for neuroregeneration. *Biochimie, 95*(12), 2246-2256. doi:10.1016/j.biochi.2013.07.013

Peterson, B. S., Vohr, B., Staib, L. H., Cannistraci, C. J., Dolberg, A., Schneider, K. C., . . . Ment, L. R. (2000). Regional Brain Volume Abnormalities and Long-term Cognitive Outcome in Preterm Infants. *Jama, 284*(15), 1939-1947. doi:10.1001/jama.284.15.1939

Rezaie, P., & Dean, A. (2002). Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. *Neuropathology, 22*(3), 106-132. doi:10.1046/j.1440-1789.2002.00438.x

Salmaso, N., Jablonska, B., Scafidi, J., Vaccarino, F. M., & Gallo, V. (2014). Neurobiology of premature brain injury. *Nature Neuroscience, 17*(3), 341-346. doi:10.1038/nn.3604

Scafidi, J., Hammond, T. R., Scafidi, S., Ritter, J., Jablonska, B., Roncal, M., . . . Gallo, V. (2014). Intranasal epidermal growth factor treatment rescues neonatal brain injury. *Nature, 506*(7487), 230-234. doi:10.1038/nature12880

Schmidt-Kastner, R., Wietasch, K., Weigel, H., & Eysel, U. T. (1993). Immunohistochemical staining for glial fibrillary acidic protein (GFAP) after deafferentation or ischemic infarction in rat visual system: Features of reactive and damaged astrocytes. *International Journal of Developmental Neuroscience, 11*(2), 157-174. doi:10.1016/0736-5748(93)90076-P

Schmitz, T., Ritter, J., Mueller, S., Felderhoff-Mueser, U., Chew, L.-J., & Gallo, V. (2011). Cellular Changes Underlying Hyperoxia-Induced Delay of White Matter Development. *The Journal of Neuroscience, 31*(11), 4327. doi:10.1523/JNEUROSCI.3942-10.2011

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods, 9*(7), 671-675. doi:10.1038/nmeth.2089

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., & Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology, 106-107*, 1-16. doi:10.1016/j.pneurobio.2013.04.001

Shen, Y., Plane, J. M., & Deng, W. (2010). Mouse Models of Periventricular Leukomalacia. *Journal of Visualized Experiments, 39*(e1951), 1-4. doi:10.3791/1951

Stoll, B. J., Hansen, N. I., Bell, E. F., Walsh, M. C., Carlo, W. A., Shankaran, S., . . . Higgins, R. D. (2015). Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm Neonates, 1993-2012. *Jama, 314*(10), 1039-1051. doi:10.1001/jama.2015.10244

Umekawa, T., Osman, A. M., Han, W., Ikeda, T., & Blomgren, K. (2015). Resident microglia, rather than blood-derived macrophages, contribute to the earlier and more pronounced inflammatory reaction in the immature compared with the adult hippocampus after hypoxia-ischemia. *Glia, 63*(12), 2220-2230. doi:10.1002/glia.22887

Vaes, J. E. G., Vink, M. A., de Theije, C. G. M., Hoebeek, F. E., Benders, M., & Nijboer, C. H. A. (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

van Tilborg, E., Achterberg, E. J. M., van Kammen, C. M., van der Toorn, A., Groenendaal, F., Dijkhuizen, R. M., . . . Nijboer, C. H. A. (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*(1), 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., . . . Nijboer, C. H. (2018b). Origin and dynamics of oligodendrocytes in the developing brain: Implications for perinatal white matter injury. *Glia, 66*(2), 221-238. doi:10.1002/glia.23256

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., & Nijboer, C. H. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Progress in Neurobiology, 136*(1873-5118 (Electronic)), 28-49. doi:10.1016/j.pneurobio.2015.11.002

van Tilborg, E., van Kammen, C. M., de Theije, C. G. M., van Meer, M. P. A., Dijkhuizen, R. M., & Nijboer, C. H. (2017). A quantitative method for microstructural analysis of myelinated axons in the injured rodent brain. *Sci Rep, 7*(1), 16492-16492. doi:10.1038/s41598-017-16797-1

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010a). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain, Behavior and Immunity, 24*(3), 387-393. doi:10.1016/j.bbi.2009.10.017

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010b). Nasal administration of stem cells: a promising novel route to treat neonatal ischemic brain damage. *Pediatric Research, 68*(5), 419-422. doi:10.1203/PDR.0b013e3181f1c289

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2011). Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. *Brain, Behavoir and Immunity, 25*(7), 1342-1348. doi:10.1016/j.bbi.2011.03.021

Verney, C., Pogledic, I., Biran, V., Adle-Biassette, H., Fallet-Bianco, C., & 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*(3), 251-264. doi:10.1097/NEN.0b013e3182496429

Viganò, F., Schneider, S., Cimino, M., Bonfanti, E., Gelosa, P., Sironi, L., . . . Dimou, L. (2016). GPR17 expressing NG2-Glia: Oligodendrocyte progenitors serving as a reserve pool after injury. *Glia, 64*(2), 287-299. doi:10.1002/glia.22929

Vincze, A., Mázló, M., Seress, L., Komoly, S., & Ábrahám, H. (2008). A correlative light and electron microscopic study of postnatal myelination in the murine corpus callosum. *International Journal of Developmental Neuroscience, 26*(6), 575-584. doi:10.1016/j.ijdevneu.2008.05.003

Volkman, R., & Offen, D. (2017). Concise Review: Mesenchymal Stem Cells in Neurodegenerative Diseases. *Stem Cells, 35*(8), 1867-1880. doi:10.1002/stem.2651

Volpe, J. J. (2009a). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurology, 8*(1), 110-124. doi:10.1016/s1474-4422(08)70294-1

Volpe, J. J. (2009b). The encephalopathy of prematurity--brain injury and impaired brain development inextricably intertwined. *Seminars in Pediatric Neurology, 16*(4), 167-178. doi:10.1016/j.spen.2009.09.005

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., & Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *International Journal of Developmental Neuroscience, 29*(4), 423-440. doi:10.1016/j.ijdevneu.2011.02.012

Wagenaar, N., de Theije, C. G. M., de Vries, L. S., Groenendaal, F., Benders, M., & Nijboer, C. H. A. (2018). Promoting neuroregeneration after perinatal arterial ischemic stroke: neurotrophic factors and mesenchymal stem cells. *Pediatric Research, 83*(1-2), 372-384. doi:10.1038/pr.2017.243

Wang, L. Q., Lin, Z. Z., Zhang, H. X., Shao, B., Xiao, L., Jiang, H. G., . . . Jin, K. L. (2014). Timing and dose regimens of marrow mesenchymal stem cell transplantation affect the outcomes and neuroinflammatory response after ischemic stroke. *CNS Neuroscience & Therapeutics, 20*(4), 317-326. doi:10.1111/cns.12216

Weil, M. T., Ruhwedel, T., Meschkat, M., Sadowski, B., & Möbius, W. (2019). Transmission Electron Microscopy of Oligodendrocytes and Myelin. *Methods in Molecular Biology, 1936*, 343-375. doi:10.1007/978-1-4939-9072-6_20

Wilson, H. C., Onischke, C., & Raine, C. S. (2003). Human oligodendrocyte precursor cells in vitro: phenotypic analysis and differential response to growth factors. *Glia, 44*(2), 153-165. doi:10.1002/glia.10280

Xing, Y. L., Röth, P. T., Stratton, J. A., Chuang, B. H., Danne, J., Ellis, S. L., . . . Merson, T. D. (2014). Adult neural precursor cells from the subventricular zone contribute significantly to oligodendrocyte regeneration and remyelination. *Journal of Neuroscience, 34*(42), 14128-14146. doi:10.1523/jneurosci.3491-13.2014

Zanier, E. R., Fumagalli, S., Perego, C., Pischiutta, F., & De Simoni, M.-G. (2015). Shape descriptors of the "never resting" microglia in three different acute brain injury models in mice. *Intensive care medicine experimental, 3*(1), 39-39. doi:10.1186/s40635-015-0039-0

Zhao, J., Chen, Y., Xu, Y., & Pi, G. (2013). Effect of intrauterine infection on brain development and injury. *International Journal of Developmental Neuroscience, 31*(7), 543-549. doi:10.1016/j.ijdevneu.2013.06.008

Zonouzi, M., Scafidi, J., Li, P., McEllin, B., Edwards, J., Dupree, J. L., . . . Gallo, V. (2015). GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury. *Nature Neuroscience, 18*(5), 674-682. doi:10.1038/nn.3990

SUPPLEMENTAL DATA S1

Synthesis of gold core-mesoporous and lipid coated silica nanoparticles (AuNP-MSN-LIP)

60nm AuNP's were synthesized by adaption of previously reported methods.[1,2] First, 18nm seeds were synthesized by adding a citric acid solution (3mL, Sigma-Aldrich) to chloroauric acid (97mL, 0.3mM, Sigma-Aldrich) at 100°C under reflux. The reaction was continued for 15minutes until a deep ruby red color was achieved. To grow 60nm AuNP's, 1.65×10^{12} AuNP seeds were added to chloroauric acid solution (2.5×10^{-5}M) under rapid stirring. Immediately afterwards, citric acid (50mM, 680 µL) and hydroquinone (50mM, 454µL, Sigma-Aldrich) were added under continuous stirring and the solution was left to react for 1h. To replace the citrate with hexadecyltrimethylammonium bromide (CTAB, Sigma-Aldrich) a 1 mL (0.1M) was added to the solution and left to stir at room temperature overnight. The next day, particles were collected by centrifugation, washed and redispersed in H_2O (100mL) to remove excess CTAB. The size and homogeneity of 60nm AuNP's was characterized by dynamic light scattering (DLS, zetasizer) and ICP-MS (iCAP™ RQ ICP-MS, Thermo Scientific) to calculate the moles of Au in a solution of 60nm AuNP's.

The synthesis of silica coated gold nanoparticles (AuNP-MSNs) was conducted following a reported protocol with adaption.[4,5] First 6.5×10-6 moles of CTAB stabilized 60 nm AuNP's were concentrated by centrifugation and redispersed in 5mL of H_2O. Then CTAB (0.273g, 7.5×10-4mol) was dissolved in a mix of 75 mL absolute ethanol (VWR) and 170mL H_2O, and stirred at 35°C. Once the solution was transparent, NH_3 (100µL, 25% vol, Carl Roth) was added and stirred for 5 minutes. Then the concentrated AuNP's were added and left to stir for a further 5 minutes. For -SH core functionalization, a mixture of (3-Mercaptopropyl) trimethoxysilane (MPTES) (5µL, 5.3µmol, Sigma-Aldrich) and Tetraethylorthosilicate (TEOS) (65µL, 60.6µmol, Sigma-Aldrich) was added dropwise and temperature increased to 60°C. After 20 minutes TEOS was added in three equal increments (20µL, 18.6µmol) every 3 minutes, and the mixture was left to stir for another 30 minutes. Then, for –NH2 surface functionalization a mixture of TEOS (10µL, 9.3µmol) and (3-aminopropyl)triethoxysilane (APTES) (5µL, 4.7µmol; Sigma-Aldrich, 99%) were added to the mixture and left to stir overnight. The particles were collected by centrifugation and washed twice with ethanol. CTAB removal was conducted by ion-exchange and acid extraction as we reported previously.[6] AuNP-MSN's were stored at −20°C until further use. Next, AuNP-atto647-MSNs were coated with a lipid layer to enhance the cell-uptake of the NPs. The

lipid coating was made by the hydration method according to previously reported protocols.[6] AuNP-atto647-MSN-LIP solution was stored at 4°C until further use.

Cell particle labeling experiments

To quantitatively determine the optimal cell-particle labelling procedure flow cytometry was employed. Particle uptake was assessed in biological triplicates in both adherent cells and cells in suspension. On day one, mMSC's were seeded in 12 well plates at 15,000cells/cm2. To prepare particle functionalized media the as-prepared AuNP-Atto647-MSN-LIP were pelleted and redispersed in culture media to a concentration of 25µg/mL. mMSC's were exposed incrementally to AuNP-Atto647-MSN-LIP. For adherent cells particles were exposed to mMSC's for 48, 24, 6 and 2 hours and samples were prepared together for flow cytometry on day 3. To prepare the samples, cells were washed with PBS, detached by 0.05% trypsin/EDTA and redispersed in cell culture medium. For cells in suspension mMSC's were detached on day 3 and exposed for particles for 10 and 30 minutes. All samples were collected by centrifugation, resuspended in 500µL PBS and kept on ice for flow cytometry analysis. Flow cytometry was performed using a BD Accuri C6. Cells were detected in the red channel under λ640nm excitation. For each sample 10,000 cells (gated) were collected. FlowJo version 10 was used for data analysis.

1. Zabetakis, K., Ghann, W. E., Kumar, S. & Daniel, M.-C. Effect of high gold salt concentrations on the size and polydispersity of gold nanoparticles prepared by an extended Turkevich–Frens method. Gold Bulletin 45, 203-211, doi:10.1007/s13404-012-0069-2 (2012).
2. Kumar, D., Mutreja, I. & Sykes, P. Seed mediated synthesis of highly monodispersed gold nanoparticles in the presence of hydroquinone. Nanotechnology 27, 355601, doi:10.1088/0957-4484/27/35/355601 (2016).
3. Haiss, W., Thanh, N. T. K., Aveyard, J. & Fernig, D. G. Determination of Size and Concentration of Gold Nanoparticles from UV–Vis Spectra. Analytical Chemistry 79, 4215-4221, doi:10.1021/ac0702084 (2007).
4. Sanz-Ortiz, M. N., Sentosun, K., Bals, S. & Liz-Marzán, L. M. Templated Growth of Surface Enhanced Raman Scattering-Active Branched Gold Nanoparticles within Radial Mesoporous Silica Shells. ACS Nano 9, 10489-10497, doi:10.1021/acsnano.5b04744 (2015).
5. Möller, K. & Bein, T. Talented Mesoporous Silica Nanoparticles. Chemistry of Materials 29, 371-388, doi:10.1021/acs.chemmater.6b03629 (2017).

6. Rosenbrand, R. et al. Lipid Surface Modifications Increase Mesoporous Silica Nanoparticles Labeling Properties in Mesenchymal Stem Cells. International Journal of Nanomedicine 2018, 7711—7725, doi:10.2147/IJN.S182428 (2018).

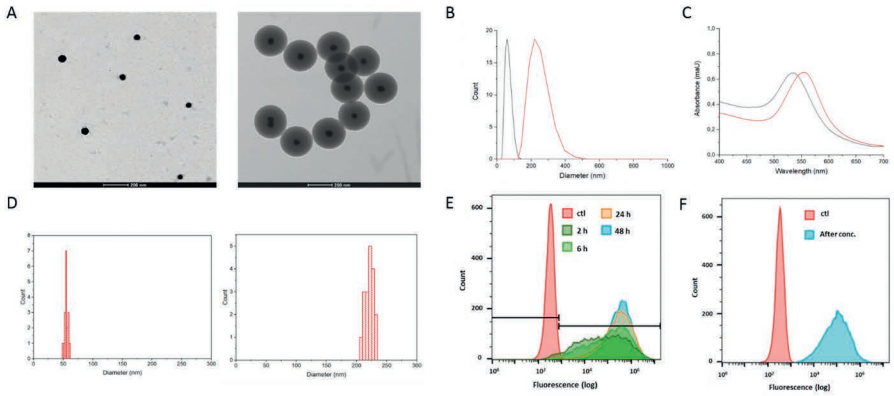


Figure S1. AuNP-MSN nanoparticle characterization and rMSC nanoparticle uptake analysis. **A.** Transmission electron microscopy (TEM) image of AuNP (left) and AuNP-MSN (right). Scale bar is 200nm. **B.** Size analysis of AuNP (black) and AuNP-MSN (red) by dynamic light scattering (DLS). **C.** UV-Vis spectroscopy of AuNP (black) and AuNP-MSN (red) showing a slight shift in absorbance after silica coating. **D.** Size analysis of 20 particles using TEM images by ImageJ; AuNP (left) and AuNP-MSN (right). **E.** Internalization of AuNP-MSN-LIP nanoparticles in rMSCs after 2, 6, 24 and 48 hours of incubation, analyzed by flow cytometry. **F.** Internalization of AuNP-MSN-LIP after 48h incubation with rMSCs and concentration to 62.5×106 cells/mL.

SUPPLEMENTAL DATA S2

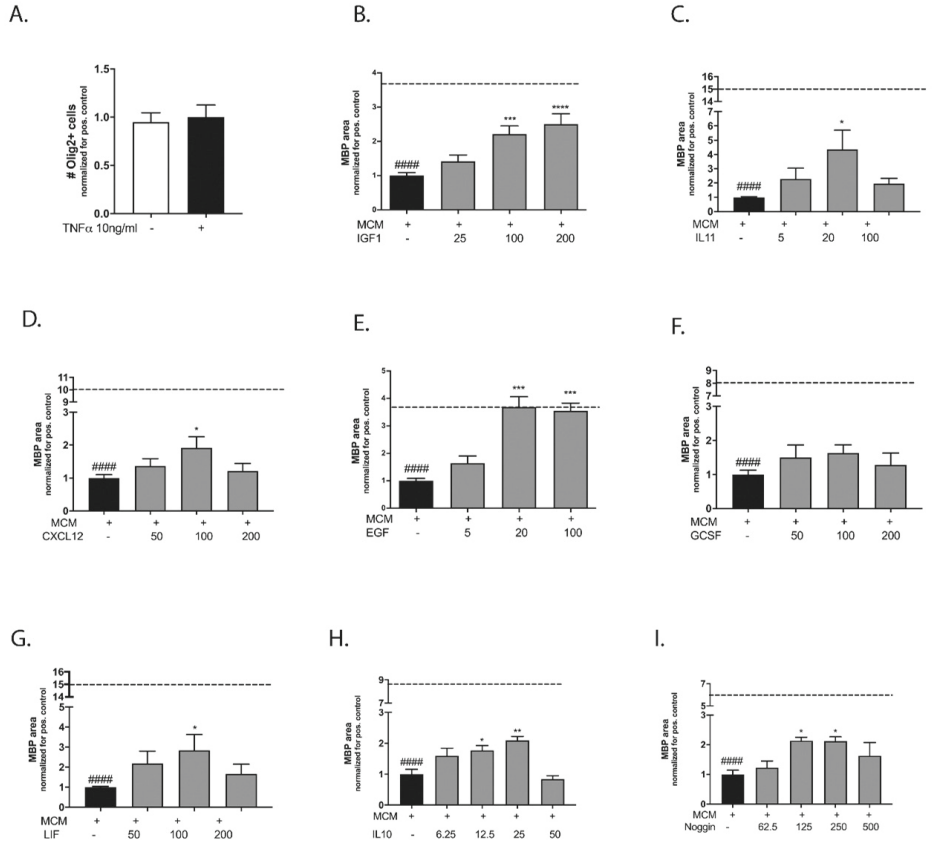


Figure S2. MSC-secreted factors boost myelin production in a dose-dependent manner in primary OL cultures. **A.** Exposure to 10ng/ml TNFα does not lead to OL death indicated by a similar amount Olig2+ cells in medium with TNFα+ compared to medium without TNFα (n=3 independent experiments, 3-4 observations per experiment; normalized for the positive control, e.g. cells exposed to TNFα). **B-I.** Addition of IGF1 (**B**), IL11 (**C**), CXCL12 (**D**), EGF (**E**),LIF (**G**), IL10 (**H**) and Noggin (**I**), but not GCSF (**F**), significantly improved myelin production by primary cultured oligodendrocytes in an dose-dependent manner following MCM+LPS exposure (dashed line represents MBP+ area in MCM-LPS control condition) (n=2 independent experiments, 2-3 observations per experiment, normalized for the positive control, e.g. cells exposed to MCM+LPS without addition of a factor). ####: p<0.0001 MCM+ (black bars) vs MCM- (dashed line) respectively; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 factor-exposed MCM+ condition (gray bars) vs MCM+ control condition (black bars).

SUPPLEMENTAL DATA S3

Table S3. Gene expression profile of MSCs after culture with SHAM or dWMI brain extracts

RefSeq	Gene symbol	2^ΔCt array MSC + SHAM extract	MSC + dWMI extract	Fold Regulation array	Fold Regulation validation
NM_009755	Bmp1	0,202782	0,072897	-2,7818	
NM_173404	Bmp3	0,000855	0,000132	-6,48	
NM_007554	Bmp4	0,138504	0,063902	-2,1675	
NM_007559	Bmp8b	0,000096	0,000615	6,4264	
NM_013653	Ccl5	1,344124	0,155681	-8,6338	
NM_016960	Ccl20	0,0006	0,002625	4,3772	
NM_170786	Cntf	0,002995	0,008125	2,7132	
NM_009969	Csf2	0,000671	0,040723	60,7158	
NM_009971	Csf3	0,000024	0,000128	5,2927	2,636229913
NM_008176	Cxcl1	0,046974	0,020503	-2,291	
NM_009141	Cxcl5	1,194715	0,014246	-83,8652	
NM_008599	Cxcl9	0,000274	0,0006	2,1886	
NM_021274	Cxcl10	0,04259	0,199805	4,6913	
NM_021704	Cxcl12	1,78757	0,473685	-3,7738	-9,427829735
NM_023158	Cxcl16	0,009596	0,020287	2,114	
NM_010113	Egf	0,000962	0,000971	1,0098	not expressed in SHAM
NM_007950	Ereg	0,010511	0,027054	2,574	
NM_010177	Fasl	0,000274	0,000872	3,1821	
NM_010198	Fgf11	0,00024	0,000632	2,6281	
NM_008003	Fgf15	0,0006	0,002635	4,3893	
NM_008005	Fgf18	0,010511	0,001452	-7,2401	
NM_010203	Fgf5	0,000019	0,000131	6,84	
NM_008008	Fgf7	0,194521	0,04395	-4,426	
NM_010205	Fgf8	0,000053	0,000528	9,8765	
NM_013518	Fgf9	0,000024	0,000192	7,9668	
NM_010216	Figf	0,499307	0,061299	-8,1455	
NM_008109	Gdf5	0,001112	0,000444	-2,5071	
NM_010275	Gdnf	0,007911	0,017973	2,2721	
NM_010427	Hgf	0,044133	0,003289	-13,4171	
NM_010512	Igf1	0,231326	0,006443	-35,9022	-72,11973123
NM_031167	Il1rn	0,035321	0,010285	-3,4343	
NM_008366	Il2	0,000019	0,000301	15,7142	
NM_010556	Il3	0,000019	0,000059	3,061	

Table S3. Continued.

RefSeq	Gene symbol	2^ΔCt array MSC + SHAM extract	MSC + dWMI extract	Fold Regulation array	Fold Regulation validation
NM_021283	Il4	0,000212	0,000606	2,856	
NM_008371	Il7	0,000251	0,001048	4,1814	
NM_008373	Il9	0,002017	0,000837	-2,4116	
NM_010548	Il10	0,000424	0,000242	-1,7532	-2,346867579
NM_008350	Il11	0,001561	0,004063	3,0823	2,468171665
NM_010551	Il16	0,018283	0,004798	-3,8106	
NM_008360	Il18	0,002954	0,001175	-2,514	
NM_010564	Inha	0,002816	0,005807	2,0619	
NM_008380	Inhba	0,012762	0,032173	2,521	
NM_008381	Inhbb	0,016154	0,001452	-11,1271	
NM_177099	Lefty2	0,00005	0,002096	41,7586	
NM_008501	Lif	0,001023	0,002553	2,7587	1,574161912
NM_008518	Ltb	0,000723	0,001922	2,6574	
NM_010798	Mif	1,737083	5,802468	3,3404	
NM_010834	Mstn	0,000019	0,000056	2,9363	
NM_013609	Ngf	0,001629	0,006054	3,7166	
-	Noggin	-	-	-	1,13250499
NM_008742	Ntf3	0,000047	0,000094	2,0056	
NM_008827	Pgf	0,000128	0,000741	5,7918	
NM_023785	Ppbp	0,000866	0,000308	-2,8089	
NM_019400	Rabep1	0,0366	0,076521	2,0907	
NM_031199	Tgfa	0,000055	0,000325	5,9546	
NM_033622	Tnfsf13b			2,0139	
NM_009505	Vegfa	0,05625	0,160651	2,856	
NM_011697	Vegfb	0,047961	0,149892	3,1253	

6

Modifying the secretome of MSCs prolongs the regenerative treatment window for encephalopathy of prematurity

Josine E.G. Vaes, Suzanne M. Onstwedder*, Chloe Trayford*, Eva Gubbins, Mirjam Maas, Sabine H. van Rijt, Cora H. Nijboer

**Both authors contributed equally to this manuscript and share the second authorship*

Submitted

ABSTRACT

Clinical treatment options to combat Encephalopathy of Prematurity (EoP) are still lacking. We and others have proposed (intranasal) mesenchymal stem cells (MSCs) as a potent therapeutic strategy to boost white matter repair in the injured preterm brain. Using a double-hit mouse model of diffuse white matter injury, we showed earlier that efficacy of MSC treatment was time-dependent, with a significant decrease in functional and histological improvement after postponement of cell administration. In this follow-up study, we aimed to investigate the mechanisms underlying this loss in therapeutic efficacy. Additionally, we optimized the regenerative potential of MSCs by means of genetic engineering with transient hypersecretion of beneficial factors, in order to prolong the treatment window. Though cerebral expression of known chemoattractants was stable over time, migration of MSCs to the injured brain was partially impaired. Moreover, using a primary oligodendrocyte (OL) culture, we showed that rescue of injured OLs was reduced after delayed MSC coculture. Cocultures of modified MSCs, hypersecreting IGF1, LIF, IL11 or IL10, with primary microglia and OLs revealed a superior treatment efficacy over naïve MSCs. Additionally, we showed that intranasal delayed administration of IGF1-, LIF- or IL11- hypersecreting MSCs improved myelination and functional outcome in EoP mice. In conclusion, impaired migration and regenerative capacity of intranasally applied MSCs likely underlie the observed loss of efficacy after delayed treatment. Intranasal administration of IGF1-, LIF- or IL11- hypersecreting MSCs is a promising optimization strategy to prolong the window for effective MSC treatment in preterm infants with EoP.

INTRODUCTION

Encephalopathy of Prematurity (EoP) is a major cause of neurological morbidity in (extreme) preterm neonates (Volpe, 2009b). In these infants particularly white matter development is impacted, characterized by widespread hypomyelination in absence of cystic lesions (*diffuse white matter injury*, dWMI) (Back, 2017; Volpe, 2009a). An arrest in oligodendrocyte (OL) lineage maturation, due to preterm birth-related insults is believed to underlie the observed myelination deficits (van Tilborg et al., 2016; Volpe et al., 2011). To date, no clinically approved treatment options to restore dWMI in preterm infants are available.

Preclinical evidence supporting a beneficial role for mesenchymal stem cell (MSC) therapy in dWMI has grown (Vaes et al., 2019). We and others have demonstrated in experimental models that (intranasal) administration of MSCs after dWMI effectively improves myelination and functional outcome, whilst attenuating neuroinflammation (Oppliger et al., 2016; Paton et al., 2018; Vaes et al., 2021). Using a range of neonatal brain injury models, we and others have shown that transplanted MSCs are unlikely to integrate into brain parenchyma but rather modulate their secretome, contributing to a cerebral environment permissive for repair and neurogenesis through paracrine signaling (Kassis et al., 2011; Liang et al., 2014; van Velthoven et al., 2010). Most recently, we showed that intranasal MSC therapy potently restored myelination after early administration (i.e. 3 days (D3) after dWMI) in newborn mice (Vaes et al., 2021). However the therapeutic potential of intranasal MSCs decreased significantly when treatment was postponed until D6 (Vaes et al., 2021). A narrow treatment window could potentially limit the clinical applicability of intranasal MSC therapy in extreme preterm infants, as early identification of dWMI is challenging due to its multiple-hit pathophysiology, first MRI possibility, and a lack of reliable biomarkers (Back, 2017; Douglas-Escobar & Weiss, 2012; Ment et al., 2009). Here, we hypothesized that reduced efficacy of postponed intranasal MSC treatment could be the result of either impaired MSC homing at a later treatment timepoint or to a limited regenerative potential of MSCs in later stages of dWMI pathophysiology. In the first case a possible lack of chemotactic factors crucial for MSC homing to areas of dWMI at later timepoints could be responsible. In the latter case, optimization of the MSC secretome, i.e. boosting their trophic and anti-inflammatory properties, could potentially prolong the treatment window.

As a follow-up of our previous work (Vaes et al., 2021), we assessed cerebral chemotactic signals between D3 and D6 after dWMI using ex vivo PCR arrays. Furthermore, we studied MSC homing using nanoparticle-based cell tracing. We used primary glial cultures to investigate the potential superior capacity of MSCs overexpressing IGF1, EGF, LIF, IL10 or IL11, secreted factors previously identified beneficial for OL maturation or dampening of microglia activation in vitro (Vaes et al., 2021). Finally, we explored whether intranasal administration of IGF1-, IL11-, LIF- or IL10-overexpressing MSCs could prolong the treatment window for dWMI in our mouse model.

MATERIALS AND METHODS

All procedures were carried out according to the Dutch and European guidelines (Directive 86/609, ETS 123, Annex II) and were approved by the Experimental Animal Committee Utrecht (Utrecht University, Utrecht, Netherlands) and the Central Authority for Scientific Procedures on Animals (the Hague, the Netherlands). Detailed materials and methods can be found in the supporting information (SI).

MSC culture

GIBCO® mouse (C57BL/6) bone marrow-derived MSCs (Invitrogen, S1502-100; Carlsbad, California, USA) were cultured in D-MEM/F-12 medium with 10% fetal bovine serum (10565-018 and 12662-029, Invitrogen) according to the supplier's protocol. MSCs were passaged once (from P2 to P3) prior to *in vivo* administration or in *in vitro* experiments.

MSC transfection

MSCs were modified to transiently overexpress growth factor or cytokines with ready-to-use recombinant adenoviral vectors encoding a murine IGF1, EGF, LIF, IL11 or IL10 transgene, combined with a control eGFP vector to assess infection efficacy (Vector Biolabs, Malvern, US). MSCs were plated 24h before infection at 2.0×105 cells per well in 6-wells plates, followed by exposure to viral particles for 6 hours. Thereafter, cells were recultured for 24 hours followed by *in vitro* gel embedment or *in vivo* administration. The optimal multiplicity of infection (MOI) was determined per adenovirus, by assessment of the secreted protein using ELISA at 2 days after infection.

In vivo model of diffuse white matter injury

dWMI was induced in postnatal day 5 (P5) C57BL/6j mouse pups as described previously (Vaes et al., 2021). Hyaluronidase was administered to the nasal cavity 30 min prior to administration of 0.5×10^6 MSCs (Vaes et al., 2021). MSCs were administered at P8 (i.e. D3) or P11 (i.e. D6). Previous dose-response experiments identified 0.5×106 MSCs as the lowest effective dose (Vaes et al., 2021). Vehicle-treated dWMI animals received dPBS. Mice were euthanized at P8, P11, or P26 (i.e. 3 weeks) by an i.p. overdose pentobarbital. For PCR arrays sham-control or dWMI (untreated) brains were collected, cerebellum was discarded and hemispheres were separately snap-frozen in liquid nitrogen and stored at -80°C until further processing.

Cerebral chemokine expression profiles

Sham-control and dWMI (untreated) brains were collected at D3 and D6 after dWMI. RNA was isolated from the ipsilateral hemispheres and cDNA transcription was carried out. cDNA of sham-control or dWMI animals was pooled per time point (D3 n=5 and D6 n=4 per experimental condition) and PCR array (PAMM-150Z, Qiagen) was performed. Chemokine/cytokine gene expression changes were calculated: 1) in dWMI mice versus sham-control mice at D3 to identify chemokines that are differentially regulated following injury and 2) in dWMI mice at D6 versus D3 to study the stability of the chemotactic signals over time. A fold regulation threshold of 3.0 was considered as either down- or upregulation. PCR array results were validated by quantitative PCR analyses in the individual cDNA samples for selected genes (table S2). Primer sequences can be found in table S1. Mean expression of GADPH and β-actin were used for data normalization.

MSC gene expression profiles after exposure to brain extracts

Brains were collected at D3 (n=5) and D6 (n=4) and brain extracts were made. MSCs were cultured and seeded at 2.0×10^5 cells per well. After 24 hours, culture medium was replaced with knock-out DMEM containing either D3 or D6 brain extract at a concentration of 1mg protein/ml. After 48h MSC RNA was isolated and transcribed to cDNA and PCR arrays were performed (Qiagen; PAMM-041Z and PAMM-150Z). Gene expression changes in MSCs exposed to D6 dWMI brain extracts were calculated relative to D3 dWMI brain extract exposure. A fold regulation threshold of 3.0 was considered as either down- or upregulation. PCR arrays results were validated for selected genes (table S3). Primer sequences can be found in table S1.

MSC labeling and cell tracing

MSCs were labelled using gold core-mesoporous and lipid-coated silica nanoparticles (AuNP-MSN-LIP). A detailed description of nanoparticle synthesis, characterization and labeling efficiency can be found in our previous paper (Vaes et al., 2021). In short, 2 hours after cell passaging, MSCs were incubated with 25µg/ml AuNP-MSN-LIP in culture medium over 48 hours. Following cell labeling, dWMI animals received intranasally 0.5×10^6 MSCs at D3 or at D6. 12 hours after treatment mice were sacrificed by overdose pentobarbital, brains were dissected and frozen in liquid nitrogen, as well as spleen, lung and liver. For details on inductively coupled plasma mass spectrometry (ICP-MS) to quantitatively assess MSC biodistribution by detection of gold in mouse tissue homogenates, we refer to (Vaes et al., 2021) and the SI.

Immunohistochemistry

At P26 animals were sacrificed by overdose pentobarbital followed by transcardial perfusion with PBS and 4% PFA. Brains were post-fixed for 24 hours in 4% PFA followed by dehydration in ethanol. Brains were paraffin-embedded and coronal sections (8µm) were cut at hippocampal level (-1.80mm from bregma in adult mice). For 3,3'-Diaminobenzidine (DAB) staining, sections were deparaffinized and rehydrated, blocked in 20% normal rabbit serum (NRS) in PBS/0.1% Tween and incubated overnight with rat-anti-MBP (MAB386, Merck Millipore; 1:500) in 10% NRS/PBS/0.1% Tween. As visualization we used biotinylated rabbit-anti-rat (BA-4000, Vector laboratories, 1:400) with vectastain ABC kit (Vector laboratories) and 0.5mg/ml DAB (Sigma) followed by embedment in depex (Serva). For immunofluorescent stainings, sections were deparaffinized and rehydrated, heated to 95°C in sodium citrate buffer (0.01M, pH 6) for antigen retrieval, blocked with 10% normal goat serum in PBS+0.1% Tween20 for MBP/NF200 or 2% bovine serum albumin (BSA)/0.1% saponin in PBS for Iba1 staining, followed by overnight incubation with rat-anti-MBP (MAB386, Merck Millipore; 1:500), rabbit-anti-NF200 (N-4142, Sigma; 1:400) and with rabbit-anti-Iba1 (019-19741, Wako; 1:500). Subsequently, sections were incubated with alexafluor-594 and -488 conjugated secondary antibodies (Life technologies, Carlsbad, CA; 1:200-500) followed by DAPI (1:5000) counterstaining and embedment in Fluorsave (Merck Millipore, 345789). In between steps we used PBS as washing buffer.

Microscopy and image analysis

Investigators were blinded for experimental conditions during image acquisition and analysis. In MBP-DAB-stained sections a 2.5x magnification was used to image the ipsilateral hemisphere using a light microscope (Zeiss, Oberkochen, Germany) with an AxioCam ICc 5 camera (Zeiss). For immunofluorescent stainings, a Cell Observer microscope with an AxioCam MRm camera (Zeiss, Oberkochen, Germany) was used to acquire images in the ipsilateral hemisphere. For MBP/NF200 stainings, 3 adjacent 40x micrographs were taken at a fixed distance of the external capsule into the cortex (for exact locations see (van Tilborg et al., 2017)). For Iba1 stainings, two 20x images were acquired in the corpus callosum of the ipsilateral hemisphere.

For both cortical myelination (2.5x) on MBP-DAB stainings and microstructural integrity of myelinated axons (40x) using MBP/NF200 stained sections we refer to (van Tilborg et al., 2017). Morphology of microglia residing in the corpus callosum was assessed after manual selection using the particle analysis function of ImageJ v.1.47 (Schneider et al., 2012) as described by (Zanier et al., 2015). Values of all acquired images were averaged per animal.

Behavioral assessment

Motor performance was evaluated using the cylinder rearing test (CRT) at P26 as described in (Vaes et al., 2021) and the SI. In short, animals were placed in a transparent cylinder. Forepaw preference was calculated as ((non-impaired – impaired)/(non-impaired + impaired + both))x100%. All CRTs were videotaped and scored by researchers blinded to the experimental conditions.

In vitro models of dWMI

Primary rat glial cultures

A mixed glial culture was acquired from P1-2 Sprague Dawley rat pup cortices, as described by (Chen et al., 2007), with small changes by our group (Vaes et al., 2021). To mimic the *in vivo* inflammatory situation to induce maturation arrest in immature oligodendrocytes, microglia were plated at a cell density of 0.5×10E6 cells per well in poly-L-ornithine (Sigma Aldrich, P3655)-coated 24 wells plates and microglia-conditioned medium (MCM) was produced as described previously. OPCs were isolated and plated at 4.0×10E4 cells/well on poly-D,L-ornithine (Sigma Aldrich, P0421)-coated 24-wells plates for the OL differentiation experiments.

Primary mouse microglia culture

A primary microglia culture was prepared from P1 C57BL/6 mice cortices for co-cultures as described previously (Vaes et al., 2021). After isolation, microglia were seeded in poly-L-ornithine-coated 24-wells plates at a density of 1.5×10^5 cells per well. Cocultures (see below) were started 24 hours later.

Non-contact MSC-glia cocultures

At 24 hours prior to the start of cocultures (and 24 hours after MSC transfection) 4.0×10^4 modified MSCs (MSC-eGFP (control, empty vector (EV)-MSC), MSC-IGF1, MSC-EGF, MSC-LIF, MSC-IL10 and MSC-IL11) were embedded in Hydromatrix gel (Sigma, A6982) transwell inserts (Merck Millipore, MCHT24H48) according to supplier's protocol.

For the OL differentiation experiment, OPC medium containing pro-proliferation factors (see SI) was replaced with either MCM+LPS or MCM-LPS when the majority of OLs displayed an immature pre-OL morphology (i.e. 4 days after OPC plating). Pro-differentiation factors (see SI) were added to MCM+LPS or MCM-LPS to start differentiation of OPCs. Transwell inserts containing modified MSCs, with EV-MSCs serving as a negative control, or no MSCs as an empty insert control, were added to the wells directly or 24 hours (delayed) after induction of differentiation. Inserts were removed 72 hours after addition of MCM and OLs were fixated with 4% PFA in PBS during 10 minutes.

For the microglia experiment, at 24 hours after plating, co-cultures of MSCs and mouse microglia were started by adding 50ng/ml LPS (Sigma, L4515) and putting transwell inserts containing modified MSCs to the wells. After 48 hours of coculture, the inserts were removed and the microglia supernatant was collected, aliquoted and stored at -80C° for ELISA.

ELISA

Tnfα concentrations in the supernatant of microglia were measured using an ELISA kit for murine Tnfα (Ucytech, Utrecht, The Netherlands) according to manufacturer's protocol. Tnfα data of different experiments were normalized to positive control conditions (i.e. 50ng/ml LPS plus empty insert without MSCs).

Luminex assay

The concentrations of 31 cytokines/chemokines in pooled microglia supernatant (n=3 per condition) were measured using a bioplex pro mouse chemokine assay

(12009159, Biorad) according to the supplier's protocol. All concentrations were normalized to the EV-MSC condition (i.e. 50ng/ml LPS plus an insert with EV-MSCs).

Immunocytochemistry of primary oligodendrocyte cultures

After fixation, nonspecific binding was blocked using 2% BSA and 0.1% saponin in PBS, followed by overnight incubation with primary antibodies (rabbit-anti-Olig2, AB9610. Merck Millipore; 1:1000, mouse-anti-MBP, SMI-94, Biolegend, 1:1000). Subsequently, wells were incubated with alexafluor-594 and -488 conjugated secondary antibodies (Life technologies; 1:1000), followed by Hoechst 33342 (Sigma) for nuclear counterstaining and embedment in Fluorsave (Merck Millipore, 345789). In between steps PBS was used as washing buffer.

Six adjacent fields were imaged (10x), starting at a fixed distance of the well edges. The number of Olig2- and Hoechst- positive cells were counted using the analyze particles function in ImageJ v.1.47. The area of MBP+ staining was measured using manual thresholding analyses in ImageJ. To compare independent experiments, all results were normalized for the positive control (MCM+LPS; empty insert without MSCs).

Statistics

All data are shown as mean ± standard error of the mean (SEM). Statistics were performed using Graphpad Prism 8.3. For details on the statistics see SI. p-values <0.05 were considered statistically significant. Sample sizes are mentioned in the figure captions.

RESULTS

Intranasal MSC treatment efficacy in dWMI: timing matters

In our recent study we showed that intranasal application of MSCs restores dWMI on both a functional and anatomical level when MSCs are applied relatively early (i.e. D3) after the insult. In the current study we confirm the limited treatment window of intranasal MSCs when administration was delayed until D6 after dWMI. Figure 1A/B shows that the dWMI-induced reduction of cortical myelination ($p=0.0075$, compared to sham) at P26 was potently restored after MSC treatment at D3 ($p=0.027$, compared to vehicle treatment). However, when treatment was postponed until D6 MSCs failed to restore cortical myelination. In our previous study, similar conclusions were drawn after assessment of the myelin microstructure (figure 1C).

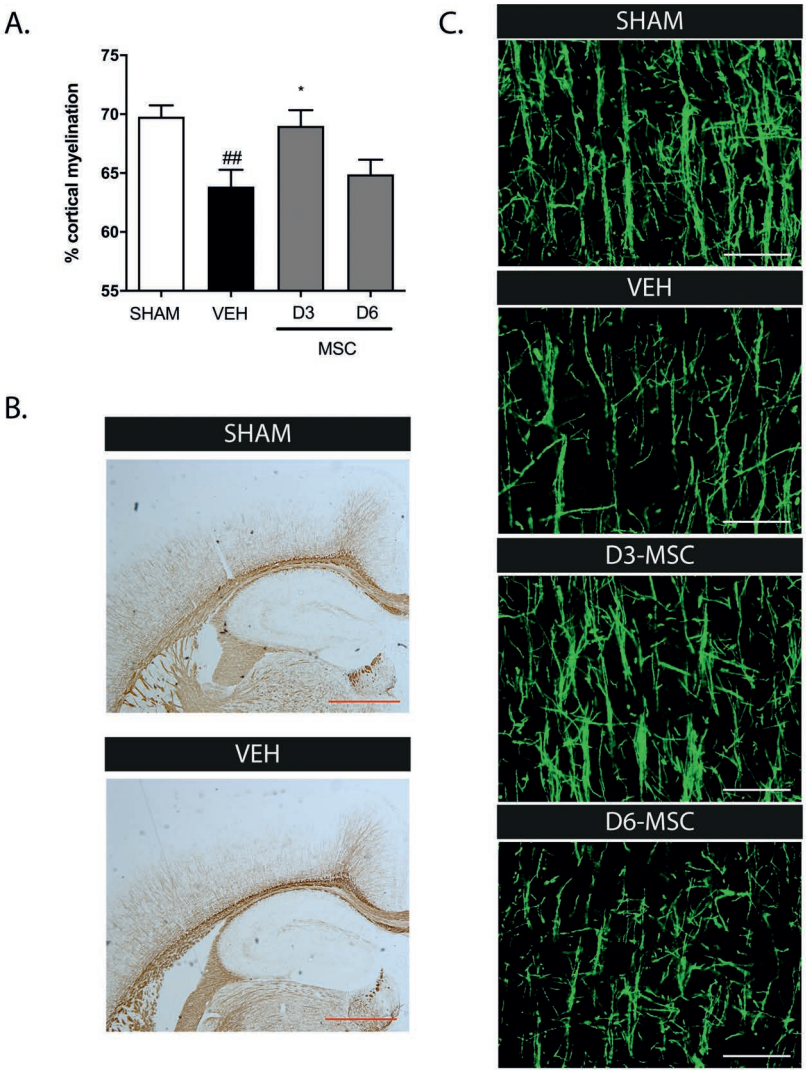


Figure 1. Delayed intranasal MSC administration reduces treatment efficacy. **A.** Cortical myelination was restored in dWMI animals that received intranasal MSC therapy at D3. Delay in MSC administration to D6 reduced treatment efficacy (SHAM n=9, VEH n=7, MSC-D3 n=8, MSC-D6=9). **B.** Representative MBP-DAB stained images (2.5x) of the cortex of a sham-control (upper) and dWMI (lower) mouse at P26. Scale bars: 200µm. **C.** Representative fluorescent images (40x) of MBP+ axons in the ipsilateral cortex of a sham-operated control mouse, dWMI-vehicle treated mouse and dWMI mice treated with MSCs at D3 and D6 (from top to bottom). Scale bars: 50µm. ##: $p<0.01$; vehicle-treated dWMI animals vs sham-controls; *: $p<0.05$; MSC-D3-treated dWMI vs vehicle-treated dWMI animals.

Expression of chemotactic signals in the brain following dWMI induction

To assess which chemotactic factors may be involved in MSC migration to the brain, cerebral gene expression profiles in dWMI mice versus sham-control mice at D3 were analyzed. We identified differential expression of 6 chemokines (Ccl4, Cxcl10, Ccl3, Cxcl3, Cxcl5 and Cxcl1), associated with migration of MSCs or other cell types in literature (table 1) (Donega et al., 2014; Kalwitz et al., 2010; Sordi et al., 2005). Subsequently, we compared the expression of these cerebral chemokines in dWMI mice sacrificed at D6 versus D3. Expression of Cxcl10 and Cxcl1 were (further) up-regulated at D6 compared to D3 after injury. The expression of Ccl4, Ccl3, Cxcl3 and Cxcl5 remained as high at D6 compared to D3 after injury (table 1). Expression of 2 factors (i.e. Cxcl10 and Ccl3) were confirmed by real-time RT-PCR on the individual samples, and showed similar fold regulation changes compared to the arrays (table S2). Our data indicate that the loss of efficacy of D6 MSC treatment is probably not primarily caused by a lack of chemotactic signals in the brain at D6 compared to D3.

Table 1. Gene expression changes (fold regulation) following dWMI induction

	D3 (P8)	dWMI
Symbol	dWMI vs. SHAM	D6 (P11) vs D3 (P8)
Ccl4	9.09	1.22
Cxcl10	6.47	5.21
Ccl3	3.74	-2.86
Cxcl3	3.62	1.51
Cxcl5	3.13	1.02
Cxcl1	-3.57	16.45

MSCs change their secretome in situ after treatment delay

To investigate changes in paracrine functioning of MSCs after delayed administration, we analyzed the gene expression profiles of MSCs exposed to brains of dWMI mice at D6 versus D3. We identified a difference in 42 MSC-expressed factors after exposure to the cerebral milieu at D6 versus D3 (table 2). The expression of trophic factors secreted by MSCs after D6 vs D3 brain extract exposure remained unchanged (Vaes et al., 2021). Interestingly, D6 brain extract exposure resulted in upregulation of pro-inflammatory cytokines and Bmps, including Tnf, IL1b, IL2 and Bmp2 in MSCs (table 2). Expression of 2 factors (i.e. IL1b and Ccl3) were confirmed by real-time RT-PCR on the individual samples (table S3).

Table 2. MSC secretome gene expression changes (fold regulation) after treatment delay

Symbol	D6 vs. D3	Symbol	D6 vs. D3
Adipoq	3.54	*Il9*	14.53
Bmp2	3.20	*Il10*	8.64
Bmp7	9.13	*Il12b*	13.52
Ccl19	5.07	*Il17a*	3.97
Ccl22	12.58	*Il17f*	7.44
Ccl24	17.64	*Il22*	4.33
Ccl3	4.41	*Il23a*	3.33
Ccl4	6.04	*Il24*	6.30
Csf2	3.42	*Mstn*	7.99
Cxcl13	12.79	*Nodal*	7.20
Cxcl3	3.08	*Ntf3*	3.94
Fasl	-6.64	*Osm*	309.49
Fgf13	4.72	*Tnf*	7.60
Ffg3	5.86	*Tnfrsf11b*	5.24
Fgf4	3.59	*Tnfsf10*	31.85
Fgf5	3.61	*Tdgf1*	12.51
Fgf8	3.42	*Xcl1*	10.41
Hc	3.07		
IfnA2	6.34		
IfnG	3.86		
Il1b	3.50		
Il2	13.65		
Il3	3.92		
Il4	4.12		
Il5	3.04		

Treatment delay limits MSC migration after intranasal administration

MSCs labeled with mesoporous silica-coated gold nanoparticles were used to study migration following intranasal administration at D3 and D6 after dWMI. Distribution of cells was measured by detection of gold signal in tissue homogenates using ICP-MS. Postponement of treatment to D6 tended to reduce (~50%) the total amount of gold found in the injured brain at 12 hours after administration, compared to D3 treatment (*p=0.065*) (figure 2A). In line with our previous findings at D3, the majority of gold was detected in the brain following intranasal delivery, with minimal loss in the liver, lungs or spleen (*p=0.075, p=0.074, p=0.035* brain compared to liver, lungs and spleen respectively) (figure 2B). Moreover, we observed dispersed distribution of cells throughout the diffusely injured brain (figure 2C).

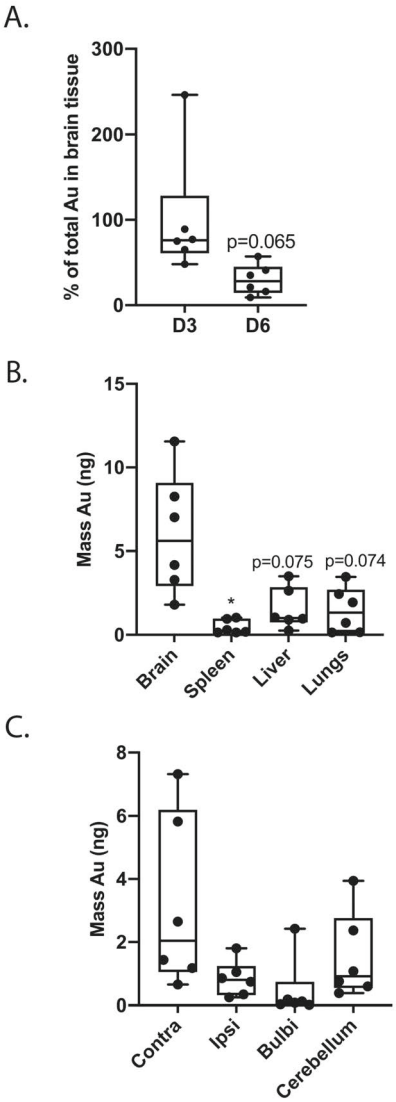


Figure 2. Delay of intranasal MSC treatment is associated with a reduction in migration of silica coated gold nanoparticle-labeled MSCs to the brain. **A.** A reduction (~50%) of the total amount of gold in the injured brain was observed after delay of intranasal MSC treatment to D6 vs D3 (MSC-D3 n=6, MSC-D6= n=6). **B.** Intranasal MSC administration at D6 is associated with minimal loss of cells in the liver, lungs of spleen as the majority of the gold nanoparticles were detected within the brain. **C.** The amount of gold measured in the brain was evenly distributed throughout the different parts of the injured brain. *: $p<0.05$; peripheral organs vs. brain. Nearly significant p values are indicated in A and B.

MSC modification leads to hypersecretion of selected factors

To optimize the MSCs' secretome, we genetically engineered MSCs to transiently overexpress a selected factor previously identified as beneficial for OL maturation and/or dampening of microglia activation (Vaes et al., 2021; Vaes et al., 2019). MSCs were transduced at different multiplicity of infections (MOIs) (see S4). Successful adenoviral vector infection of MSCs was confirmed visually using the eGFP signal (figure S1).

Modification of the MSC's secretome enhances myelination and prolongs the treatment window in vitro

To study the possible superiority of modified MSCs to boost OL maturation and subsequent myelin production, we cultured primary pre-OLs and challenged these with medium of LPS-stimulated microglia (MCM+LPS) in a non-contact coculture with MSCs. A 24-hour treatment interval (i.e. adding the MSC transwell inserts 24 hours after MCM+LPS) was used to mimic delay of MSC treatment in vivo. Pre-OLs exposed to MCM+LPS demonstrated a strong reduction in MBP+ area compared to pre-OLs exposed to MCM-LPS (dotted line) (*p=0.002*), indicating impaired maturation (figure 3A/B). Direct coculture with EV -MSCs partially restored MBP+ area in pre-OL cultures exposed to MCM+LPS (*p=0.002*) (figure 3A). The beneficial effect of EV-MSCs was identical to that of naïve, non-modified MSCs, as observed in our previous study (figure 3C) (Vaes et al., 2021). IGF1-, LIF- or IL11-MSCs demonstrated a superior treatment efficacy on MBP+ area when compared to EV-MSCs (*p=0.015, p=0.0089 and p=0.024 vs EV-MSCs* respectively) (figure 3A/B). Coculture with EGF-MSCs or IL-10-MSCs did significantly boost myelin production compared to empty gel inserts (*p= 0.0082 and p=0.041* vs empty gels respectively), but failed to significantly outperform EV-MSCs (*p=0.063 and p=0.275* vs EV-MSCs respectively) (figure 3A/B). When start of coculture was delayed for 24 hours, EV-MSCs were not able to improve myelin production by OLs after MCM+LPS. Furthermore, we observed an overall reduction in efficacy of modified MSCs, though IGF1- and IL10- MSCs were still able to significantly improve myelination compared to the empty gel inserts (IGF1 *p=0.044* and IL10 *p=0.020*) and to EV-MSCs (IL10 MSCs *p=0.032* and a trend for IGF1-MSCs (*p=0.066*) (figure 3D).

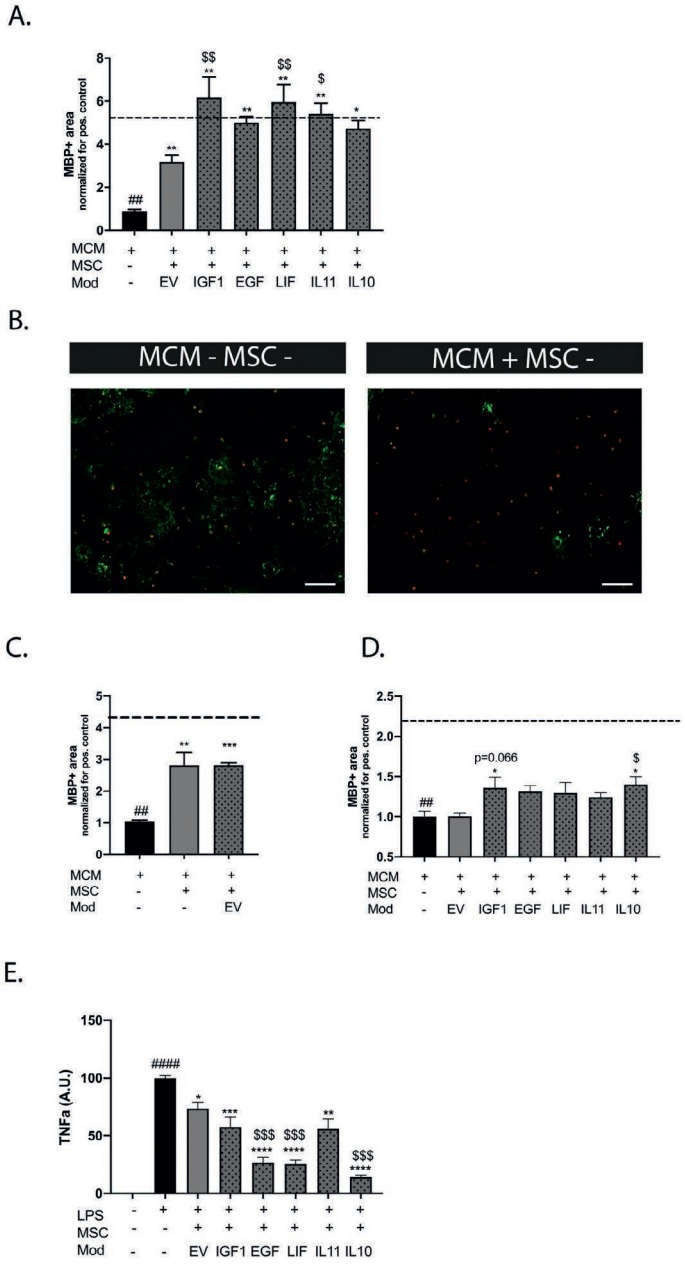


Figure 3. Modification of the MSC secretome enhances myelination in vitro **A.** MCM+LPS leads to a reduction of MBP+ area (dashed line represents MBP+ area in MCM-LPS control condition). Treatment with 4×104 EV-MSCs, EGF-MSCs or IL10-MSCs in a noncontact co-culture significantly enhances MBP expression. IGF1-MSCs, IL11-MSCs or LIF-MSCs display a superior ability to boost OL maturation compared to EV-MSCs (n=3 independent experiments, 3-4 observations per experiment, normalized for the positive control, e.g. cells exposed to

MCM+LPS, which was set at 1). Cocultures were started at the same time as MCM exposure. **B.** Representative fluorescent images (10x) of primary cultured oligodendrocytes stained for oligodendrocyte marker Olig2 (red) and myelin component MBP (green). Cells were exposed to MCM-LPS (MCM-) or MCM+LPS (MCM+) in a noncontact gel-insert. Scale bars: 100µm. **C.** Transfection of MSCs with EV did not affect the capacity to restore MBP+ area, compared to naïve, non-transfected MSCs (n= 2 independent experiments, 2 observations per experiment, normalized for the positive control, e.g. cells exposed to MCM+LPS, which was set at 1). **D.** A 24-hour delay in the start of coculture impairs treatment efficacy of EV-MSCs after in vitro maturation arrest of OLs (MCM+LPS). Coculture with IGF1- and IL10-overexpressing MSCs significantly improves MBP expression, however, with a lower efficacy compared to direct coculture (see **A**). Only IL10-MSCs significantly outperformed EV-MSCs in restoration of MBP+ area, IGF1-MSCs had a borderline significant superior effect (n=2 independent experiments, 3-4 observations per experiment, normalized for the positive control, e.g. cells exposed to MCM+LPS, which was set at 1). **E.** LPS stimulation evokes a strong increase in Tnf-α secretion by microglia. Treatment with 4×104 EV-MSCs, IGF1-MSCs or IL11-MSCs in a noncontact gel-insert partially attenuates microglial Tnf-α production. Noncontact coculture with EGF-MSCs, LIF-MSCs or IL10-MSCs leads to additional dampening of Tnf-α secretion compared to EV-MSCs (n=2 independent experiments, 2 observations per experiment, normalized for the positive control, e.g. cells exposed to LPS, which was put at 100). ##: p<0.01; ####: p<0.0001 MCM+ (black bars) vs MCM- control (dashed line (A/C/D) or versus no LPS in E); *: p< 0.05; **: p<0.01; ***: p<0.001 MSC conditions vs MCM+ control (black bar A/C/D) or LPS control (black bar E) $: p< 0.05; $$; p<0.01; $$$: p<0.001 modified MSC conditions (gray dotted bars) vs EV-MSC condition (gray bars). Nearly significant p values are indicated in **D.**

Modified MSCs display superior anti-inflammatory properties on microglia in vitro

To assess the direct effects of the secretome modification of MSCs on microglia activation, we exposed primary LPS-stimulated microglia to non-contact coculture with modified MSCs. LPS stimulation strongly increased Tnfα production by microglia compared to non-stimulated cells (p<0.0001) (figure 3E). Coculture with EV-MSCs significantly decreased Tnfα production indicating attenuation of neuroinflammation (p=0.011). The secretome of EGF- LIF- and IL10-MSCs displayed a superior dampening effect on Tnfα production by microglia (*p=0.0007*, *p=0.0006* and *p=0.0004* respectively versus EV-MSCs) (figure 3E). Coculture with IGF1- or IL11-MSCs significantly did not reduce Tnfα secretion to a superior level to EV-MSCs (*p=0.4932* and *p=0.4537* respectively; *p=0.0008* and *p=0.0011* respectively versus empty inserts).

To study the environmental changes provoked by coculture with modified MSCs, we measured the concentration of 31 different cytokines and chemokines in the microglia supernatants using Luminex. We observed distinct and specific micro-environmental changes in 13 factors when comparing exposure of microglia to EV-MSCs to the different types of modified MSCs (table S5, in bold).

Intranasal administration of modified MSCs prolongs the treatment window after dWMI

To investigate the therapeutic potential of modified MSCs after treatment delay, dWMI animals received modified MSCs intranasally at D6. Based on the *in vitro* findings on OL maturation, we selected IGF1-, LIF-, IL11- and IL10-overexpressing MSCs as the most promising candidates to prolong the treatment window. Complexity of myelin microstructure was assessed using segmentation analyses at P26 as described before (van Tilborg et al., 2017). EV-MSC treatment at D6 failed to significantly restore dWMI-induced reduction in fiber length and number of intersections (*p= 0.228* and *p=0.168* respectively), indicating persistent myelination failure after delayed MSC treatment as we observed before (figure 4A/B, (Vaes et al., 2021)). Interestingly, treatment with IGF1-, LIF- or IL11-MSCs at D6 significantly improved fiber length and number of intersections (IGF1-MSC: *p=0.024* and *p=0.003*, IL11-MSC: *p=0.041* and *p=0.047*, LIF-MSC: *p=0.011* and *p=0.0003,* versus vehicle) (figure 4A/B). Intranasal administration of IL10- MSCs did not significantly improve myelin microstructure (*p=0.908* and *p=0.483* versus vehicle).

In line with the histological findings and our earlier study (Vaes et al., 2021), intranasal EV-MSCs at D6 failed to significantly improve motor outcome at P26 (*p=0.7778*) (figure 4D). Treatment with IGF1-, IL11- or LIF-MSCs at D6 potently reduced forepaw preference (*p=0.*0002, *p=0.*0024 and *p=0.0013* respectively, versus vehicle) (figure 4D). Similar to our histological findings, IL10- MSCs did not improve motor performance (*p>0.999* versus vehicle). Taken together, these data indicate a superior therapeutic efficacy of IGF1-, IL11- and LIF- MSCs on dWMI after a delayed treatment onset.

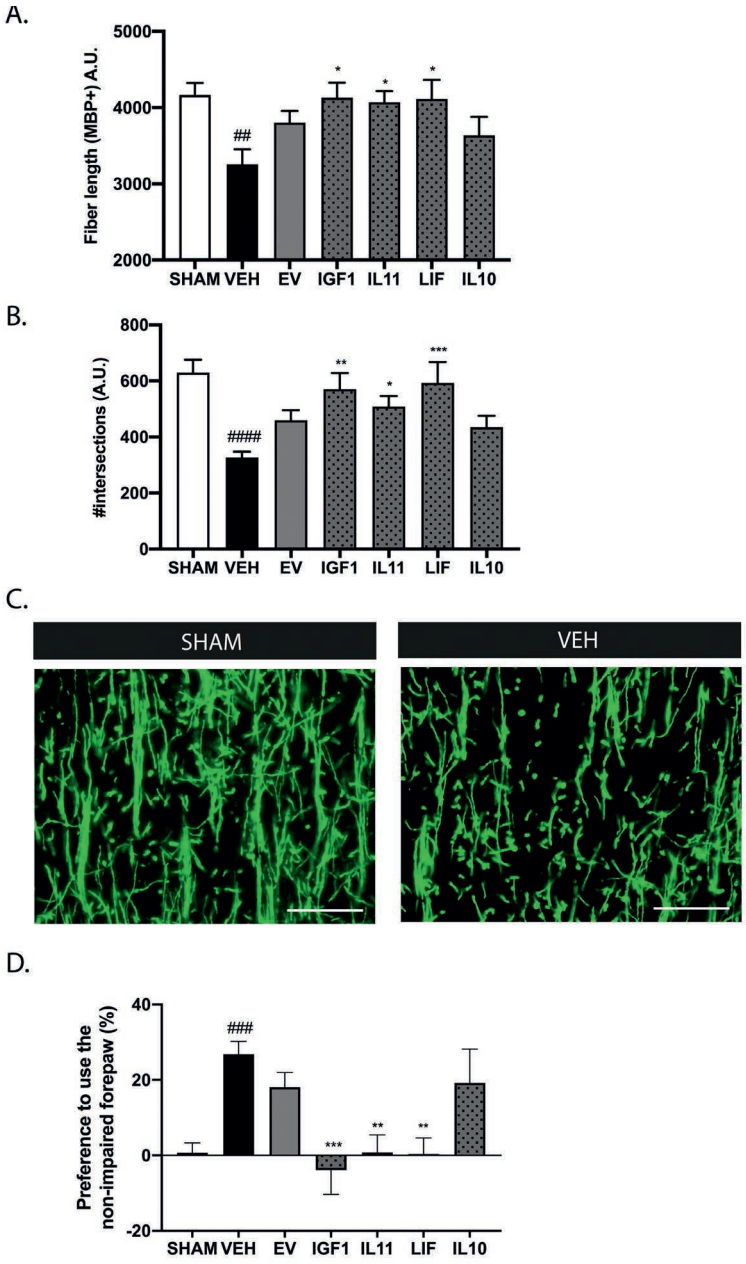


Figure 4. Intranasal administration of modified MSCs prolongs the treatment window in dWMI mice. **A/B.** Intranasal EV-MSC treatment at D6 fails to restore the dWMI-induced reduction in fiber length (**A**) and the number of intersections (**B**), as measures for myelin microstructure. Treatment with IGF1-, LIF- or IL11-MSCs significantly improved the microstructural myelin parameters (SHAM n=15, VEH n=15, EV- MSCs n=13, IGF1- MSCs n=9, IL11-MSCs n=9, LIF-MSCs n=13 and IL10=MSCs n=11). **C.** Representative fluorescent images (40x) of MBP+ axons in

the ipsilateral cortex of a sham-operated control mouse (upper) and dWMI mouse (lower). Scale bars: 50µm **D.** Intranasal administration of EV-MSCs at D6 after dWMI induction fails to reduce forepaw preference in the cylinder rearing test. Intranasally administered IGF1-, IL11- or LIF-MSCs significantly improved motor outcome at D6 (SHAM n=15, VEH n=15, EV- MSCs n=15, IGF1- MSCs n=10, IL11-MSCs n=9, LIF-MSCs n=10 and IL10=MSCs n=8). ##: p<0.01; ###: p<0.001; ####: p<0.0001 vehicle-treated dWMI animals vs sham-controls; *: p<0.05; **: p<0.01; ***: p<0.001; modified MSC-treated vs vehicle-treated animals.

IL10- and LIF-overexpressing MSCs attenuate microglia activation after delayed administration following dWMI

In line with previous findings in this model, we observed an increase in the number of Iba+ cells in the corpus callosum of vehicle-treated dWMI animals compared to sham-control animals at P26 (*p=0.033*) (Vaes et al., 2021). Treatment with EV- or modified MSCs did not significantly reduce Iba+ numbers, though we observed a trend with IL11-MSCs (*p=0.08*) (figure 5A/B). More detailed analyses of microglial morphology revealed an amoeboid (activated) phenotype in vehicle-treated dWMI animals, displayed by an increase in circularity and solidity (*p<0.0001 and p<0.0001 vs sham-control respectively*). Administration of IL10- and LIF- MSCs reduced cell circularity and solidity (circularity: *p=0.012* and *p=0.041* and solidity: *p=0.005* and *p=0.026* respectively), while other modified MSCs or EV-MSCs did not significantly reduce the activation state of microglia (figure 5C/D).

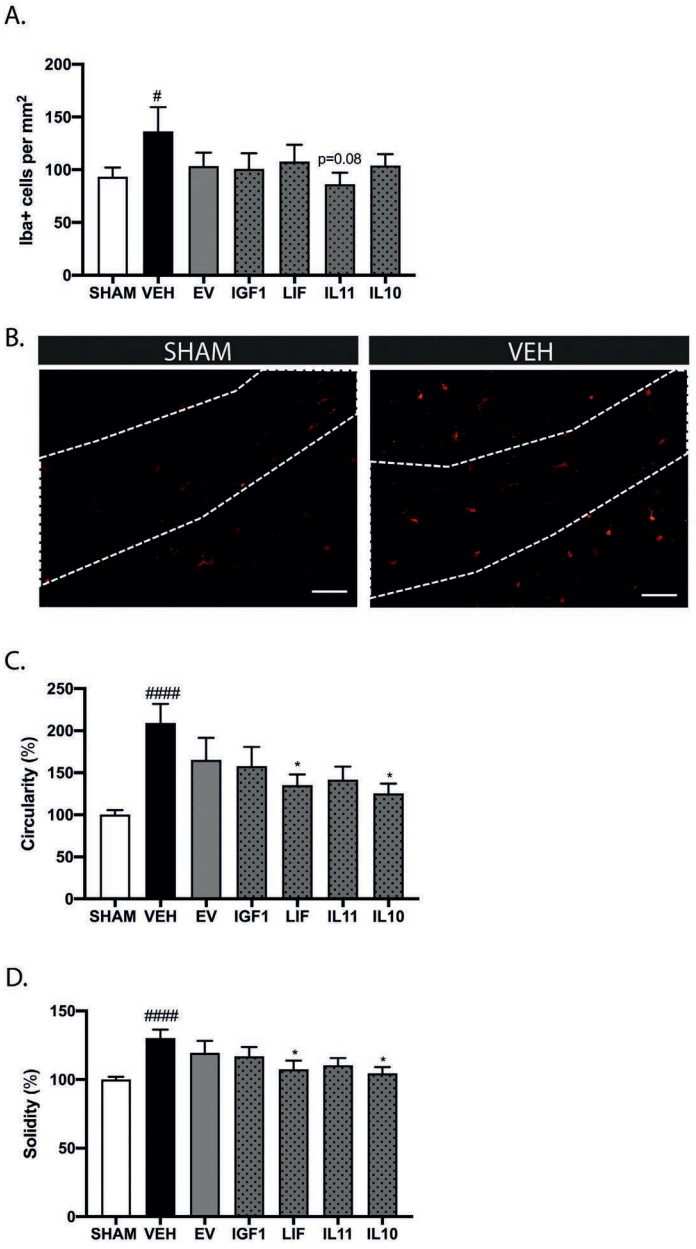


Figure 5. Modified MSCs attenuate microglia activation following delayed administration. **A.** Quantification of microglia cell numbers in the corpus callosum reveals a reduction of Iba+ cells (trend) after IL11-MSC treatment compared to vehicle treatment at D6. Treatment with EV-MSC or other modified MSCs at D6 did not affect microglia density in the injured brain (SHAM n=15, VEH n=14, EV- MSCs n=13, IGF1- MSCs n=10, IL11-MSCs n=9, LIF-MSCs n=12 and IL10-MSCs n=10). **B.** Representative fluorescent images (20x) of Iba+ cells in the corpus callosum (white

outline) in a sham-control (left) and vehicle-treated dWMI animal right). Scale bars: 100µm. **C/D.** Assessment of microglia circularity (**C**) and solidity (**D**), morphological parameters of the microglia activation state, shows a less pro-inflammatory phenotype following intranasal administration of LIF- or IL10-MSCs compared to vehicle treatment at D6 (SHAM n=14, VEH n=12, EV- MSCs n=9, IGF1- MSCs n=8, IL11-MSCs n=9, LIF-MSCs n=10 and IL10=MSCs n=11). #: p<0.05; ####: p<0.0001 vehicle-treated dWMI animals vs sham-controls; *: p<0.05; modified MSC-treated vs vehicle-treated dWMI animals. Nearly significant p values are indicated in A.

DISCUSSION

In this study we investigated the potential superior capacity of genetically modified MSCs (i.e. hypersecreting IGF1, EGF, LIF, IL10 or IL11) to prolong the treatment window for dWMI in newborn mice after intranasal application. We confirm here that the treatment window of intranasal MSCs in our mouse model of dWMI is limited, with a strong reduction in treatment efficacy when MSCs are administered at D6 versus D3 after dWMI. Though the cerebral chemotactic signals after dWMI appear to remain largely intact between D3 and D6, we show that migration of MSCs after intranasal administration is hampered at D6. Furthermore, we show that naïve MSCs exposed to D6 brain extract ex vivo respond with similar secreted GFs profiles but a more pro-inflammatory profile when compared to MSCs exposed to D3 brain extract. Moreover, *in vitro* assays using primary OL cultures reveal a limited potential of naïve MSCs to boost myelination after delayed coculture. Taken together, these results indicate that both impaired cell homing as well as a limited regenerative potential of naïve MSCs in the later stages of dWMI pathophysiology could underlie the observed loss of treatment efficacy. To optimize the treatment window of intranasal MSC treatment, MSCs were successfully modified to transiently overexpress IGF1, EGF, IL11, LIF or IL10. We report here a superior capacity of selected modified MSCs to directly boost OL maturation and attenuate microglia activation in vitro, with unique environmental changes provoked by the different modified MSC types. Moreover, we show that intranasal administration of IGF1-, LIF- or IL11- MSCs restore myelination and improves behavioral outcome when applied at D6 after dWMI. In addition, LIF- and IL10-MSCs dampen microglia activation after D6 treatment. Collectively, these data imply that modified MSC treatment is a potent strategy to prolong the treatment window in preterm dWMI, using cells with a superior regenerative potential to compensate for impaired cell migration and enduring injury.

A broad therapeutic window is essential for clinical translation of novel treatments for preterm infants, as pinpointing the exact timeframe in which dWMI develops is challenging. The pathophysiology of dWMI is believed to be multifactorial, with multiple (potentially) detrimental insults occurring in the perinatal and (early) postnatal period (van Tilborg et al., 2016; Volpe, 2009a). Currently, clinical diagnosis of dWMI is often based on neuro-imaging around term-equivalent age when myelination is progressing (de Vries et al., 2013). Moreover, validated biomarkers for early identification of preterm neonates at risk for developmental brain injury are lacking. Thus, while *early* administration of MSCs could be vital for optimal treatment efficacy, selecting patients that possibly benefit from MSC therapy could be difficult in an early phase. Therefore, prolongation of the therapeutic window by using modified MSCs might prove to be very relevant in this group of patients. Previous studies in the field of (neonatal) brain injury have reported a superior treatment efficacy of genetically engineered MSCs that (transiently) hypersecrete a beneficial factor versus naïve MSCs (Liu et al., 2010; Lu et al., 2009; van Velthoven et al., 2014). However, genetic engineering of cells is often met with some safety concerns. The adenoviruses used here do not integrate into the MSCs' DNA and thus induce only transient overexpression of the gene of interest (Park et al., 2018; Schäfer et al., 2016). Moreover, it is believed that intranasally administered MSCs are short-lived and do not integrate into the brain, but temporarily aid endogenous repair through paracrine signaling (van Velthoven et al., 2011). Pioneer clinical studies, using modified human MSCs in adult stroke did not report safety concerns (Steinberg et al., 2016). Additional preclinical studies assessing long-term outcome and conventional clinical safety studies are needed to confirm safety in preterm neonates.

Chemokine gradients in tissues are crucial to regulate migration of MSCs to sites of injury (Ullah et al., 2019). Moreover, in order for MSCs to exert their regenerative capacities, close proximity to the lesion site is suggested to be of importance (Liang et al., 2014; Oppliger et al., 2017; Paliwal et al., 2018). Here, we identified changes in expression of 6 chemokines associated with chemotaxis of MSCs or other cells at D3 after dWMI (Donega et al., 2014; Kalwitz et al., 2010; Sordi et al., 2005). When comparing the cerebral expression profiles of these chemokines at D6 versus D3, we observed an even further upregulation of Cxcl10 and C1 at D6, while expression of Ccl3, Ccl4, Cxcl3 and Cxcl5 remained unchanged. Importantly, murine MSCs have been reported previously to express the receptors for most of these ligands, i.e. CCR3, CCR5 and CXCR3 (Chamberlain et al., 2008). However, expression of CXCR2

was reported to be low in murine MSCs, implying a limited role for Cxcl1 in MSC chemotaxis (Chamberlain et al., 2008). Even though cerebral chemokine levels were stabile between D3 and D6, gold-labeled MSC tracing experiments did reveal a borderline significant reduction in the amount of MSCs reaching the brain at D6 versus D3 after dWMI. The observed discrepancy between the cerebral chemokine levels and cell tracing might be explained by several things. It is possible that changes in other chemoattractants important for MSC homing, ie.g. basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF1), platelet-derived growth factor (PDGF) and osteopontin, that were not included in our PCR array, play a role in impairing MSC migration (De Becker & Riet, 2016; Li & Jiang, 2011; Ullah et al., 2019). Aside from limitations in the chemotactic factors assessed in the array, it is possible that factors that did not reach our cut-off in up- or downregulation could have an essential role in MSC migration after dWMI. From the current data it is unclear if MSCs truly migrate to a lesser extent after intranasal administration at D6, leading to a smaller proportion of cells responsible for regeneration, or if the migration process is slowed down. In the latter case, the observed reduction in treatment efficacy at D6 might be explained by impaired regenerative capacity of an equal amount of MSCs arriving in later stages of dWMI pathophysiology.

As suggested above, an impaired regenerative potential of MSCs in later stages of OL injury could underlie the observed reduction in therapeutic efficacy after delayed MSC treatment. Previous studies in other (neonatal) brain pathologies report similar findings, with MSC therapy being most effective during acute inflammation and a reduction in efficacy after disease stabilization (Park et al., 2016; Zappia et al., 2005). We and others have shown that MSCs adapt their secretome based on the microenvironment of the target tissue, which indicates that insufficient endogenous production of inflammatory cytokines in situ at later timepoints after dWMI might not elicit essential secretome changes in MSCs (Park et al., 2016; Vaes et al., 2021; van Velthoven et al., 2010). In line with this hypothesis, we observed changes in the secretome of MSCs exposed to the D6 intracerebral milieu compared to D3, with largely unchanged expression of trophic factors but an upregulation of known OL differentiation-inhibiting factors, such as Tnfa, Bmp2, IL1b, IL2, IL17 and IL9 (Ding et al., 2015; van Tilborg et al., 2016).

To boost the potential of MSCs for delayed treatment, we modified the secretome. *In vitro* we show that IGF1-, LIF- and IL11-MSCs were superior in boosting OL maturation compared to control EV-MSCs. Similar to our *in vivo* findings, EV-MSCs did not significantly improve OL maturation after *in vitro* delay of MSC coculture. Interestingly, IL10- and IGF1- MSCs were able to (borderline) significantly boost OL maturation compared to control EV-MSCs after an initial delay in coculture. Apart from affecting OL maturation, we assessed the impact of modified MSCs on microglia activation, another key pathophysiological hallmark of dWMI. EGF-, LIF- and IL10- MSCs were shown to outperform EV-MSCs in attenuating microglial activation *in vitro*. Based on the combined *in vitro* findings, we selected IGF1-, IL11-, LIF- and IL10- MSCs for *in vivo* administration at D6. EGF-MSCs were excluded as these cells did not outperform EV-MSCs in boosting OL maturation at either time points of the culture. IGF1-, IL11- and LIF-MSCs were able to significantly improve myelination and functional outcome after administration at D6, thereby prolonging the treatment window. IL10-MSCs failed to significantly improve myelination and motor performance, however these cells were able to attenuate microglia activation at D6. These data imply that attenuation of neuroinflammation plays a less prominent role in dWMI repair in a later stage of injury. However, considering the effects of IL10-MSCs on OL maturation *in vitro*, it is also possible that the secreted levels of IL10 after modification of MSCs are insufficient for repair at later time points.

Our current data indicate a promising potential for modified MSC treatment in the (delayed) repair of dWMI. However, the exact mechanisms underlying the superior treatment efficacy after postponed treatment remain unclear. Investigation of microglial supernatant after coculture with different hypersecreting MSCs revealed specific composition of factors present in the culture medium per MSC type, implying that different modified MSCs could elicit unique environmental changes in situ. It is unclear if these changes are solely evoked by selective hypersecretion of the factor of interest secreted by MSCs. The observed net equal regenerative repair response after modified MSC treatment at D6 compared to repair by naïve MSCs at D3 could be the result of a superior, high dose of the overexpressed trophic factor as such or of a superior cocktail of (multiple) secreted beneficial factors evoked by autocrine actions, leading to increased OL maturation though a smaller amount of MSCs reached the lesion at D6. Aside from a superior regenerative capacity, improved migration of modified MSCs could also play a role in the observed therapeutic efficacy after delayed treatment. Multiple strategies for MSC priming (for example with hypoxic or

inflammatory stimuli) and modification have been shown to influence cell migration with an upregulation of receptors vital for MSC migration (De Becker & Riet, 2016; Ullah et al., 2019).

CONCLUSION

To summarize, this study shows that the therapeutic window of intranasal MSC therapy in a mouse model of preterm dWMI is relatively limited. The observed reduction in treatment efficacy likely results from impaired migration of MSCs towards the brain and a limited regenerative capacity in later stages of dWMI. Modified MSCs, transiently hypersecreting IGF1, IL11 and LIF, possess a superior capacity to boost white matter development after dWMI and thereby extent the therapeutic window.

Acknowledgements

We would like to thank Caren van Kammen, Rebecca Kleisen, Rik van Vliet, Roeland Lokhorst and Diederick Slange for their (bio)technical support.

Funding

This work was funded by Brain Foundation the Netherlands and the Gravitation Program “Materials Driven Regeneration”, funded by the Netherlands Organization for Scientific Research (024.003.013) and the Dutch Province of Limburg (LINK project).

REFERENCES

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathologica, 134*(3), 331-349. doi:10.1007/s00401-017-1718-6

Chamberlain, G., Wright, K., Rot, A., Ashton, B., & Middleton, J. (2008). Murine Mesenchymal Stem Cells Exhibit a Restricted Repertoire of Functional Chemokine Receptors: Comparison with Human. *PLoS One, 3*(8), e2934. doi:10.1371/journal.pone.0002934

Chen, Y., Balasubramaniyan, V., Peng, J., Hurlock, E. C., Tallquist, M., Li, J., & Lu, Q. R. (2007). Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc, 2*(5), 1044-1051. doi:10.1038/nprot.2007.149

De Becker, A., & Riet, I. V. (2016). Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy? *World J Stem Cells, 8*(3), 73-87. doi:10.4252/wjsc.v8.i3.73

de Vries, L. S., Benders, M. J. N. L., & Groenendaal, F. (2013). Imaging the premature brain: ultrasound or MRI? *Neuroradiology, 55*(2), 13-22. doi:10.1007/s00234-013-1233-y

Ding, X., Cao, F., Cui, L., Ciric, B., Zhang, G. X., & Rostami, A. (2015). IL-9 signaling affects central nervous system resident cells during inflammatory stimuli. *Exp Mol Pathol, 99*(3), 570-574. doi:10.1016/j.yexmp.2015.07.010

Donega, V., Nijboer, C. H., Braccioli, L., Slaper-Cortenbach, I., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2014). Intranasal administration of human MSC for ischemic brain injury in the mouse: in vitro and in vivo neuroregenerative functions. *PLoS One, 9*(11), e112339-e112339. doi:10.1371/journal.pone.0112339

Douglas-Escobar, M., & Weiss, M. D. (2012). Biomarkers of brain injury in the premature infant. *Front Neurol, 3*, 185. doi:10.3389/fneur.2012.00185

Kalwitz, G., Andreas, K., Endres, M., Neumann, K., Notter, M., Ringe, J., . . . Kaps, C. (2010). Chemokine Profile of Human Serum from Whole Blood: Migratory Effects of CXCL-10 and CXCL-11 on Human Mesenchymal Stem Cells. *Connective Tissue Research, 51*(2), 113-122. doi:10.3109/03008200903111906

Kassis, I., Vaknin-Dembinsky, A., & Karussis, D. (2011). Bone marrow mesenchymal stem cells: agents of immunomodulation and neuroprotection. *Curr Stem Cell Res Ther, 6*(1), 63-68. doi:10.2174/157488811794480762

Li, L., & Jiang, J. (2011). Regulatory factors of mesenchymal stem cell migration into injured tissues and their signal transduction mechanisms. *Frontiers of Medicine, 5*(1), 33-39. doi:10.1007/s11684-011-0114-1

Liang, X., Ding, Y., Zhang, Y., Tse, H. F., & Lian, Q. (2014). Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant, 23*(9), 1045-1059. doi:10.3727/096368913×667709

Liu, A. M., Lu, G., Tsang, K. S., Li, G., Wu, Y., Huang, Z. S., . . . Poon, W. S. (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*(2), 357-365; discussion 365-356. doi:10.1227/01.neu.0000371983.06278.b3

Lu, Z., Hu, X., Zhu, C., Wang, D., Zheng, X., & Liu, Q. (2009). Overexpression of CNTF in Mesenchymal Stem Cells reduces demyelination and induces clinical recovery in experimental autoimmune encephalomyelitis mice. *J Neuroimmunol, 206*(1-2), 58-69. doi:10.1016/j.jneuroim.2008.10.014

Ment, L. R., Hirtz D Fau - Huppi, P. S., & Huppi, P. S. (2009). Imaging biomarkers of outcome in the developing preterm brain. *Lancet Neurol, 8*(1474-4465 (Electronic)), 1042-1055. doi:10.1016/S1474-4422(09)70257-1

Oppliger, B., Joerger-Messerli, M., Mueller, M., Reinhart, U., Schneider, P., Surbek, D. V., & Schoeberlein, A. (2016). Intranasal Delivery of Umbilical Cord-Derived Mesenchymal Stem Cells Preserves Myelination in Perinatal Brain Damage. *Stem Cells and Development, 25*(16), 1234-1242. doi:10.1089/scd.2016.0027

Oppliger, B., Joerger-Messerli, M. S., Simillion, C., Mueller, M., Surbek, D. V., & 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*(7), 829-838. doi:10.1016/j.jcyt.2017.03.075

Paliwal, S., Chaudhuri, R., Agrawal, A., & Mohanty, S. (2018). Regenerative abilities of mesenchymal stem cells through mitochondrial transfer. *J Biomed Sci, 25*(1), 31. doi:10.1186/s12929-018-0429-1

Park, W. S., Ahn, S. Y., Sung, S. I., Ahn, J. Y., & Chang, Y. S. (2018). Strategies to enhance paracrine potency of transplanted mesenchymal stem cells in intractable neonatal disorders. *Pediatr Res, 83*(1-2), 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., . . . Chang, Y. S. (2016). Optimal Timing of Mesenchymal Stem Cell Therapy for Neonatal Intraventricular Hemorrhage. *Cell Transplant, 25*(6), 1131-1144. doi:10.3727/096368915×689640

Paton, M. C. B., Allison, B. J., Li, J., Fahey, M. C., Sutherland, A. E., Nitsos, I., . . . Miller, S. L. (2018). Human Umbilical Cord Blood Therapy Protects Cerebral White Matter from Systemic LPS Exposure in Preterm Fetal Sheep. *Dev Neurosci, 40*(3), 258-270. doi:10.1159/000490943

Schäfer, R., Spohn, G., & Baer, P. C. (2016). Mesenchymal Stem/Stromal Cells in Regenerative Medicine: Can Preconditioning Strategies Improve Therapeutic Efficacy. *Transfusion Medicine and Hemotherapy, 43*(4), 256-267. doi:10.1159/000447458

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods, 9*(7), 671-675. doi:10.1038/nmeth.2089

Sordi, V., Malosio, M. L., Marchesi, F., Mercalli, A., Melzi, R., Giordano, T., . . . Piemonti, L. (2005). Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood, 106*(2), 419-427. doi:10.1182/blood-2004-09-3507

Steinberg, G. K., Kondziolka, D., Wechsler, L. R., Lunsford, L. D., Coburn, M. L., Billigen, J. B., . . . Schwartz, N. E. (2016). Clinical Outcomes of Transplanted Modified Bone Marrow-Derived Mesenchymal Stem Cells in Stroke: A Phase 1/2a Study. *Stroke, 47*(7), 1817-1824. doi:10.1161/strokeaha.116.012995

Ullah, M., Liu, D. D., & Thakor, A. S. (2019). Mesenchymal Stromal Cell Homing: Mechanisms and Strategies for Improvement. *iScience, 15*, 421-438. doi:10.1016/j.isci.2019.05.004

Vaes, J. E. G., van Kammen, C. M., Trayford, C., van der Toorn, A., Ruhwedel, T., Benders, M., . . . Nijboer, C. H. (2021). Intranasal mesenchymal stem cell therapy to boost myelination after encephalopathy of prematurity. *Glia, 69*(3), 655-680. doi:10.1002/glia.23919

Vaes, J. E. G., Vink, M. A., de Theije, C. G. M., Hoebeek, F. E., Benders, M., & Nijboer, C. H. A. (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

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., & Nijboer, C. H. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Progress in Neurobiology, 136*(1873-5118 (Electronic)), 28-49. doi:10.1016/j.pneurobio.2015.11.002

van Tilborg, E., van Kammen, C. M., de Theije, C. G. M., van Meer, M. P. A., Dijkhuizen, R. M., & Nijboer, C. H. (2017). A quantitative method for microstructural analysis of myelinated axons in the injured rodent brain. *Sci Rep, 7*(1), 16492-16492. doi:10.1038/s41598-017-16797-1

van Velthoven, C. T., Braccioli, L., Willemen, H. L., Kavelaars, A., & Heijnen, C. J. (2014). Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage. *Mol Ther, 22*(3), 645-654. doi:10.1038/mt.2013.260

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain, Behavior and Immunity, 24*(3), 387-393. doi:10.1016/j.bbi.2009.10.017

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2011). Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. *Brain, Behavoir and Immunity, 25*(7), 1342-1348. doi:10.1016/j.bbi.2011.03.021

Volpe, J. J. (2009a). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurology, 8*(1), 110-124. doi:10.1016/s1474-4422(08)70294-1

Volpe, J. J. (2009b). The encephalopathy of prematurity--brain injury and impaired brain development inextricably intertwined. *Seminars in Pediatric Neurology, 16*(4), 167-178. doi:10.1016/j.spen.2009.09.005

Volpe, J. J., Kinney, H. C., Jensen, F. E., & Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *International Journal of Developmental Neuroscience, 29*(4), 423-440. doi:10.1016/j.ijdevneu.2011.02.012

Zanier, E. R., Fumagalli, S., Perego, C., Pischiutta, F., & De Simoni, M.-G. (2015). Shape descriptors of the "never resting" microglia in three different acute brain injury models in mice. *Intensive care medicine experimental, 3*(1), 39-39. doi:10.1186/s40635-015-0039-0

Zappia, E., Casazza, S., Pedemonte, E., Benvenuto, F., Bonanni, I., Gerdoni, E., . . . Uccelli, A. (2005). Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood, 106*(5), 1755-1761. doi:10.1182/blood-2005-04-1496

SUPPORTING INFORMATION

Supporting Materials and Methods

MSC culture

GIBCO® mouse (C57BL/6) bone marrow-derived MSCs (Invitrogen, S1502-100; Carlsbad, California, USA) were cultured in D-MEM/F-12 medium (10565-018, Invitrogen) with 10% fetal bovine serum (12662-029, Invitrogen) according to the supplier's protocol. In all experiments, MSCs were passaged once (from P2 to P3) prior to *in vivo* administration or in *in vitro* experiments.

MSC transfection

MSCs were modified to transiently overexpress growth factor or cytokines with ready-to-use recombinant adenoviral vectors encoding a murine IGF1, EGF, LIF, IL11 or IL10 transgene, combined with a control eGFP vector to assess infection efficacy (Vector Biolabs, Malvern, US). Transgenes were placed under control of a CMV promotor, including a separate promotor for eGFP. To stimulate cell entry, an Arg-Gly-Asp (RGD) motif was included, due to poor basal expression of the coxsackievirus-adenovirus receptor (CAR) in MSCs. 24 hours prior to viral particle exposure, MSCs were plated at 2.0×10^5 cells per well in 6-wells plates to allow cell adherence. The following day, MSCs were exposed to viral particles diluted in MSC culture medium for 6 hours. Subsequently, wells were washed with culture medium and recultured for 24 hours followed by *in vitro* gel embedment or *in vivo* administration. Transfection was visually confirmed by evaluation of the eGFP signal. The optimal multiplicity of infection (viral particles needed per cell; MOI) was determined per adenovirus, by assessment of secreted protein concentrations by MSCs in supernatants using ELISA (at 2 days after infection). All ELISAs were performed according to manufacturer's protocol (mouse IGF1: MG100, R&D systems; mouse EGF: EMEGF, Invitrogen; mouse LIF: ABIN5526767, antibodies online; mouse IL11: RAB0251, Sigma; mouse IL10: ABIN2114255, antibodies online).

In vivo model of diffuse white matter injury

All procedures were carried out according to the Dutch and European guidelines (Directive 86/609, ETS 123, Annex II) and were approved by the Experimental Animal Committee Utrecht (Utrecht University, Utrecht, Netherlands) and the Central Authority for Scientific Procedures on Animals (the Hague, the Netherlands). Diffuse white matter injury was induced as described previously (Vaes et al., 2020). In short,

hypoxia-ischemia (HI) was induced in postnatal day 5 (P5) C57BL/6j mouse pups by permanent unilateral occlusion of the right common carotid artery and exposure to hypoxia (6% O_2) for 35 minutes under temperature-controlled conditions. Directly after hypoxia, pups received an intraperitoneal (i.p.) injection with 1mg/kg LPS (List Biological Laboratories, Campbell, CA) dissolved in 0.9% NaCl. Sham-control littermates underwent surgical incision only, without carotid artery occlusion, nor hypoxia or LPS injection. Prior to MSC administration at P8 (i.e. D3 after dWMI induction) or P11 (i.e. D6) nasal mucosa permeability was improved by administration of 2 dosages of 2µl Hyaluronidase (12.5 U/µl in total, Sigma-Aldrich, St. louis, MO) dissolved in H_2O in each nostril (total of 8 µl). Thirty minutes after hyaluronidase treatment, 0.5×10^6 MSCs were administered intranasally in dPBS (Thermo-fisher, 14190-169, Waltham, MA) in 2 dosages of 2µl in each nostril (total of 8µl). Previous dose-response experiments identified 0.5×10^6 MSCs as the lowest effective dose (Vaes et al., 2020). Vehicle-treated dWMI animals received 8µl dPBS (2 dosages of 2µl in each nostril). Mice were euthanized at P8 (i.e. 3 days), P11 (i.e. 6 days) or P26 (i.e. 3 weeks) by an i.p. overdose pentobarbital. For PCR arrays sham-control or dWMI (untreated) brains were collected, cerebellum was discarded and hemispheres were separately snap-frozen in liquid nitrogen and stored at -80°C until further processing.

Cerebral chemokine expression profiles

For cerebral chemokine expression analysis sham-control and dWMI (untreated) brains were collected at two time points after dWMI induction, P8 (i.e. 3 days) and P11 (i.e. 6 days). The ipsilateral hemisphere of each brain was crushed using a mortar and pestle chilled on liquid nitrogen. Brain tissue was lysated in RLT lysis buffer using a TissueLyser LT Adapter and stainless-steel beads (all Qiagen, Hilden, DE), at 50Hz during 2 minutes according to manufacturer's protocol. RNA was isolated using the RNeasy minikit (Qiagen), including on-column DNase digestion with the RNase-free DNase set (79254, Qiagen). RNA quantity and quality were assessed by spectrophotometry (NanoDrop 2000, Thermo-scientific, Waltham, MS) at 260mm and OD 260/280 ratio. cDNA transcription was carried out using the RT2 first strand kit (Qiagen), following manufacturer's protocol. cDNA of sham-control or dWMI animals were pooled per time point (D3 n=5 and D6 n=4 per experimental condition) and the expression of 84 chemokine and cytokine-related genes were assessed in a commercially-available PCR array (PAMM-150Z, Qiagen). PCR arrays were carried out following suppliers' protocol, using the RT2 Real-Time SYBR green PCR Master Mix (Qiagen) on a Biorad MyIQ. PCR array data were normalized using multiple house-

keeping genes incorporated per array, and analyzed by comparing 2-ΔCt using software provided by Qiagen. Chemokine gene expression changes were calculated: 1) in dWMI mice relative to sham-control mice at P8 (i.e. 3 days) to identify chemokines that are differentially regulated following injury induction and 2) in dWMI mice at P11 (i.e. 6 days) versus dWMI mice at P8 (i.e. 3 days) to study the stability of the chemotactic signals over time. A fold regulation threshold of 3.0 was considered as either down- or upregulation. PCR array results were validated by quantitative PCR analyses in the individual cDNA samples for selected genes (CXL10 and Ccl3). Real time RT-PCR was carried out using the QuantStudio 3 (Applied Biosystems) with SYBR select master mix (Applied Biosystems). Primer sequences can be found in table S1. Mean expression of GADPH and β-actin were used for data normalization.

MSC gene expression profiles after exposure to brain extracts

To evaluate the response of the MSC secretome to the cerebral milieu at two time points after dWMI induction, dWMI brains were collected at D3 (n=5) and D6 (n=4). The ipsilateral hemispheres were crushed on liquid nitrogen using a mortar and pestle. After weighing of the tissue pieces, tissue of both time points was pooled and homogenized at 150 mg/ml in knock-out DMEM (Thermo Fisher, 10829018) containing a protease inhibitor cocktail (1:50 dilution; Invitrogen) using a potter tissue homogenizer (10 strokes), followed by centrifugation for 10 minutes at 10,000 x g at 4°C. The supernatant 'brain extract' was collected and the protein concentration assessed using a protein assay (Biorad) with BSA as standard. Brain extracts were aliquoted and kept at -80C until use. MSCs were cultured and seeded at 2.0×105 cells per well (6-wells plate) in standard MSC medium. After allowing the cells to adhere during 24 hours, culture medium was replaced with knock-out DMEM containing either D3 or D6 dWMI brain extract at a concentration of 1mg protein/ml. Wells were washed with ice-cold PBS 48 hours after addition of the brain extract-enriched medium. MSC RNA was isolated using the RNeasy minikit (Qiagen). RNA quantity and quality were assessed by spectrophotometry (NanoDrop 2000, Thermo-scientific, Waltham, MS) at 260mm and OD 260/280 ratio was determined to evaluate quality. Transcription of RNA to cDNA was performed using the RT2 first strand synthesis kit (Qiagen) according to manufacturer's instructions. The expression profiles of 168 growth factor- and cytokine- related genes were assessed by commercially available PCR arrays (Qiagen; PAMM-041Z and PAMM-150Z). PCR array analysis was performed once following manufacturer's protocol with the RT2 Real-Time SYBR green PCR Master Mix (Qiagen) on QuantStudio 3 (Applied Biosystems). PCR data were normal-

ized using multiple housekeeping genes provided within the PCR array and analyzed by comparing 2-ΔCt using Qiagen software. Gene expression changes in MSCs exposed to D6 dWMI brain extracts were calculated relative to MSCs exposed to brain extracts obtained at D3 after injury induction. A fold regulation threshold of 3.0 was considered as either down- or upregulation. As described in the previous section, PCR arrays results were validated by quantitative PCR analyses in the individual cDNA samples for selected genes (IL1b and Ccl3). Primer sequences can be found in table S1. Mean expression of GADPH and β-actin were used for data normalization.

MSC tracing

MSC labeling

MSCs were labelled using gold core-mesoporous and lipid-coated silica nanoparticles (AuNP-MSN-LIP). A detailed description of nanoparticle synthesis, characterization and labeling efficiency can be found in our previous paper (Vaes et al., 2020). In short, 2 hours after cell passaging, MSCs were incubated with 25µg/ml AuNP-MSN-LIP in culture medium over 48 hours. Following cell labeling, dWMI animals received intranasal treatment with 0.5×10^6 MSCs in PBS at P8 or at P11.

Cell tracing in mouse tissue

Mice were sacrificed by overdose pentobarbital following by decapitation at 12 hours after intranasal MSC treatment. The brains were collected, divided in ipsilateral and contralateral hemispheres, cerebellum and olfactory bulbs and frozen separately in liquid nitrogen. Additionally, the spleens, lungs and livers were dissected and frozen in liquid nitrogen to study loss of cells in peripheral organs. Inductively coupled plasma mass spectrometry (ICP-MS) was used to quantitatively assess MSC biodistribution by detection of gold in mouse tissue homogenates, as described previously (Vaes et al., 2020). In summary, snap frozen tissue sections were weighed, lyophilized overnight and reweighed. Freshly prepared aqua regia (HCl 30% and HNO3 60%, VWR) was added to each tissue section for w/v; 1mg/50µL. Samples were disintegrated overnight at 40°C using an ultrasonic bath (Bransonic®, Thermo Scientific) and further homogenized by microwaving (5×30 seconds, 600W). When solutions were transparent, all tissue samples were diluted 1:10 in freshly prepared matrix solution (1% HNO3 functionalized with 20ppb of ruthenium (VWR)). In addition, a gold standard curve ranging from 1ng L-1 to 100µg L-1 was made by diluting gold stock solution (VWR) in the prepared matrix. An iCAP™ RQ ICP-MS (Thermo Scientific) was used to

measure ICP-MS. Due to its high molecular weight of 197 other ions will not interfere with measured gold content. The detection limit of gold was 1ng/L.

Immunohistochemistry

Animals were sacrificed by overdose pentobarbital followed by transcardial perfusion with PBS and 4% PFA in PBS at P26 (i.e. 3 weeks). Brains were post-fixed during 24 hours in 4% PFA followed by a dehydration series. Brains were then embedded in paraffin and coronal sections (8µm) were cut at hippocampal level (-1.80mm from bregma in adult mice). For 3,3'-Diaminobenzidine (DAB) staining, sections were deparaffinized in xylene followed by 100% ethanol. Endogenous peroxidase was blocked by incubation in 3% H2O2/methanol and sections were hydrated using decreasing concentrations of ethanol. Sections were blocked with 20% normal rabbit serum in PBS/0.1% Tween followed by overnight incubation with rat-anti-MBP (MAB386, Merck Millipore; 1:500) in 10% normal rabbit serum in PBS/0.1% Tween. The next day, sections were washed in PBS and incubated with biotinylated rabbit-anti-rat (BA-4000, Vector laboratories, 1:400), followed by PBS washes. Biotin was HRP-labeled using a vectastain ABC kit (Vector laboratories) according to the supplier's protocol, followed by 0.05M Tris-HCl (pH: 7.6) washing. Subsequently, sections were stained with 0.5mg/ml DAB (Sigma) in 0.05M Tris-HCl with 0.03% H_2O_2. Sections were washed in H2O, dehydrated in increasing ethanol concentrations and embedded with depex. For immunofluorescent stainings, sections were deparaffinized in xylene, followed by rehydration in decreasing concentrations of ethanol. Sections were heated to 95°C in sodium citrate buffer (0.01M, pH 6) for antigen retrieval. After cooling down and washing in PBS (+0.1%Tween20 for MBP/NF200), sections were blocked with 10% normal goat serum in PBS+0.1% Tween20 for MBP/NF200 or 2% bovine serum albumin (BSA)/0.1% saponin in PBS for Iba1 staining and incubated overnight with rat-anti-MBP (MAB386, Merck Millipore; 1:500), rabbit-anti-NF200 (N-4142, Sigma; 1:400) and with rabbit-anti-Iba1 (019-19741, Wako; 1:500). The next day, sections were washed in PBS and incubated with alexafluor-594 and -488 conjugated secondary antibodies (Life technologies, Carlsbad, CA; 1:200-500) for 1-2 hours at room temperature, followed by DAPI (1:5000) counterstaining and embedment in Fluorsave (Merck Millipore, 345789).

Microscopy and image analysis

Investigators were blinded for experimental conditions during image acquisition and analysis. In MBP-DAB-stained sections a 2.5x magnification was used to image the

ipsilateral hemisphere using a light microscope (Zeiss, Oberkochen, Germany) with an AxioCam ICc 5 camera (Zeiss). For immunofluorescent stainings, a Cell Observer microscope with an AxioCam MRm camera (Zeiss, Oberkochen, Germany) was used to acquire images in the ipsilateral hemisphere. For MBP/NF200 stainings, 3 adjacent 40x micrographs were taken at a fixed distance of the external capsule into the cortex (for exact locations we refer to (van Tilborg et al., 2017)). For Iba1 stainings, two 20x images were acquired in the corpus callosum of the ipsilateral hemisphere.

In MBP-DAB stainings, cortical myelination (2.5x) was quantified as described by van Tilborg et al. (2017). In addition, microstructural integrity of myelinated axons (40x) was analyzed in MBP/NF200 stained sections as described in van Tilborg et al. (2017). Morphology of microglia residing in the corpus callosum was assessed using the particle analysis function of ImageJ v.1.47 (Schneider et al., 2012) as described by (Zanier et al., 2015). In these analyses, microglia were manually selected followed by morphological measurements. Values of all acquired images were averaged per animal.

Behavioral assessment

Motor performance was evaluated using the cylinder rearing test (CRT) at P26. Animals were placed in a transparent cylinder (80mm diameter and 300mm height) and videotaped during at least 3 minutes. A minimum of 10 full weight-bearing rearings were recorded per animal, mice that did not meet this criterium within the timeframe were retested approximately 30 minutes later. Forepaw preference was calculated as ((non-impaired – impaired)/(non-impaired + impaired + both))x100%. All CRTs were videotaped and scored by researchers blinded to the experimental conditions.

In vitro models of dWMI

Primary rat glial cultures

A mixed glial culture was acquired from P1-2 Sprague Dawley rat pup cortices, as described by Chen et al. (2007), with small changes. In summary, brains were isolated, cortices were dissected and the meninges were removed. Subsequently, cortices were pooled and minced, followed by dissociation using DNase I (10ug/l, Sigma Aldrich, D5025) and Trypsin (0.01%, Sigma Aldrich, T1426) solution in HBSS. Tissue was collected and dissociated by pipetting. The suspension was filtered through a 0.70µm filter twice. Cells were plated in poly-D-lysine-coated (0.1mg/ml, Sigma Aldrich, P6407) T75 culture flasks. For details on the media used, we kindly refer

to the original paper by Chen et al. (2007). After a minimum of 10DIV, microglia and oligodendrocyte precursor cells (OPCs) were collected by mechanical detachment on an orbital shaker (see below for details). A second harvest was performed after a minimum of 7 addition DIV.

To mimic the *in vivo* inflammatory situation to induce maturation arrest in immature oligodendrocytes, microglia-conditioned medium (MCM) was produced. To detach microglia, culture flasks were shaken during 1 hour at 200rpm at 37°C. Culture medium containing the microglia was centrifuged (10 minutes, 1200rpm, RT) and microglia were counted. Microglia were plated at 0.5×106 cells per well in poly-L-ornithine (Sigma Aldrich, P3655)-coated 24 wells plates. At 24 hours after plating, culture medium was replaced by Basal Defined Medium (BDM) with- or without 50ng/ml LPS (Sigma, L4616) for 24 hours at 37°C. MCM-LPS and MCM+LPS was collected, filter sterilized (0.20µm) and stored at -80°C upon use.

After shaking to remove the microglia, culture flasks were refilled with fresh medium and shaken for an additional 20 hours at 200 rpm at 37°C to isolate OPCs. The cell suspension was collected and passed through a 20µm sterile screening pouch (Merck Millipore, NY2004700, Burlington, MS) to avoid potential contamination with microglia and astrocytes. OPCs were collected by centrifugation (100g during 10 minutes RT) and plated at 4.0×10E4 cells/well on poly-D,L-ornithine (Sigma Aldrich, P0421)-coated 24-wells plates in OPC medium (BDM with PDGF-aa (Peprotech, 100-13A) and bFGF (Peprotech, 100-18B)). OPC medium was changed every other day. For the OPC differentiation assay, see below at cocultures.

Primary mouse microglia culture

A primary microglia culture was prepared from P1 C57BL/6 mice cortices for cocultures. In short, cortices were dissected and meninges were removed. The tissue was minced and incubated with 0.25% trypsin (Sigma, T4799) in Gey's balanced salt solution (GBSS) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 mM D(+)-glucose during 15 minutes. The tissue suspension was dissociated by pipetting until homogenous and thereafter cultured in poly-L-ornithine-coated(Sigma, P3655) T75 flasks in DMEM/HamF10 (1:1) (Gibco, 41965-039 and 31550-023) supplemented with 10% FCS, 2mM glutamine and antibiotics (see above). At DIV10-12 culture flasks were put on an orbital shaker during 20-22 hours (130-135rpm, 37°C) to detach microglia. Subsequently, microglia were collected by centrifugation (1200 rpm during

10 minutes RT), counted and seeded in poly-L-ornithine-coated 24-wells plates at a density of 1.5×10^5 cells per well. Cocultures (see below) were started 24 hours later. After shaking the T75 flasks, new culture medium was added to each flask to allow a second microglia harvest after an additional 7-10 DIV.

Non-contact MSC-glia cocultures

To assess the effect of MSC modification on myelin production by OLs or activation of microglia, overexpressing-MSCs were embedded in gel inserts for a non-contact coculture between MSCs and primary OPCs or microglia respectively. In this coculture system, the effects of MSCs are mediated by their secretome, as MSCs in the gel insert are not able to have direct cell-contact with the primary cells in the lower well. Moreover, it allows both cell types to remain viable in their own culture medium. At 24 hours prior to the start of cocultures (and 24 hours after MSC transfection) 4.0×10^4 modified MSCs (MSC-eGFP (control), MSC-IGF1, MSC-EGF, MSC-LIF, MSC-IL10 and MSC-IL11) were embedded in Hydromatrix gel (Sigma, A6982) transwell inserts (Merck Millipore, MCHT24H48) according to supplier's protocol.

For the OL differentiation experiment, OPC medium containing PDGF-AA and bFGF was replaced with either MCM+LPS or MCM-LPS when the majority of OLs displayed an immature pre-OL morphology (i.e. 4 days after OPC plating). Pro-differentiation factors: NAC (5µg/ml Sigma, A8199), CNTF (10ng/ml Peprotech, 450-50) and T3 (122ug/ml Sigma, T2752) were added to MCM+LPS or MCM-LPS to start differentiation of OPCs. Transwell inserts containing modified MSCs (MSC-eGFP (control, EV), MSC-IGF1, MSC-EGF, MSC-LIF, MSC-IL10 and MSC-IL11) or no MSCs as an empty insert control, were added to the wells directly or 24 hours (delayed) after induction of differentiation. Inserts were removed and OLs were fixated 72 hours after addition of MCM, with 4% PFA in PBS during 10 minutes.

At 24 hours after plating, co-cultures of MSCs and mouse microglia were started by adding 50ng/ml LPS (Sigma, L4515) to the microglia and putting transwell inserts containing modified MSCs (MSC-eGFP, MSC-IGF1, MSC-EGF, MSC-LIF, MSC-IL10 and MSC-IL11) to the wells. After 48 hours of coculture, the inserts were removed and the microglia supernatant was collected, aliquoted and stored at -80C° for ELISA.

ELISA

TNFα concentrations in the supernatant of microglia were measured using an ELISA kit for murine TNFα (Ucytech, Utrecht, The Netherlands) according to manufacturer's protocol. TNFα data of different experiments were normalized to positive control conditions (i.e. 50ng/ml LPS and empty insert without MSCs).

Luminex assay

The concentrations of 31 cytokines/chemokines in pooled microglia supernatant (n=3 per condition) were measured using a bioplex pro mouse chemokine assay (12009159, Biorad) according to the supplier's protocol. All concentrations were normalized to the EV-MSC condition (i.e. 50ng/ml LPS and an insert with EV-MSCs).

Immunocytochemistry of primary oligodendrocyte cultures

After fixation and washing in PBS, 2% BSA and 0.1% saponin in PBS was added to the wells to block nonspecific binding. Wells were incubated with primary antibodies (rabbit-anti-Olig2, AB9610. Merck Millipore; 1:1000, mouse-anti-MBP, SMI-94, Biolegend, 1:1000) overnight at 4°C. The following day, wells were washed with PBS, followed by incubation with alexafluor-594 and -488 conjugated secondary antibodies (Life technologies; 1:1000) for 1 hour at room temperature. Hoechst 33342 (Sigma) was used for nuclear counterstaining and wells were embedded in Fluorsave (Merck Millipore, 345789).

Six adjacent fields were imaged (10x), starting at a fixed distance of the well edges. The number of Olig2- and Hoechst- positive cells were counted using the analyze particles function in ImageJ v.1.47. The area of MBP+ staining was measured using manual thresholding analyses in ImageJ. In order to compare independent experiments, all results were normalized for the positive control (MCM+LPS; empty insert without MSCs).

Statistics

All data are shown as mean ± standard error of the mean (SEM). Statistics were performed using Graphpad Prism 8.3. Unpaired t-tests were used for comparison of two groups, or in case of unequal variances, non-parametric mann-whitney tests. For comparison of >2 groups, one-way ANOVA with Bonferroni posthoc tests were executed. In the event of unequal variances, a non-parametric Kruskal-Wallis test with Dunn's posthoc correction was used for comparison of multiple groups. p-values <0.05 were considered statistically significant. Sample sizes are mentioned in the figure captions.

Table S1. Overview of primer sequences used in validation qPCR

Symbol	Forward primer sequence	Reverse primer sequence
Cxcl10	*GCTGCCGTCATTTTCTGC*	*TCTCACTGGCCCGTCATC*
Ccl3	*CTGCCCTTGCTGTTCTTCTCTG*	*CGATGAATTGGCGTGGAATCTTC*
IL1b	*CAACCAACAAGTGATATTCTCCATG*	*GATCCACACTCTCCAGCTGCA*
GAPDH	*TGAAGCAGGCATCTGAGGG*	*CGAAGGTGGAAGAGTGGGAG*
β-actin	*AGAGGGAAATCGTGCGTGAC*	*CAATAGTGATGACCTGGCCGT*

Table S2. Validation chemokine expression changes

RefSeq	Gene symbol	2^ΔCt SHAM D3	dWMI D3	Fold Regulation validation
NM_021274	Cxcl10	0,0000595	0,000362167	6,08
NM_011337.2	Ccl3	0,000143404	0,00037147	2,59

Table S3. Validation MSC secretome expression changes

RefSeq	Gene symbol	2^ΔCt MSC D3	MSC D6	Fold Regulation validation
NM_008361	IL1b	0,000005414	0,0000152402	2,814970144
NM_011337.2	Ccl3	0,000015817	0,0000271900	1,719075577

Table S4. Optimal multiplicity of infection (MOI) for MSC transduction

Factor	Condition	MOI	Mean concentration
IGF1 (pg/ml)	Control (no virus)	-	30,1
	EV-MSC	2000	23,9
	IGF1-MSC	500	111,9
		1000	352,1
		2000	408
EGF (pg/ml)	Control (no virus)	-	0
	EV-MSC	4000	0
	EGF-MSC	2000	2,7
		4000	9,5
IL11 (ng/ml)	Control (no virus)	-	0
	EV-MSC	4000	79,5
	IL11-MSC	2000	231,9
		4000	414
LIF (pg/ml)	Control (no virus)	-	2,5
	EV-MSC	8000	11,5
	LIF-MSC	4000	278,7
		8000	445,6
IL10 (pg/ml)	Control (no virus)	-	0
	EV-MSC	4000	420,5
	IL10-MSC	1000	2295
		2000	2634
		4000	3725

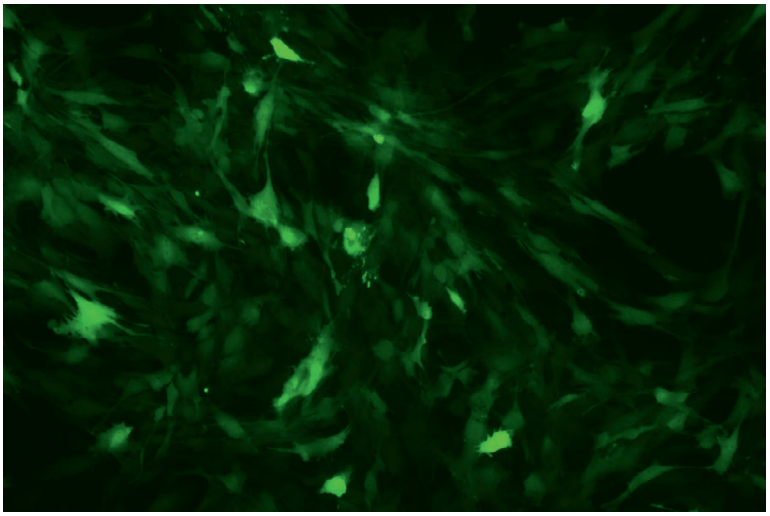


Figure S1. Representative fluorescent image (10x) of MSCs transfected with control adenoviral vector containing an eGFP transgene at a multiplicity of infection (MOI) of 4000 pfu/cell during 6 hours.

Table S5. Relative changes in cyto- and chemokine concentrations in the microglial supernatant measured using Luminex

	Cxcl13	Ccl27	Cxcl5	Ccl11	Ccl24	Cx3Cl1	GMCSF
LPS – MSC -	0.57	0.49	**0.04**	0.66	**0.17**	**0.54**	**0.09**
LPS + MSC -	0.84	0,92	**0.06**	0.96	**0.76**	**0.76**	**0.26**
LPS + EV-MSC	1	1	**1**	1	**1**	**1**	**1**
LPS + IGF1-MSC	0.95	0.88	**0.98**	1	**0.30**	**0.96**	**0.84**
LPS + EGF-MSC	1.01	0.95	**1.48**	1.03	**0.68**	**1.34**	**1.96**
LPS + LIF-MSC	0.93	0.92	**1.29**	1.11	**0.98**	**1.15**	**1.53**
LPS + IL11-MSC	1.03	0.90	**2.90**	0.99	**0.70**	**1.28**	**1.98**
LPS + IL10-MSC	1.06	0.90	**3.11**	1.02	**0.69**	**1.29**	**1.69**
	Cxcl11	**Cxcl1**	**Ccl2**	**Ccl7**	**Ccl12**	**Ccl22**	**Ccl3**
LPS – MSC -	0.48	**0.01**	**0.04**	**0.04**	**0.06**	0.57	0.05
LPS + MSC -	0.94	**0.45**	**0.61**	**0.53**	**0.89**	0.94	1.05
LPS + EV-MSC	1	**1**	**1**	**1**	**1**	1	1
LPS + IGF1-MSC	1.04	**0.84**	**0.87**	**0.83**	**0.91**	1.05	1.03
LPS + EGF-MSC	1.09	**1.42**	**1.19**	**1.18**	**0.86**	1.03	1.07
LPS + LIF-MSC	1.01	**1.18**	**1.03**	**0.95**	**0.76**	1.28	0.99
LPS + IL11-MSC	1.08	**1.60**	**1.34**	**1.37**	**0.99**	1.16	1.04
LPS + IL10-MSC	1.06	**1.43**	**1.13**	**1.21**	**1.21**	0.94	0.91

Ccl1	Ifnγ	IL1β	IL2	IL4	IL6	IL10	IL16	Cxcl10
0.66	0.56	0.31	0.45	0.56	**0**	**0.41**	0.77	**0.19**
0.89	1.01	0.84	0.88	0.96	**0.30**	**0.94**	0.92	**0.96**
1	1	1	1	1	**1**	**1**	1	**1**
1	1	0.92	1.10	1	**0.74**	**1.04**	1.08	**1.03**
0.99	0.94	1.06	1.08	1.04	**1.90**	**0.99**	1.13	**2.20**
0.95	1.04	0.93	0.99	0.97	**1.26**	**0.91**	1.38	**2.35**
0.91	1.03	0.99	0.98	1.06	**1.44**	**1.02**	1.11	**1.19**
0.96	1.07	0.87	1.10	1.02	**1.12**	**13.81**	1.11	**1.22**

Ccl4	Ccl20	Ccl19	Ccl5	Cxcl16	Cxcl12	Ccl17	Tnfα
0.16	0.47	0.46	0.01	**0.40**	0.75	0.75	**0.01**
1.03	0.94	0.93	1.21	**0.75**	0.92	0.97	**1.88**
1	1	1	1	**1**	1	1	**1**
1.03	1.22	1.03	0.98	**0.76**	0.96	1.01	**0.80**
1.03	1.09	1	0.99	**0.92**	1.02	1.02	**0.72**
0.97	1.08	0.94	1.07	**0.85**	1	1.04	**0.77**
1.08	1.12	1	1.07	**0.73**	1.02	1.04	**0.83**
0.91	1.31	0.99	0.89	**0.69**	1.01	1.04	**0.45**

REFERENCES SUPPLEMENTARY INFORMATION

Chen, Y., Balasubramaniyan, V., Peng, J., Hurlock, E. C., Tallquist, M., Li, J., & Lu, Q. R. (2007). Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc, 2*(5), 1044-1051. doi:10.1038/nprot.2007.149

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods, 9*(7), 671-675. doi:10.1038/nmeth.2089

Vaes, J. E. G., van Kammen, C. M., Trayford, C., van der Toorn, A., Ruhwedel, T., Benders, M. J. N. L., . . . Nijboer, C. H. (2020). Intranasal mesenchymal stem cell therapy to boost myelination after encephalopathy of prematurity. *Glia, n/a*(n/a). doi:10.1002/glia.23919

van Tilborg, E., van Kammen, C. M., de Theije, C. G. M., van Meer, M. P. A., Dijkhuizen, R. M., & Nijboer, C. H. (2017). A quantitative method for microstructural analysis of myelinated axons in the injured rodent brain. *Sci Rep, 7*(1), 16492-16492. doi:10.1038/s41598-017-16797-1

Zanier, E. R., Fumagalli, S., Perego, C., Pischiutta, F., & De Simoni, M.-G. (2015). Shape descriptors of the "never resting" microglia in three different acute brain injury models in mice. *Intensive care medicine experimental, 3*(1), 39-39. doi:10.1186/s40635-015-0039-0

7

Insulin-like growth factor 1 therapy to restore hypomyelination in encephalopathy of prematurity

J.E.G. Vaes, L.M. Baak*, C.M. van Kammen*, F. Groenendaal, M.J.N.L Benders, C.H. Nijboer

**Both authors contributed equally to this manuscript and share the second authorship*

Submitted

ABSTRACT

Neurodevelopmental disorders caused by Encephalopathy of Prematurity (EoP) are a major health issue after (extreme) preterm birth. A key hallmark of EoP is diffuse white matter injury (dWMI), resulting from impaired maturation of oligodendrocytes (OLs) leading to developmental myelination failure. At present, clinically-approved therapies to combat dWMI are not available. Insulin-like growth factor I (IGF1), an endogenous growth factor vital for normal white matter development, is shown to be largely lacking following extreme preterm birth. Here, we studied changes in endogenous IGF1 levels in a validated double-hit neonatal mouse model of dWMI and investigated the therapeutic potential of intranasally administered IGF1. Induction of dWMI by postnatal hypoxia-ischemia and systemic inflammation led to a transient reduction in systemic and cerebral IGF1 and IGFBP3 levels, while IGFBP2 production and IGF receptor expression were unaffected. Intranasally administered IGF1 rapidly diffused throughout the injured brain and potently improved myelination and functional outcome after dWMI. We show that improved myelination is preceded by direct IGF1-stimulated OL maturation. Moreover, IGF1 treatment dampened astrocyte activity but not microglia activation after dWMI. *In vitro,* we show that primary microglia and astrocyte cultures differentially express the IGF1 receptor leading to different responsivity to IGF1. In conclusion, experimental dWMI is associated with a transient systemic and cerebral IGF1 and IGFBP3 reduction, in line with the human preterm neonate. Intranasal IGF1 administration is a potent new strategy to restore myelination in a mouse model of dWMI. IGF1 aids in white matter development by directly boosting OL differentiation following maturation arrest.

INTRODUCTION

Encephalopathy of Prematurity (EoP), a descriptive term that encompasses an array of neurological abnormalities frequently observed after (extreme) preterm birth, is a major cause of life-long neurodevelopmental disorders in preterm neonates (Larroque et al., 2008; Volpe, 2009). Diffuse white matter injury (dWMI), characterized by diffuse hypomyelination of the brain in absence of necrotic cysts, is the most commonly observed type of brain injury after (extreme) preterm birth at present (Back, 2017; Back & Miller, 2014; Volpe, 2017). Preterm birth-related insults, such as inflammation and fluctuations in cerebral oxygenation, are thought to disturb key developmental processes in a critical window of oligodendrocyte (OL) lineage maturation, resulting in OL maturation arrest and insufficient myelination (van Tilborg et al., 2016; Volpe et al., 2011). Although dWMI is associated with considerable neurodevelopmental disorders, at the present time only supportive (intensive) care is available for prematurely born infants. Novel therapeutic strategies that support white matter development after (extreme) preterm birth are therefore urgently needed.

Insulin-like growth factor I (IGF1), a growth factor produced in virtually all tissues, has been reported to play a prominent role in normal brain development and regeneration after injury, including that of the white matter (Beck et al., 1995; Guan et al., 2001; Hellström et al., 2016; Masters et al., 1991; Ye et al., 1995). More specifically, previous studies have demonstrated that exogenous IGF1 treatment, either administered locally (intracerebroventricularly) or intranasally in other experimental models of neonatal brain injury in rodents and sheep could boost proliferation, differentiation and survival of the OL lineage and subsequent myelin production (Brywe et al., 2005; Cai et al., 2011; Cao et al., 2003; Lin et al., 2005). Interestingly, clinical studies have identified a reduction in plasma IGF1 levels in the first postnatal weeks following (extreme) preterm birth when compared to in utero blood levels of IGF1 at similar post-menstrual ages (Hansen-Pupp et al., 2007; Hellstrom et al., 2016). This relative deficiency of IGF1 during a time window critical for white matter development has been associated with a poorer neurodevelopmental outcome and volumetric deficits in multiple brain structures (Hansen-Pupp et al., 2011; Hansen-Pupp et al., 2013). It is unclear whether this transient IGF1 deficiency is also observed in clinically-relevant experimental models of dWMI.

The current study investigates the regulation of endogenous production of IGF1 and its binding proteins (IGFBPs) after preterm-birth related insults using a validated neonatal mouse model combining two clinically-relevant hits, i.e. postnatal hypoxia-ischemia and systemic inflammation, leading to dWMI, the most commonly observed form of brain injury in preterm infants (Vaes et al., 2021). Moreover, we use this dWMI mouse model to explore the therapeutic potential of different intranasally administered IGF1 regimes, with and without addition of IGFBP3, to restore anatomical dWMI and its associated functional deficits. Finally, we investigate the underlying mechanisms of IGF1-mediated repair after dWMI, by using primary glia cell cultures to assess the cell-specific effects of IGF1 on OL lineage maturation and neuroinflammation.

MATERIALS AND METHODS

dWMI mouse model

All experimental procedures were performed according to the Dutch and European guidelines (Directive 86/609, ETS 123, Annex II) and were consented by the Experimental Animal Committee Utrecht (Utrecht University, Utrecht, Netherlands) and the Central Authority for Scientific Procedures on Animals (the Hague, the Netherlands). All animals were kept under standard housing conditions with food and water available *ad libitum*, a 12-hour light/dark cycle and in a temperature-controlled environment. Males and females were included in all described experiments and randomly assigned to experimental groups with an equal distribution across groups. Induction of diffuse white matter injury was performed as described previously by our group (Vaes et al., 2021). In summary, on postnatal day 5 (P5) C57BL/6j mouse pups were subjected to hypoxia-ischemia (HI) by permanent unilateral ligation of the right common carotid artery and exposed to hypoxia (6% O_2) during 35 minutes in a temperature-controlled environment. Immediately after the hypoxic hit, mouse pups were injected with 1mg/kg LPS (intraperitoneal; i.p.) (List Biological Laboratories, Campbell, CA) dissolved in 0.9% NaCl. Sham-control littermates received surgical incision exclusively, without occlusion of the carotid artery, nor hypoxia or LPS injection.

Endogenous IGF1 kinetics *in vivo*

To study the effect of dWMI on cerebral IGF1 receptor (IGFR) expression and endogenous IGF1 production, dWMI and sham-control mice were sacrificed by an overdose of pentobarbital i.p. at 6, 24 and 72 hours after injury induction. Peripheral blood samples were drawn from the right atria directly following euthanization and collected in

Minicollect tubes containing lithium heparin (450537, GreinerBioOne). Blood samples were kept on ice for a maximum of 30 minutes until centrifugation (20 minutes at 2000g at 4°C). The upper layer of plasma was collected and stored at -80°C upon use. Directly after collection of blood samples, animals were transcardially perfused using ice-cold PBS, followed by decapitation. Brains were rapidly collected, cerebellum was removed, ipsi- and contralateral hemispheres were separated and divided in anterior and posterior parts. All samples were frozen separately in liquid nitrogen and stored at -80°C upon use. The ipsilateral posterior cerebral pieces were used for further analysis of endogenous cerebral IGF1 levels as we previously identified this part of the brain affected by dWMI (Vaes et al., 2021). The ipsilateral posterior parts were crushed on liquid nitrogen using a mortar and pestle. Brain tissue was weighed, followed by homogenization of a portion of the tissue in RIPA buffer (6 ul/mg tissue; 10 mM PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 20% SDS and 10ul/ml protease inhibitor cocktail (P8340, Sigma)) using a Tissuelyser LT (Qiagen, Hilden, DE) with pre-cooled stainless-steel beads (69989, Qiagen) during 2 minutes at 50Hz for ELISA. Subsequently, homogenized samples were centrifuged at 15.000g during 20 minutes at 4°C. Cell lysate was collected after two cycles of centrifugation followed by determination of protein concentration using a protein assay (Biorad) with BSA as standard. The concentration of IGF1, IGFBP2 and IGFBP3 was measured in both brain lysate and plasma using ELISA kits for murine IGF1 (MG100, R&D systems), IGFBP2 (ab207615, Abcam) and IGFBP3 (MGB300, R&D systems) according to manufacturers' protocols. The remaining crushed brain tissue was lysated in RLT lysis buffer using the TissueLyser LT Adapter (Qiagen, Hilden, DE) and pre-cooled stainless-steel beads (69989, Qiagen) at 50Hz during 2 minutes. RNA isolation was carried out using the RNeasy minikit (Qiagen), including on-column DNase digestion with the RNase-free DNase set (79254, Qiagen). RNA quantity and quality was assessed by spectrophotometry (NanoDrop 2000, Thermo-scientific, Waltham, MS) at 260mm and OD 260/280 ratio. cDNA transcription was performed using the RT2 first strand kit (Qiagen), following manufacturer's protocol. Real time RT-PCR for IGFR gene expression was carried out using the QuantStudio 3 (Applied Biosystems, Foster City, CA) using SYBR select master mix (4472903, Applied Biosystems). The following primer sequences were used: IGFR FW: *GGAATGAAGTCTGGCTCCGG* REV: *CGATCACCGTGCAGTTTTCC*, GAPDH FW: *TGAAGCAGGCATCTGAGGG* REV: *CGAAGGTGGAAGAGTGGGAG*, β-actin FW: *AGAGGGAAATCGTGCGTGAC* REV: *CAATAGTGATGACCTGGCCGT*. Mean expression of GAPDH and β-actin were used for normalization of the data.

Insulin-like growth factor 1 administration

Mouse pups were treated intranasally with IGF1 during 6 consecutive days, starting either directly after induction of dWMI (i.e. P5-P10) or at P7 (i.e. P7-P12). To assess the therapeutic efficacy of a short treatment protocol, a subset of animals received intranasal IGF1 from P5 to P8. Mouse pups received a daily dosage of recombinant human IGF1 (100-11, Peprotech) (different dosages; 10, 25 or 50µg) or a combination of 10 or 25 µg human recombinant IGF1 with equimolar IGFBP3 addition (100-11/100-08, Peprotech), dissolved in sterile 0.9% NaCl, in 2 nose droplets of 2µl in each nostril (total of 8µl). Vehicle-treated animals received similar intranasal volumes of 0.9% NaCl.

Cerebral IGF1 distribution

To confirm cerebral IGF1 delivery and distribution following intranasal application, dWMI mice were euthanized by an i.p. overdose of pentobarbital 30 minutes after intranasal administration of the first dose of 25µg IGF1 or vehicle- treatment. The 30 minutes timepoint to study IGF1 delivery was chosen based on other studies (Cai et al., 2011; Thorne et al., 2004). Brains were collected, cerebellum was snap-frozen separately, hemispheres were separated and cut into anterior and posterior parts and separately snap-frozen in liquid nitrogen. All brain parts were stored at -80°C until further processing. Brain tissue lysates were prepared as described for measurements of endogenous IGF1 levels described above. The concentration of exogenous human recombinant IGF1 in the mouse brain was measured using an ELISA kit for human IGF1 (DG100, R&D systems) according to the supplier's protocol.

Immunohistochemistry

Animals were sacrificed by an overdose pentobarbital i.p. followed by transcardial perfusion with PBS and 4% PFA/PBS at P19 or P26 (i.e., 2 weeks or 3 weeks after dWMI induction). The brains were post-fixed for 24 hours in 4% PFA, followed by dehydration in increasing concentrations of ethanol. Subsequently, brains were embedded in paraffin and cut in coronal sections (8µm) at hippocampal level (-1.80mm from bregma in adult mice). Hippocampal size was assessed using hematoxylin and eosin (HE) staining. For fluorescent stainings, sections were deparaffinized in xylene, followed by rehydration in decreasing concentrations of ethanol. For antigen retrieval, sections were heated to 95°C in sodium citrate buffer (0.01M, pH 6). Sections were washed in PBS (+0.1%Tween20 for MBP/NF200) after cooling down, and blocked with 5-10% normal goat serum in PBS+0.1% Tween20 for MBP/NF200 and CC1/Olig2 or 2% bovine serum albumin (BSA)+0.1% saponin in PBS for Iba1/GFAP staining and

incubated overnight with rat-anti-MBP (MAB386, Merck Millipore; 1:500), rabbit-anti-NF200 (N-4142, Sigma; 1:400), mouse-anti-CC1 (OP80, Calbiochem; 1:300), rabbit-anti-Olig2 (ab9610, Chemicon; 1:500), rabbit-anti-Iba1 (019-19741, Wako; 1:500) and/or with mouse-anti-GFAP (BM2278, Origine; 1:200). The following day, sections were washed in PBS and incubated with alexafluor-594 and -488 conjugated secondary antibodies (Life technologies, Carlsbad, CA; 1:200-500) for 1-2 hours at room temperature, followed by DAPI (1:5000) nuclear counterstaining and embedment in Fluorsave (Merck Millipore, 345789).

Microscopy and image analysis

For image acquisition and analysis the investigators were blinded for experimental conditions. For HE-stained sections a 2,5x magnification was used to visualize both hemispheres using a light microscope (Zeiss, Oberkochen, Germany) with an AxioCam ICc 5 camera (Zeiss). Hippocampal areas in both hemipsheres were measured on HE-stained sections using Zen software (Zeiss). The loss of ipsilateral hippocampal area was expressed as the ratio of ipsilateral to contralateral hippocampal areas.

For immunofluorescent stainings, micrographs of both hemispheres were acquired using a Cell Observer microscope with an AxioCam MRm camera (Zeiss, Oberkochen, Germany). For MBP/NF200 stainings, 2.5x images were taken of the cortex. Subsequently, 3 adjacent 40x images were made at a fixed distance from the external capsule in the cortex (layer III/IV), superjacent to the hippocampus. For exact locations we refer to van Tilborg et al. (2017). In MBP/NF200 stained sections, the extent of cortical myelination and microstructural integrity of myelinated axons was measured as described (van Tilborg et al., 2017). Values of all acquired images were averaged for each animal.

For Iba/GFAP and CC1/Olig2 stainings, two 20x images were taken in the corpus callosum. An additional 20x image of the CA1 region of the hippocampus was obtained in GFAP stainings. Moreover, for CC1/Olig2 staining two 20x micrographs were obtained in the cortex. Morphology of microglia in the corpus callosum was assessed using the particle analysis function of ImageJ v1.47 (Schneider et al., 2012) as described previously by Zanier et al. (2015). In this analysis, microglia were selected manually, followed by measurement of morphological description parameters. All morphological microglia parameters were normalized for sham-control values. GFAP (20x) threshold analyses to measure the positive area of staining in the corpus

callosum, were carried out using ImageJ software. All GFAP$^+$ area calculations were normalized for sham-control values. Cell counts of microglia (Iba1) and OL lineage (CC1/Olig2) markers were performed manually using Zeiss software (Axiovision and Zen; Zeiss, Oberkochen, Germany) and corrected for measured area. In this analysis, cells with a clear DAPI$^+$ nucleus in combination with clear Iba1$^+$ or CC1$^+$/Olig2$^+$ staining were counted. In the event of excessive background staining or large artefacts, images were excluded.

Behavioral assessments

The behavioral paradigms were videotaped and scored by researchers blinded to the experimental conditions. All setups were cleaned with soapy water and ethanol between runs to eliminate smell.

Cylinder rearing test

Motor impairment was assessed at P26 using the cylinder rearing test (CRT). Mice were put in a transparent cylinder (80mm diameter and 300mm height) and videotaped for at least three minutes. A minimum of 10 full weight-bearing rearings against the cylinder walls were recorded per animal. Animals that failed to perform 10 rearings within the timeframe were retested approximately 30 minutes later. The usage of the forepaws for initiation of the rearing was scored as left (impaired), right (non-impaired) or both. Preference to use the non-impaired forepaw was calculated as ((non-impaired – impaired)/(non-impaired + impaired + both))x100%.

Delayed spontaneous alternation in T-maze

Deficits in executive memory were assessed using by measuring correct alternating behavior in a T-maze at P24-P26. Each trial was comprised of two runs, a sample and a choice run, in a T-shaped maze. For the sample run, mice were put in the starting arm of the T-maze. The investigator waited (max 2 minutes) for the mouse to enter one of the two goal arms, followed by closing of the chosen arm, thereby constraining the animal in the chosen arm for 30 seconds. Animals were then returned to the home cage for two minutes, after which they were placed back into the starting arm for the choice run. A correct alternation was scored when the animal would enter the unexplored arm in the choice run, whilst entering the previously explored arm was scored as an incorrect alternation. All animals performed two trails per day, during three consecutive days (i.e. a total of 6 trials). Percentage of correct alternations was

calculated as (the total number of choice runs choosing the alternate/unexplored arm of the 6 total trials) x100%.

In vitro model of dWMI

Primary oligodendrocyte culture (rat)

A mixed glia culture was prepared from P1-2 Sprague Dawley rat pup cortices, as described previously by Chen et al. (2007) with minor modifications. In summary, after isolation of brains and dissection of the cortices, the meninges were removed. All cortices were pooled, minced and dissociated using DNase I (10µg/l, D5025, Sigma Aldrich) and Trypsin solution (0.01%, T1426, Sigma Aldrich) in HBSS. Subsequently, tissue suspensions were dissociated by pipetting and filtered through a 0.70µm filter twice. Poly-D-Lysine-coated (0.1mg/ml, P6407, Sigma Aldrich) T75 culture flasks were used for cell plating. We refer to the original paper by Chen et al. (2007) for details on the media used. Oligodendrocyte precursor cells (OPCs) were harvested by mechanical shaking of the flasks on an orbital shaker after a minimum of 10 days-*in-vitro* (DIV). Culture flaks were kept for a remaining 7 DIV for a second OPC harvest. After initial removal of microglia by a 1 hour shake at 200 rpm at 37°C, flasks were shaken during 20 hours at 200 rpm at 37°C to isolate OPCs. The cell suspension was passed through a 20µm sterile screening pouch (Merck Millipore, NY2004700, Burlington, MS) for removal of possible remaining/detached microglia and astrocytes. Subsequently, after spinning down (100g for 10 minutes at RT), OPCs were plated at 4.0×10^4 cells/well on poly-D,L-ornithine (P0421, Sigma Aldrich)-coated 24-wells plates in OPC medium (basal defined medium (BDM) with PDGFα (100-13A, Peprotech) and bFGF (100-18B, Peprotech)). Cells were fed with a complete OPC medium change every other day. Four days after OPC plating, when cells displayed a pre-OL morphology (i.e. round cell body with multiple extensions), differentiation was started by replacing the OPC medium containing bFGF and PDGFα with BDM, containing the differentiation growth factors (NAC (A8199, Sigma), CNTF (450-50, Peprotech) and T3 (T2752, Sigma)) as described by Chen et al. (2007). TNFα (10ng/ml, rat recombinant 400-19, Peprotech) was added to induce an OL maturational arrest (+TNFα). To study the potential of IGF1 to boost OL maturation, IGF1 (100ng/ml, murine recombinant, 250-19, Peprotech) was added directly or 24 hours after the medium change. The optimal concentration of IGF1 was obtained in previous dose-response experiments (Vaes et al., 2021). After 72 hours of culture, OLs were fixated with 4% PFA in PBS during 10 minutes. For Western Blot experiment on pre-OLs exposed to IGF1, we added 100ng/ml IGF1 to the wells after induction of differ-

entiation and collected protein lysates 10 minutes later by adding 1x sample buffer to the wells.

Immunocytochemistry

After fixation of OL cultures, non-specific binding was blocked using 2% BSA and 0.1% saponin. Wells were incubated with primary antibodies (rabbit-anti-Olig2, AB9610, Merck Millipore; 1:1000 and mouse-anti-MBP, SMI-94, Biolegend; 1:1000) overnight at 4°C. The next day, wells were washed with PBS and incubated with alexafluor-594 and -488 conjugated secondary antibodies (Life technologies; 1:1000) for 1 hour at room temperature, followed by nuclear counterstaining using Hoechst 33342 (Sigma) and embedment of wells in Fluorsave (345789, Merck Millipore). To assess OL maturation, six adjacent fields were imaged (10x), starting at a fixed distance of the well edges. The analyze particles function in ImageJ v.1.47. was used to count the number of Olig2- and Hoechst- positive cells. The area of MBP^+ staining was measured using manual thresholding in ImageJ. To compare independent experiments, all results were normalized for the positive control (-TNFα; - IGF1).

Primary glia culture (mouse)

A primary microglia and astrocyte culture was obtained from P1 C57BL/6 mice cortices. In short, meninges were removed after dissection of the cortices. The tissue was minced and incubated with 0.25% trypsin (T4799, Sigma) in Gey's balanced salt solution (GBSS) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 mM D(+)-glucose for 15 minutes. Tissue was dissociated until homogenous by pipetting and cultured in poly-L-ornithine-coated (P3655, Sigma) T75 flasks in DMEM/HamF10 (1:1) (41965-039 and 31550-023, Gibco) supplemented with 10% FCS, 2mM glutamine and antibiotics (see above). At DIV10-12, culture flasks were placed on an orbital shaker during 20-22 hours (130-135rpm at 37°C) to detach microglia. Subsequently, microglia were collected by centrifugation (1200 rpm during 10 minutes at RT), counted and seeded in poly-L-ornithine-coated 24-wells plates at a density of 1.4×10^5 cells per well. After shaking the T75 flasks, new culture medium was added to each flask to allow a second microglia harvest after an additional 7-10 DIV. After the second harvest of microglia, astrocytes were harvested from the flasks, centrifuged and plated at a density of 2.0×10^5 cells per well. At 24 hours after plating of microglia or astrocytes, cells were activated using 50ng/ml LPS (L4515, Sigma). To study endogenous IGF1 production after LPS exposure, at 48 hours after LPS stimulation supernatant was harvested and used for IGF1 ELISA. To study the potential

of IGF1 to dampen neuroinflammation, IGF1 (100ng/ml, murine recombinant, 250-19, Peprotech) was added to wells with astrocytes or microglia directly after induction of neuroinflammation. After 48 hours in culture, microglia supernatant was collected for TNFα measurements. For Western Blot experiments on activated microglia and astrocytes exposed to IGF1, protein lysates were collected 10 mins after addition of LPS and IGF1 in 1x sample buffer.

Western blotting

Protein homogenates from pre-OL, microglia and astrocyte cultures were pooled from two wells. Proteins were separated using SDS-PAGE and transferred to Amersham Protan membranes. Membranes were blocked with 5% BSA and stained using primary antibodies (rabbit-anti-P-Akt, 1:1000, 4060S, Cell signaling; rabbit-anti-Akt, 1:1000, 4691S, Cell signaling; P-IGFRβ, 1:1000, 2969S, Cell signaling; β-actin, 1:2000, SC47778, Santa Cruz) overnight, followed by incubation with peroxidase-labeled secondary antibodies, and visualization by chemiluminescence. β-actin or total Akt were used as a loading control. Images were analyzed by Image J software.

ELISA

For microglia and astrocyte cultures, TNFα concentrations in the supernatant (after LPS and IGF1 exposure) were measured using an ELISA kit for murine TNFα (Ucytech, Utrecht, The Netherlands) according to manufacturer's instructions. To study endogenous IGF1 production by microglia and astrocytes after LPS exposure, IGF1 concentrations in the supernatant were assessed using for murine IGF1 (MG100, R&D systems) following supplier's protocol. To compare independent experiments, all ELISA data were normalized (for TNFα ELISA to positive control conditions (50ng/ml LPS; no IGF1) and for IGF1 ELISA to negative control (no LPS)).

Statistics

The data are presented as mean ± standard error of the mean (SEM). All statistical analysis were carried out using Graphpad Prism 8.3. When comparing two groups, unpaired t-tests, or in the event of unequal variances, non-parametric Mann-Whitney tests were used. For comparison of >2 groups, one-way ANOVA with Bonferroni posthoc tests was carried out. For comparison of multiple groups with unequal variances, a non-parametric Kruskal-Wallis test with Dunn's posthoc correction was used. P-values <0.05 were considered statistically significant. Specific sample sizes are mentioned in the figure captions.

RESULTS

Induction of experimental dWMI transiently reduces endogenous IGF1 and IGFBP3 production in brain and plasma

Human studies have identified a relative deficiency of circulatory IGF1 levels in the first postnatal weeks after extreme preterm birth (Hansen-Pupp et al., 2007; Hellström et al., 2016). To explore whether our validated dWMI mouse model also leads to changes in endogenous production of IGF1 and its binding proteins, the concentrations of these factors were measured in plasma and ipsilateral cerebrum at 6, 24 and 72 hours after induction of dWMI. At 6 and 24 h after dWMI, a significant reduction of circulatory IGF1 was found in the plasma of dWMI animals compared to sham-controls ($p<0.0001$ (6h) and $p=0.013$ (24h)) (figure 1A). The reduction in circulatory IGF1 was restored at 72 hours post-WMI ($p=0.791$ compared to sham-controls) (figure 1A). Similar to plasma IGF1, a transient deficit in local cerebral IGF1 was observed at 6 hours post-dWMI in ipsilateral hemispheres, with normalization of cerebral IGF1 levels from 24h post-dWMI (6h $p=0.038$; 24h $p=0.287$; 72h p=0.996, compared to sham-controls) (figure 1B). For IGFBP3, a significant reduction in plasma level was found only at 24 hours post-dWMI induction ($p=0.0028$, compared to sham-controls) (figure 1C). In contrast, dWMI mice displayed an increase in cerebral IGFBP3 concentration at 24 hours (p=0.0012, ipsilateral dWMI compared to sham-controls), while cerebral IGFBP3 levels were unaffected at 6 or 72 hours post-dWMI induction ($p=0.486$ and $p=0.104$ respectively, ipsilateral dWMI compared to sham controls) (figure 1D). dWMI did not affect circulating or cerebral IGFB2 levels at any of the timepoints (plasma: 6h $p=0.460$; 24h $p=0.831$; 72h $p=0.623$ and brain: 6h $p=0.841$; 24h $p=0.444$; 72h $p=312$, compared to sham-controls) (figure 1E-F).

To investigate whether experimental dWMI also affected expression of the IGF receptor in the brain *in vivo*, thereby possibly limiting the efficacy of exogenous IGF1 therapy in our model, we compared mRNA expression levels of the IGF receptor at 6, 24 and 72 hours post-dWMI using quantitative real time RT-PCR. We did not observe any changes in IGF receptor expression at any of the time points after dWMI induction (6h $p=0.100$; 24h $p=0.124$; 72h $p=0.513$, compared to sham-controls) (figure 1G).

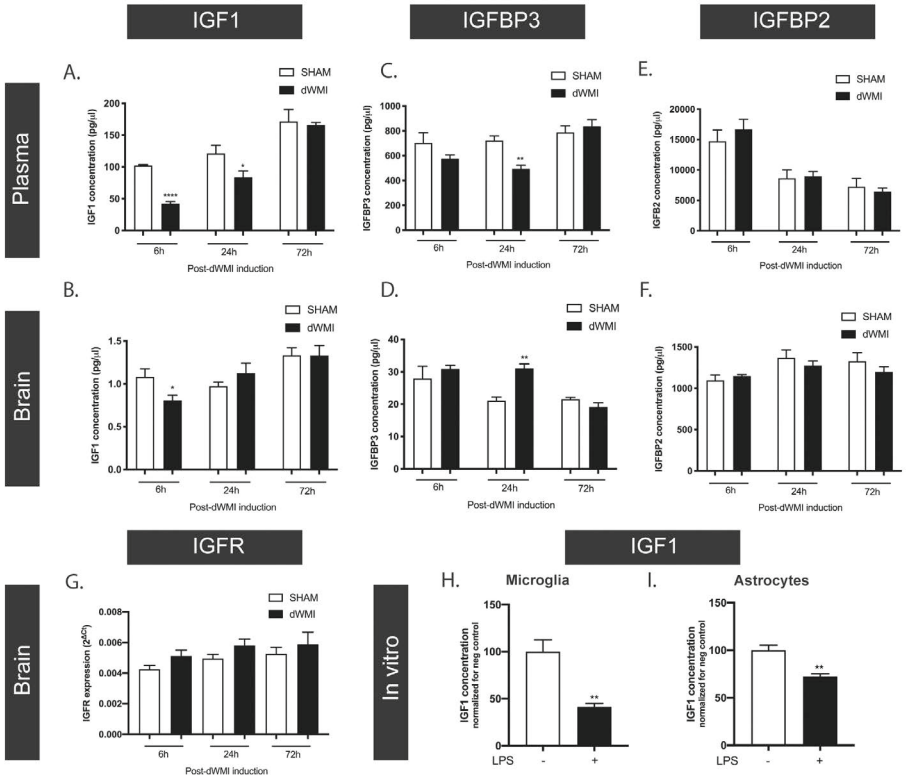


Figure 1. Preterm birth-related hits transiently reduce endogenous IGF1 and IGFBP3 production in newborn mice. **A/B.** Induction of dWMI reduces IGF1 concentrations for at least 24 hours in plasma (**A**) and for at least 6 hours in the ipsilateral hemisphere (**B**) of neonatal mice. **C/D**. Mice subjected to postnatal hypoxia/ischemia and systemic inflammation displayed lower plasma IGFBP3 levels (**C**) and increased cerebral IGFBP3 levels (**D**) at 24 hours post-induction compared to sham-controls **E/F**. dWMI induction did not affect IGFBP2 levels in plasma (**E**) or brain (**F**). **G.** No significant changes in IGF receptor (IGFR) expression were observed post-dWMI induction in the ipsilateral hemisphere. Animal numbers for plasma: sham-controls 6h n=4, 24h n=4, 72h n=5; dWMI 6h n=4, 24h n=4, 72h n=5. Animal numbers for the brain (ipsilateral hemisphere): sham-controls 6h n=5, 24h n=4, 72h n=5; dWMI 6h n=5, 24h n=4, 72h n=5. **H/I**. Exposure to LPS for 48 hours reduced IGF1 secretion by primary cultured microglia (**H**) and astrocytes (**I**) (n= 2 independent experiments, 2 observations per experiment; data normalized for cells not exposed to LPS that were put at 100). *: p< 0.05; **: p<0.01; ****p<0.0001 dWMI animals (black bar) vs sham-control animals (white bar), or LPS+ (black bar) vs. LPS- control (white bar) condition.

Local cerebral production of IGF1 reduced after inflammation in vitro

We showed previously that our experimental mouse model of dWMI induces activation of microglia and astrocytes in the brain (Vaes et al., 2021). As microglia and astrocytes are an essential source of cerebral IGF1 production (Labandeira-Garcia et al., 2017), we here investigated whether dWMI affected the endogenous IGF1 production by glial cells. To do so, we subjected primary cultured mouse microglia and astrocytes to an LPS stimulus and subsequently measured IGF1 concentration in the culture medium. In line with our in vivo findings in Figure 1B, we observed a reduction in endogenous IGF1 production by both microglia (p=0.0026) (figure 1H) and astrocytes (p=0.0035, compared to unstimulated cells) after LPS stimulation (figure 1I).

IGF1 diffuses throughout the brain after intranasal application

To assess cerebral delivery and distribution of IGF1 after intranasal administration in dWMI mouse pups, the concentration of human IGF1 (hIGF1) was measured in the ipsi- and contralateral anterior and posterior cerebrum and cerebellum at 30 minutes after intranasal application. Figure 2A shows an even distribution of hIGF1 in both hemispheres and the cerebellum of dWMI animals, indicating rapid and dispersed distribution of IGF1 throughout the injured brain. As a control, we were unable to detect any hIGF1 in dWMI animals treated with vehicle solution (data not shown).

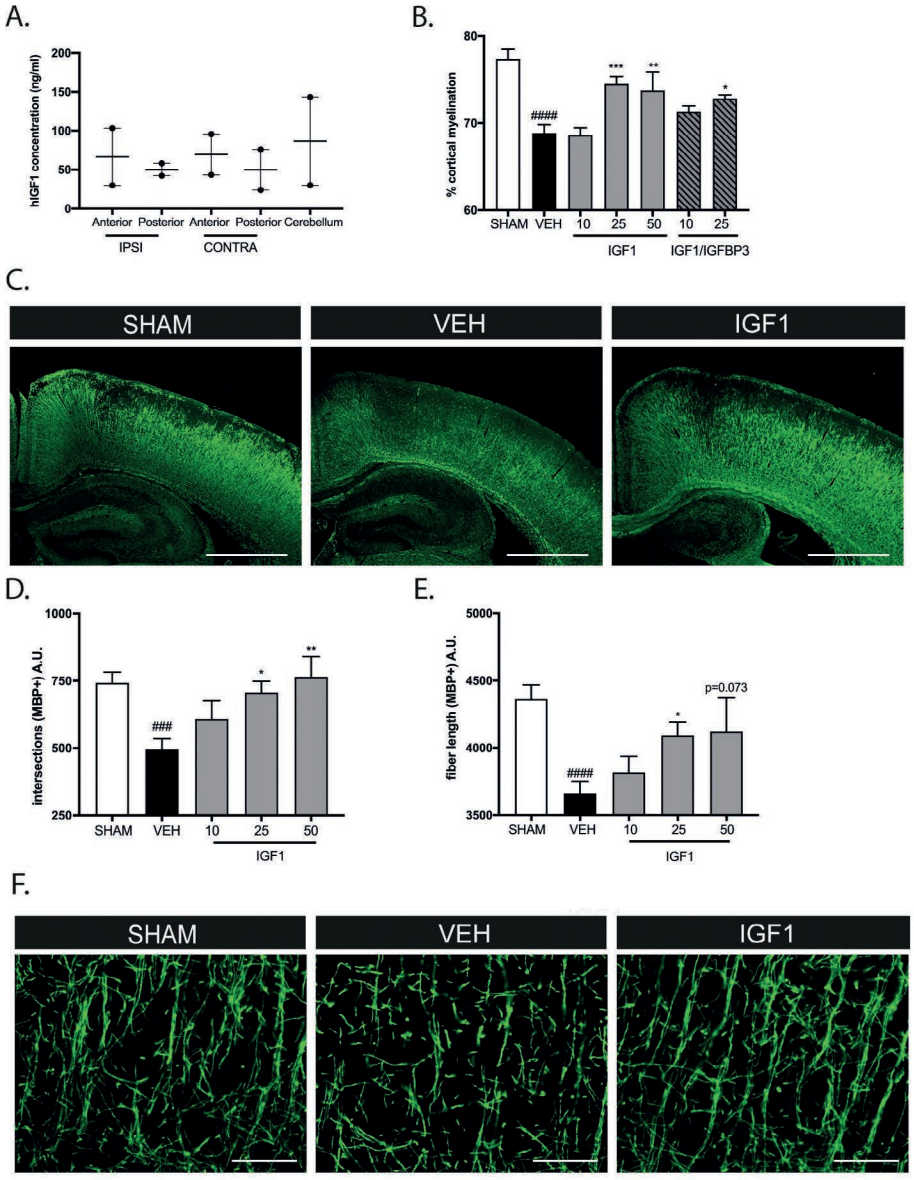


Figure 2 (see overleaf).

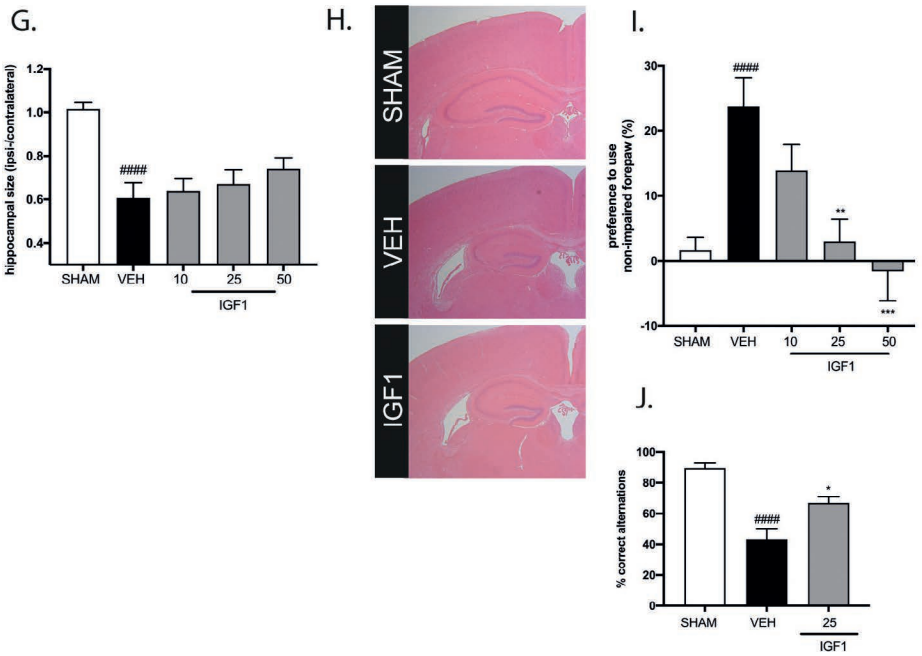


Figure 2 (Continued). Intranasal IGF1 therapy potently rescues myelination and restores motor and cognitive impairments in dWMI mice at P26. **A.** Intranasally administered hIGF1 (25 µg) distributes evenly and rapidly throughout the injured P5 brain as measured at 30 min after gift (n=2 IGF1-treated dWMI mice). As control, n=2 vehicle-treated dWMI brain parts were also measured and were negative for hIGF1. IPSI: ipsilateral hemisphere, CONTRA: contralateral hemisphere. **B.** Intranasal administration of 25µg and 50µg IGF1 for 6 consecutive days significantly restored MBP^+ coverage of the cortex in dWMI animals when compared to vehicle (VEH) treatment. Addition of IGFBP3 did not enhance IGF1's regenerative potential after dWMI. Animal numbers: sham-control (SHAM) n=13, vehicle-treated dWMI n=13, dWMI animals treated with i.n. IGF1 10µg n=11, 25µg n=12 and 50µg n=8 or with IGF1/IGFBP3 10µg n=9, 25µg n=12. **C.** Representative fluorescent images (2.5x) of the ipsilateral cortex of a sham-operated control mouse (left), vehicle-treated dWMI mouse (middle) and dWMI mouse treated with 25µg IGF1 (right) stained for myelin marker MBP (green). Scale bars: 500 µm. **D/E.** Intranasal treatment with 25µg or 50µg IGF1 improved dWMI-induced aberrations in myelin microstructure assessed by the number of intersections (**D**) and fiber length (**E**) (SHAM n=13, VEH n=13, 10µg n=9, 25µg n=12, 50µg n=7). **F.** Representative fluorescent micrographs (40x) of MBP^+ axons in the ipsilateral cortex of a sham-operated control animal (left), vehicle-treated dWMI animal (middle) and dWMI animal treated with 25µg IGF1 (right). Scale bars: 100µm **G.** Intranasal IGF1 therapy does not restore dWMI-induced ipsilateral reduction of hippocampal size (SHAM n=13, VEH n=13, 10µg n=11, 25µg n=12, 50µg n=10) **H.** Representative images of the ipsilateral HE-stained hippocampus of a sham-operated control mouse (upper), vehicle-treated dWMI mouse (middle) and dWMI mouse treated with 25µg IGF1 (lower). **I.** Motor performance, assessed with the cylinder rearing test, improved potently after IGF1 treatment (SHAM n=13, VEH n=13, 10µg n=10, 25µg n=11, 50µg n=9). **J.** IGF1 therapy partially restored the number of correct alternations in the T-maze compared to vehicle- treatment (SHAM n=7, VEH n=7, 25µg n=6). ###: $p<0.001$; ####: $p<0.0001$ vehicle-treated dWMI animals vs sham-controls; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ IGF1-treated dWMI animals vs vehicle-treated dWMI animals. Nearly significant p values are indicated in E.

Intranasal IGF1 therapy repairs hypomyelination and improves functional outcome

To investigate the potential of IGF1 treatment to reduce myelination deficits, dWMI animals were treated intranasally with different dosages of IGF1 or an equimolar IGF1/IGFBP3 mixture on P5 to P10 (6 consecutive days; once daily). At P26, a significant reduction in cortical myelination was observed in vehicle-treated dWMI animals compared to sham-control animals ($p<0.0001$), which could be rescued with a daily dose of intranasal 25µg and 50µg IGF1, indicated by increased MBP^+ coverage of the cortex ($p=0.0003$ and $p=0.0079$ dWMI-veh vs dWMI-IGF1 25µg or 50µg respectively) (figure 2B-C). The lowest dose of 10µg did not enhance cortical myelination after dWMI ($p>0.999$, dWMI-veh vs dWMI-IGF1 10µg) (figure 2B). Although addition of IGFBP3 to IGF1 slightly enhanced the therapeutic efficacy of the lowest dose of 10µg IGF1 ($p=0.024$ dWMI-10µg IGF1 vs dWMI-10µg IGF1/IGFBP3), it failed to significantly restore cortical myelination compared to vehicle-treated animals ($p=0.434$) (figure 2B). Addition of IGFBP3 to 25µg IGF1 did restore cortical myelination compared to vehicle treatment ($p=0.023$, dWMI-veh vs dWMI-25µg IGF1/IGFBP3) but did not increase the therapeutic efficacy of 25µg IGF1 as mono-therapy ($p=0.105$ dWMI-25µg IGF1 vs dWMI-25µg IGF1/IGFBP3) (figure 2B). Based on these findings, we concluded that addition of IGFBP3 to IGF1 did not enhance the therapeutic efficacy of IGF1 treatment and was therefore not assessed further in this study. In addition, we tested whether intranasal IGF1 treatment in the lowest effective dose of 25µg in sham-operated animals had any effect on white matter development, compared to untreated sham-controls. We did not find any differences in cortical myelination between untreated and IGF1-treated sham-controls (data not shown).

To assess myelination in more detail, we performed microstructural analysis of MBP^+ axons, revealing a negative effect of dWMI on complexity of myelinated fibers in vehicle-treated dWMI animals (intersections: $p=0.0002$ and fiber length: $p<0.0001$) (figure D-F). Intranasal IGF1 treatment with a daily dose of 25µg or 50µg restored the number of intersections (25µg $p=0.012$ and 50µg $p=0.004$, dWMI-veh vs dWMI-IGF1 dose) and fiber length (25µg $p=0.042$ and 50µg trend: $p=0.073$, dWMI-veh vs dWMI-IGF1 dose), while the lowest dose of 10µg IGF1 did not restore myelin complexity (intersections $p=0.423$ and fiber length $p>0.999$, dWMI-veh vs dWMI-IGF1 dose) (figure 2D-F). In contrast to the white matter, intranasal IGF1 treatment did not significantly restore developmental gray matter deficits after dWMI, with persistent dWMI-induced loss of hippocampal size after IGF1 treatment at all dosages ($p<0.0001$, dWMI-veh

vs sham-control; 10µg $p>0.999$; 25µg $p>0.999$ and 50µg $p=0.420$, dWMI-veh vs dWMI-IGF1 dose) (figure 2G-H).

Next, we assessed the effect of intranasal IGF1 therapy on motor outcome after dWMI using the CRT at P26. Vehicle-treated dWMI animals exhibited an impairment in motor functioning, indicated by a preference to use their non-impaired forepaw ($p=0.0001$, dWMI-veh vs sham-control) (figure 2I). Intranasal treatment with 25µg or 50µg potently improved motor outcome (25µg $p=0.0041$ and 50µg $p=0.0010$, dWMI-veh vs dWMI-IGF1), while treatment with 10µg IGF1 failed to restore motor behavior ($p=0.303$, dWMI-veh vs dWMI-10µg IGF1) (figure 2I). In line with our histological findings on cortical myelination, addition of IGFBP3 did not enhance the therapeutic efficacy of intranasal IGF1 treatment on motor outcome (data not shown). Based on the histological and functional outcome parameters shown in Figure 2A-H, we determined a daily intranasal dose of 25µg IGF1 to be the lowest effective dose for optimal recovery after dWMI. Subsequently, the effect of 25µg IGF1 on executive memory functioning at P26 was assessed. Vehicle-treated dWMI animals displayed a reduction in the percentage of correct alternations in the T-maze compared to sham-controls ($p<0.0001$) (figure 2J). Intranasal 25µg IGF1 treatment potently restored executive memory functioning, i.e. increasing the level of correct alternations ($p=0.010$, dWMI-veh vs dWMI-IGF1) (figure 2J).

The optimal timing of intranasal IGF1 treatment for dWMI

Based on our data in Figure 1 showing restoration of endogenous IGF1 levels at 72 hours post-dWMI compared to sham-controls, we hypothesized that shortening the intranasal IGF1 treatment regime to P5-8 might be sufficient to induce therapeutic efficacy of exogenous IGF1 administration. In addition, to gain more insight in the possibility to postpone start of intranasal IGF1 therapy (i.e. enlarge the therapeutic window), we delayed the start of IGF1 administration to P7 for 6 consecutive days (i.e. P7-12). Figure 3A confirms that daily administration of 25µg IGF1 from P5-10 restored the dWMI-induced deficits in fiber length ($p<0.0001$, dWMI veh vs dWMI-IGF1 P5-10). Interestingly, shortening of the treatment protocol to only P5-8 or postponement of treatment-start to P7 (i.e. P7-12) led to a strong reduction in therapeutic efficacy of intranasal IGF1 (P5-8 $p=0.340$ and P7-12 $p>0.999$, dWMI veh vs dWMI-IGF1) (figure 3A). Assessment of motor outcome by using CRT supported these histological findings. The beneficial effect of intranasal IGF1 treatment at P5-10 ($p=0.0014$) on the reduction of forepaw preference after dWMI was largely lost when IGF1 treatment

was shortened (p=0.754, dWMI veh vs dWMI-IGF1 P5-8) or postponed (p>0.999, dWMI veh vs dWMI-IGF1 P7-12) (figure 3B). Collectively, these data indicate that the treatment window for intranasal IGF1 in our dWMI mouse model is most optimal after timely start and prolonged administration.

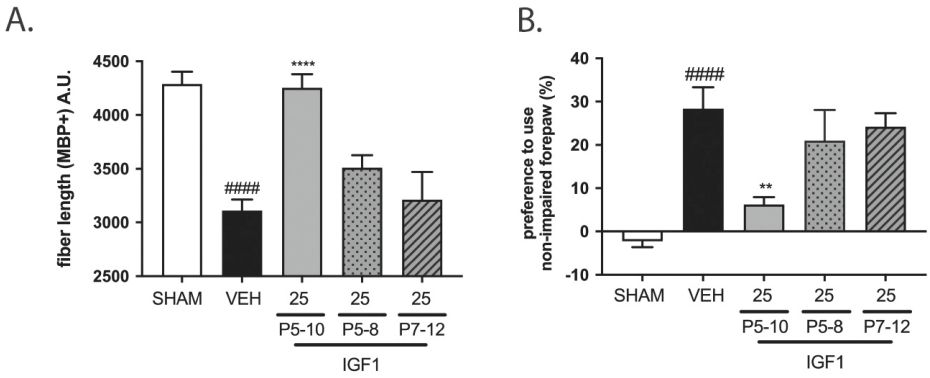


Figure 3. A shortened or delayed IGF1 treatment protocol reduces the regenerative potential of intranasal IGF1 therapy on myelination and functional outcome at P26. **A.** Fiber length, a marker of myelin microstructure, was restored in dWMI mice that received IGF1 treatment from P5-10. Shortening IGF1 treatment to P5-8 or delaying treatment to P7-12 led to a reduction in treatment efficacy (SHAM n=9, VEH n=9, IGF1-P5-10 n=9, IGF1-P5-8 n=6, IGF1-P7-12 n=8). **B.** The restorative effect of intranasal P5-P10 IGF1 treatment on motor outcome, assessed with the cylinder rearing test, is largely lost after shortening or postponement of IGF1 treatment (SHAM n=9, VEH n=9, IGF1-P5-10 n=9, IGF1-P5-8 n=6, IGF1-P7-12 n=9)**.** Dosing used for all treatment regimes: 25 µg IGF1. ####: p<0.0001 vehicle-treated dWMI animals vs sham-controls; **: p<0.01; ****: p<0.001 IGF1-treated dWMI animals (P5-P10 regime) vs vehicle-treated dWMI animals.

IGF1 directly boosts OL maturation

Myelination failure in dWMI is believed to be the consequence of a maturation arrest of the OL lineage. In line with this hypothesis, we previously observed that cortical hypomyelination following induction of dWMI was accompanied by a deficit in mature OLs at P19 in our mouse model (Vaes et al., 2021). To examine if the beneficial effects of intranasal IGF1 therapy on myelination were associated with recovered numbers of mature Ols after dWMI, double-stainings for CC1 and Olig2 were performed on brain sections of vehicle- and IGF1 treated dWMI animals at P19. Similar to our previous observations, dWMI induced a reduction in $CC1^+/Olig2^+$ cell density in the cortex in vehicle-treated dWMI animals (p=0.0065 compared to sham-controls), which was restored by daily intranasal treatment with 25µg IGF1 from P5 to P10, indicating recovery

of OL lineage maturation by IGF1 (p=0.0001, dWMI-veh vs dWMI-IGF1) (figure 4A-B). CC1 analyses were performed at P19 as we previously demonstrated endogenous recovery of mature OL numbers at P26 in this model (Vaes et al., 2021).

To investigate whether IGF1 can act on OLs directly, activation of the IGFR was assessed by western blots for phosphorylated Akt, one of the downstream signaling pathways, in a primary pre-OL culture. Our results confirmed that exposure of OLs to IGF1 increased P-Akt levels (1.25x, compared to OLs without IGF1) after 10 min, indicative of IGF1R activation (figure 4C). To confirm that IGF1 can boost OL maturation and subsequent myelination in a direct manner, we subjected primary cultured pre-OLs to TNFa to mimic dWMI *in vitro*, and measured the MBP$^+$ area per OL as a maturational readout. Pre-OLs exposed to TNFa demonstrated a strong reduction in MBP$^+$ area/cell compared to pre-OLs cultured in absence of TNFa (p=0.0037). Addition of 100 ng/ml IGF1 to TNFa-exposed pre-OLs potently restored MBP$^+$ area, indicating a boosting effect of IGF1 on OL differentiation (p=0.0094, vs +TNFa) (figure 4D-E).

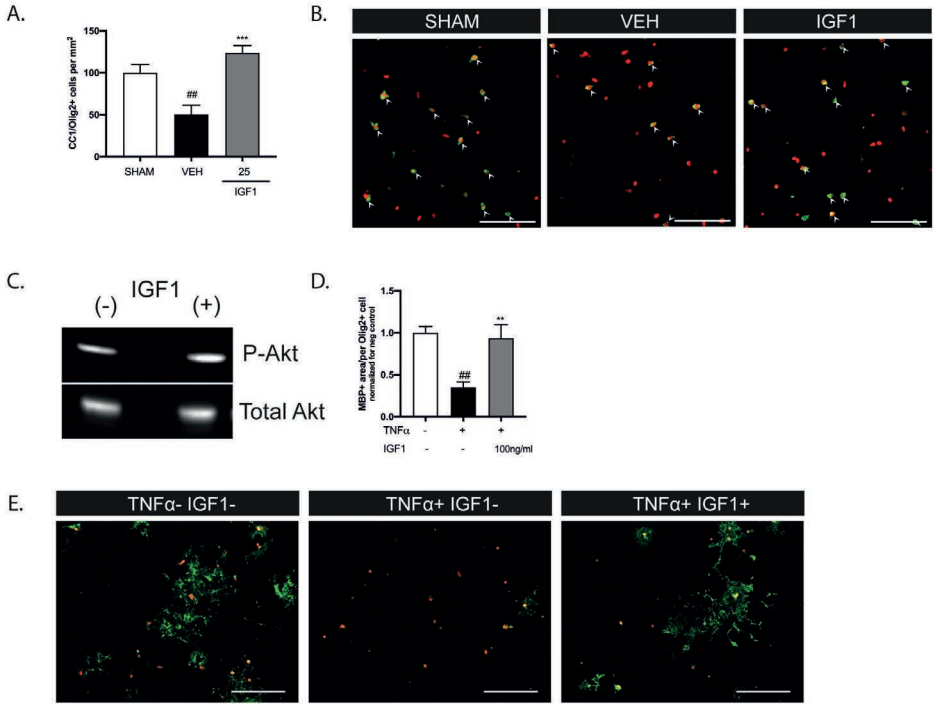


Figure 4. IGF1 can directly boost OL maturation. **A.** Intranasal treatment with 25µg IGF1 daily from P5-10 significantly restored CC1⁺/Olig2⁺ cells numbers at P19 compared to vehicle-treatment, even up to sham-control levels, indicating a boost in OL lineage maturation (SHAM n=6, VEH n=6, IGF1 n=7). **B.** Representative fluorescent images of the P19 cortex (20x), double-stained for CC1 (green) and Olig2 (red), of a sham-control mouse (left), vehicle-treated dWMI mouse (middle) and dWMI mouse treated with 25µg IGF1 (right). Double-positive cells are marked with an arrowhead. Scale bars: 100µm. **C.** Western blot reveals increased phosphorylation of Akt, a downstream target of the IGFR, in pre-OLs exposed to IGF1 (2 pooled wells, in n=1 independent experiment). **D.** IGF1 treatment (100 ng/ml) directly boosts OL differentiation after TNFa- induced maturation arrest in primary cultured OL precursors (n=3 independent experiments, 2-3 observations per experiment; negative control condition (i.e. no stimulation with TNFa) was put at 1.0). **E.** Representative fluorescent images (10x) of primary cultured oligodendrocytes stained for oligodendrocyte marker Olig2 (red) and myelin component MBP (green). Cells were exposed to TNFa to induce a maturation arrest and IGF1 was added as rescue therapy. Scale bars: 100µm. ##: p<0.01 vehicle-treated dWMI animals vs sham-controls or TNFa (black bar) vs. no TNFa (white bar) condition. **: p<0.01; ***: p<0.001; IGF1-treated dWMI animals vs vehicle-treated dWMI animals or TNFa (black bar) vs. TNFa +IGF1 (gray bar) condition.

IGF1 dampens astrocyte activation, but not microgliosis

Induction of dWMI at P5 was previously shown to be associated with neuroinflammation, a key etiological hallmark of dWMI pathophysiology (Vaes et al., 2021). To investigate the potential of intranasal IGF1 treatment to dampen neuroinflammation, brain sections of sham-control, dWMI-vehicle and dWMI-IGF1 mice were stained for the microglia marker Iba1 and astrocyte marker GFAP at P26. In line with previous findings, an increase in the number of Iba1$^+$ cells was found in the corpus callosum of vehicle-treated dWMI animals compared to sham-controls ($p<0.0001$), which could not be dampened by intranasal IGF1 treatment ($p=0.850$, dWMI veh vs dWMI-IGF1) (figure 5A-B). Detailed assessment of microglial morphology revealed an active (amoeboid) phenotype in vehicle-treated dWMI animals compared to sham-control mice, indicated by increased cell circularity ($p=0.0007$) and a reduction in cell perimeter ($p=0.0004$) (figure 5C-D). Intranasal treatment with IGF1 did not alter the activation state of Iba$^+$ cells (circularity $p>0.999$ and perimeter $p>0.999$, dWMI-veh vs dWMI-IGF1) (figure 5C-D). In addition, astrocyte reactivity was assessed in the corpus callosum and hippocampus at P26. In contrast to the microglia data, intranasal IGF1 treatment dampened dWMI-induced astrocyte reactivity (corpus callosum $p=0.029$ and hippocampus $p=0.0009$, dWMI-veh vs sham-control), indicated by a reduction in GFAP$^+$ area in both the corpus callosum ($p=0.0145$, dWMI veh vs dWMI-IGF1) and hippocampus ($p=0.0001$, dWMI veh vs dWMI-IGF1) (figure 5E-G).

Primary glia cultures were used to further examine the observed differences in *in vivo* IGF1 responsiveness between microglia and astrocytes. Western blot analysis revealed a strong increase in phosphorylation of Akt, a downstream target of the IGFR, in primary reactive astrocytes treated with IGF1 for 10 min (2.65x, compared to astrocytes without IGF1), but not in IGF1-stimulated microglia (1.05x, compared to microglia without IGF1) (figure 5H-I). Additionally, we checked activation of the IGFR in activated microglia and astrocytes by western blot for phosphorylated IGFR-1b protein. We detected phosphorylated IGFR in activated astrocytes stimulated with IGF1, while we confirmed absence of activation of IGFR after IGF1 exposure in cultured mouse microglia (figure 5J). In line with our *in vivo* findings, *in vitro* IGF1 exposure did not dampen LPS-induced microglia activation (*p<0.0001*, LPS activation vs no LPS) indicated by persistent high-level production of TNFa measured by ELISA (p=0.975, LPS activation and IGF1 vs no LPS) (figure 5K). Altogether, the data in Figure 5 show that IGF1 potently dampens astrocyte activity but has little to no effect on microglia activation, possibly by unresponsiveness or lack of the IGFR on the latter cell type.

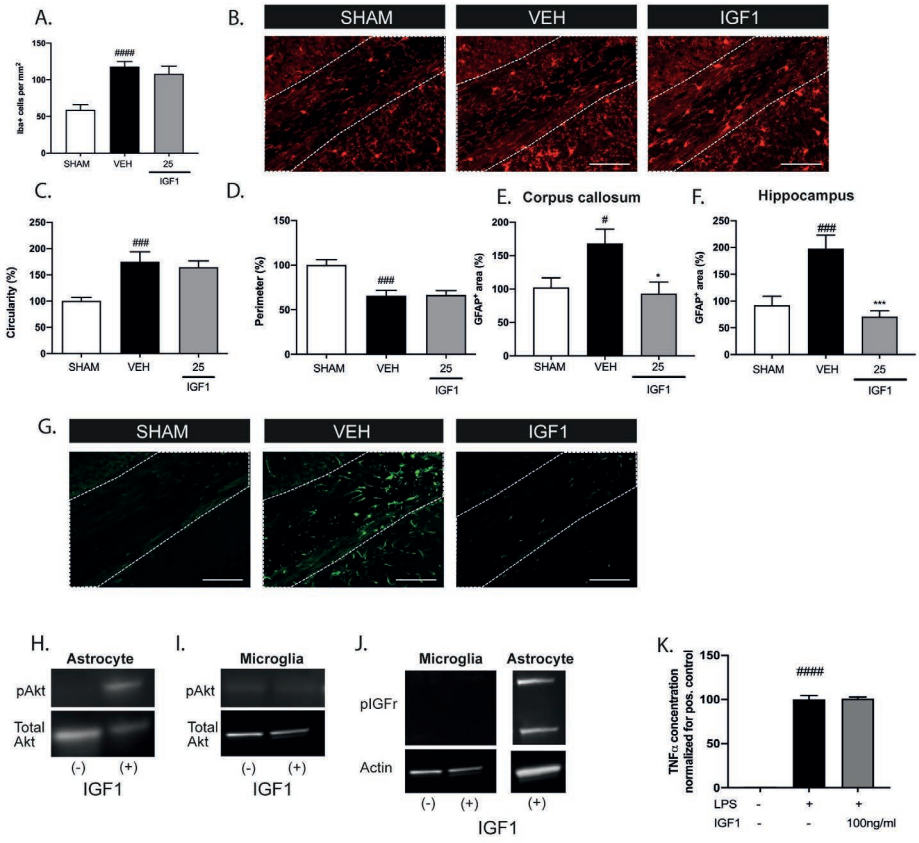


Figure 5. IGF1 treatment dampens astrocyte activation but not microglia activation. **A.** Intranasal IGF1 treatment (25 µg P5-P10) failed to reduce Iba$^+$ cell density in the corpus callosum after dWMI induction (SHAM n=10, VEH n=9, IGF1 n=10). **B.** Representative fluorescent images (20x) of Iba1$^+$ cells in the corpus callosum (white outline) in a sham-control animal (left), vehicle-treated dWMI animal (middle) and dWMI animal treated with IGF1 (right). Scale bars: 100µm **C/D.** Intranasal IGF1 treatment did not affect the activation state of microglia, assessed using morphological parameters i.e. cell circularity (**C**) and perimeter (**D**) (SHAM n=10, VEH n=9, IGF1 n=10). **E/F.** A strong reduction in GFAP$^+$ area was observed in the corpus callosum (**E**) and hippocampus (**F**) of dWMI animals treated with IGF1 compared to vehicle-treated dWMI animals, indicative of reduced astrocyte reactivity (SHAM n=10, VEH n=10, IGF1 n=9). **G.** Representative fluorescent images (40x) of GFAP$^+$ staining in the corpus callosum in a sham-control animal (left), vehicle-treated dWMI animal (middle) and dWMI mouse treated with 25µg IGF1 (right). Scale bars: 100µm. **H/I**. Western blots reveal an increase in phosphorylation of Akt, a downstream target of the IGFR, in LPS-activated primary astrocytes exposed to IGF1 (2 pooled wells, in n=1 independent experiment), but not in LPS-activated primary microglia in vitro (2 pooled wells, in n=1 independent experiment). **J.** IGFR-1b phosphorylation, indicative for IGFR activation, was detected in activated astrocytes after IGF1 administration, but not in activated microglia in vitro (2 pooled wells, in n=1 independent experiment). **K.** IGF1 treatment failed to

attenuate TNFa production by LPS-exposed primary microglia in vitro (n=2 two independent experiments, 2 observations per experiment). #: p=0.05; ###: p<0.001; ####: p<0.0001 vehicle-treated dWMI animals vs sham-controls or LPS (black bar) vs. no LPS (white bar, not visible) condition; *: p<0.05; ***: p<0.001; IGF1-treated dWMI animals vs vehicle-treated dWMI animals.

DISCUSSION

In the present study, we evaluated whether induction of experimental dWMI in newborn mice would affect systemic and cerebral production of IGF1 and its binding proteins. We show that postnatal hypoxia-ischemia and systemic inflammation in P5 mice transiently reduces IGF1 and IGFBP3 levels in the plasma and brain, comparable to the human preterm neonate. Moreover, we investigated the regenerative potential of intranasal IGF1 treatment after dWMI. We report that intranasal application of IGF1 leads to rapid diffusion of the protein throughout the injured brain. Intranasal IGF1 treatment potently restores myelination deficits and improves functional outcome after daily treatment from P5-P10. Furthermore, we demonstrate that IGF1 therapy supports white matter development after dWMI by directly boosting OL differentiation following maturation arrest. Moreover, we show that IGF1 treatment selectively dampens astrocyte reactivity, but not microglia activation after dWMI. These cell type-specific disparities in IGF1 responsivity observed in our dWMI model are likely the result of limited IGF receptor responsiveness or availability in microglia. Collectively, these data imply that intranasal IGF1 administration is a potent therapeutic strategy to restore myelination in a clinically relevant double-hit mouse model of dWMI, aiding in white matter regeneration by boosting OL lineage development after maturation arrest.

Clinically relevant animal models of EoP are vital for translation of novel therapeutic options for (extreme) preterm infants. In our double-hit mouse model, induction of dWMI at P5 was associated with a transient decrease in endogenous IGF1 production in both the brain and systemic circulation. Plasma IGF1 levels normalized between 24 and 72 hours post-insult, while cerebral IGF1 levels normalized to sham-control levels prior to 24 hours. This temporary early IGF1 deficiency between P5-P6 coincidences with a critical postnatal window in rodent OL development similar to that of the human preterm neonate (approximately 27-30 weeks of gestation) (Craig et al., 2003; Hellstrom et al., 2016; Salmaso et al., 2014). Our data show that cerebral IGF1 levels appeared to recover prior to systemic IGF1 levels. Although IGF1, bound to IGFBP's, is able to cross the blood-brain-barrier (BBB) via active transport, the observed rise in

cerebral IGF1 is likely a reflection of upregulated *local* IGF1 production (Pan & Kastin, 2000) rather than a redistribution from the periphery as systemic IGF1 levels were lower for a longer period after dWMI. In the central nervous system, microglial cells and astrocytes are an important local source of IGF1 production (Labandeira-Garcia et al., 2017). In the current study we observed a reduction in IGF1 production by primary cultured microglia and astrocytes at 48 hours after LPS stimulation in vitro, indicating that inflammatory insults could affect locally produced IGF1 levels. Our *in vitro* data are in line with a study by Sivakumar et al. (2010) that showed similar reductions in IGF1 production by microglia after using glutamate as stimulus. *In vivo*, Lee et al. (1996) showed that an initial downregulation of IGF1 after a hypoxic-ischemic insult in near-term rats was followed by a compensatory upregulation of astrocytic IGF1 production. In line with the study by Lee et al. (1996), we also observed an early recovery of local cerebral IGF1 levels in our mouse model. It is currently not known which cells are responsible for the recovery of local IGF1 production as neurons and infiltrating macrophages could also contribute to this early compensatory mechanism. Responding to brain injury with an initial decrease in endogenous IGF1 production seems to be distinctive for the neonatal period as (hypoxic)-ischemic brain injury in adolescent/adult rodents leads to IGF1 upregulation (Gluckman et al., 1992; Lee et al., 1992). It is important to note that the role of peripherally versus locally produced IGF1 in brain development and plasticity is still under debate (Torres-Aleman, 2010). Selective elimination of serum IGF1 in adult rodents led to central nervous system abnormalities, while elimination of brain IGF1 production did not produce any major changes in the adult brain (Davila et al., 2007). It has been suggested that the autocrine and paracrine function of locally produced IGF1 primarily plays a role in prenatal and early postnatal brain development, as local IGF1 expression decreases shortly after birth (Wrigley et al., 2017).

IGF-binding proteins (IGFBPs) regulate the biological function of IGF1, for example by acting as carrier proteins for BBB transport and by prolonging IGF1's half-life. IGFBP3 is the most common IGFBP accounting for about 80% of IGF1 binding in the circulation. Though IGF1 has been shown to cross the BBB, the proportion of protein that reaches the brain after systemic administration is likely limited and IGFBP3 might play an essential role in regulation of this process (Pan & Kastin, 2000; Reinhardt & Bondy, 1994; Thorne et al., 2004). Similar to IGF1, systemic IGFBP3 levels have been reported to be low after preterm birth in human infants (Hellstrom et al., 2016). In line with clinical observations, we observed a transient IGFBP3 deficiency in the plasma

at 24 hours after induction of dWMI in newborn mice. Interestingly, early feasibility and pharmacokinetic studies in human preterms use a protein-complex of IGF1/IGFBP3. IGFBP3 was added to the treatment product because of persistently low protein levels of IGF1 and IGFBP3 after preterm birth, and IGF1-independent IGFBP3 actions, including modulation of cell proliferation and survival (Chesik et al., 2008; Ley et al., 2013). In our study, we tested whether the therapeutic potential of intranasal IGF1 could be enhanced by equimolar co-administration of IGFBP3. However, we observed that adding IGFBP3 to the IGF1 complex did not lead to superior effects on dWMI compared to IGF1 alone. The discrepancy between our findings and the apparent need of IGFBP3 addition in other (clinical) studies could be explained by the mode of administration. While it is possible that IGFBP3 addition is essential for the delivery of systemically administered IGF1 to the CNS, intranasally administered IGF1 was previously shown to bypass the BBB. Thorne et al. (2004) confirmed rapid diffusion of IGF1 throughout the entire brain after intranasal application, entering the brain via the olfactory system and trigeminal nerve within 30 minutes. Intranasal IGF1 administration might provide a less invasive, rapid and direct route to target the preterm brain. Alternatively, the observed increase in local IGFBP3 levels after induction of experimental dWMI could account for the limited additive effect of IGFBP3 when administrated intranasally.

One of the challenges for future clinical translation of IGF1 treatment is the determination of the optimal treatment protocol. In this study, we propose a lowest effective dose of 25µg daily applied intranasally during six consecutive days from P5 to P10, based on histological and functional outcome parameters. Earlier studies on IGF1 treatment have reported on a slightly higher dose of 50µg IGF1 to be effective in reducing pre-OL loss and improvement of myelination in other models of (neonatal) brain injury, using either intranasal or more invasive intracranial or intracerebroventricular administration routes (Brywe et al., 2005; Cai et al., 2011; Guan et al., 1993; Lin et al., 2005; Lin et al., 2009; Wood et al., 2007). In our study we did not observe a superior effect of 50 µg IGF1 treatment compared to 25 µg dosing. This is in line with studies by Pang et al. (2010) and Cao et al. (2003) showing that higher doses of IGF1 were ineffective or even exacerbated brain injury. Interestingly, the concentration of IGF1 that was detected in the frontal and posterior brain after intranasal administration of 25 µg IGF1 in our model did fall into the range reported in previous studies with intranasal dose of 50 µg IGF1 (Cai et al., 2011). However, direct comparison between our study and these previous studies is challenging due to essential differences in

the mode and duration of administration and/or pathophysiology of WMI. For example, the optimal IGF1 treatment protocol in earlier studies has been aimed at early neuroprotection after hypoxic-ischemic encephalopathy in *term* neonates or severe cystic WMI in preterm neonates, with the majority of studies applying a single IGF1 dose shortly after injury (within 16 hours) to prevent OL lineage cell death and subsequent myelination deficits (Cai et al., 2011; Lin et al., 2009; Wood et al., 2007). In contrast, in diffuse forms of WMI myelination deficits are believed to result from impaired development or maturational arrest of the OL lineage, without overt loss of cells. We therefore suggest that the underlying pathophysiological mechanisms of specific types of neonatal brain injury might determine the optimal dose, length and timing of IGF1 treatment. Moreover, in the current study we observed a specific optimal treatment regime of daily intranasal dosing of 25 µg IGF1 on P5-P10, with partial loss of treatment efficacy after shortening or postponement of IGF1 treatment. Though we showed that the drop in endogenous systemic and cerebral IGF1 levels was recovered at P8, shortening of treatment to P5-8 reduced histological and functional recovery compared to IGF1 treatment from P5-10. These data could imply that instead of restoring IGF1 levels to physiological levels, supraphysiological levels of IGF1 might be needed for effective white matter regeneration after dWMI for an extended period of time. Delay of IGF1 treatment, starting treatment at P7 instead of P5, also led to a reduction in treatment efficacy, implying a limited treatment window of intranasal IGF1 therapy after dWMI and indicating that IGF1 therapy should be started very early after (extremely) preterm birth. Towards clinical translation, this could imply that all infants born extremely preterm should receive intranasal IGF1 treatment, as selecting patients most at risk to develop dWMI in the upcoming weeks of life is still impossible. This is the result of the multifactorial etiology of dWMI, with multiple perinatal and (early) postnatal insults that potentially interfere with early brain development, eventually leading to insufficient myelination at 32 weeks of gestation and onwards (van Tilborg et al., 2016). At this time, validated biomarkers for early identification of preterm neonates at risk for dWMI, and thus useful in selection of patients that could benefit from IGF1 therapy, are still lacking. Additional *in vitro* and *in vivo* studies are needed to explore the treatment window and feasibility of intranasal IGF1 therapy in even more detail as well as the mechanisms underlying reduced treatment efficacy.

To gain more insight in the repair mechanisms of IGF1 treatment on the white matter, primary cultures of glial cells were used. Over the years, a wide range of *in vitro* studies have reported an important role for IGF1 in healthy development of the OL

lineage, by boosting proliferation and differentiation (Masters et al., 1991; McMorris et al., 1986; Roth et al., 1995; Wilson et al., 2003). Moreover, multiple *in vitro* studies have shown that IGF1 can directly boost OL survival after exposure to WMI-associated stimuli, such as hypoxia or inflammation (Ness et al., 2004; Pang et al., 2007; Wood et al., 2007; Ye & D'Ercole, 1999). In line with these studies, we report here that IGF1 can directly promote differentiation of OL lineage cells after an inflammation-induced maturation arrest. Aside from a direct effect of IGF1 on the OL lineage, IGF1 has also been reported to affect neuroinflammation. Hence, IGF1 treatment could contribute to a more favorable microenvironment for white matter development thereby indirectly promoting OL maturation. Here we show that intranasal IGF1 treatment after dWMI selectively dampens astrocyte reactivity, while microglia activation is not affected. Our western blot data confirm that IGF1 exposure of LPS-challenged primary astrocytes resulted in phosphorylation of the receptor and downstream activation, whilst IGF1 exposure of LPS-activated microglia did not result in activation of the IGFR nor downstream signaling, implying that IGF1 might not directly act on mouse microglia. Whether the limited effect of IGF1 on microglia is caused by very low expression of the IGFR or by inability of the receptor to be activated is not clear. Other *in vitro* studies report conflicting results on IGF1 effects on microglia and astrocytes. For instance, Cai et al. (2011) showed that though activation of microglia was dampened after intranasal IGF1 application, the expression profile of inflammatory cytokines was not altered, implying that IGF1 did not have a direct anti-inflammatory effect but rather prevents further inflammation induced by OL cell death. For primary mouse astrocytic cultures, Genis et al. (2014) demonstrated a decrease in astrocytic ROS production after IGF1 treatment. Similarly, Bellini et al. (2011) showed that IGF1 reduced the expression of toll-like-receptor 4, IL1b, IL6 and TNFa in LPS-activated primary astrocytes. In contrast, Grinberg et al. (2013) observed reduced microglial, but not astrocytic, ROS and TNFa production in rat hippocampal slices after IGF1 treatment. The differences in IGF1 effects on neuroinflammation might be explained by the different species or cell origins used and by the differences in IGFR expression and IGFR signaling. Collectively, these data imply that the regenerative effect of IGF1 on the white matter in our dWMI model is likely mediated by primarily a direct effect of IGF1 on OL lineage maturation together with a beneficial dampening of astrocyte activation.

In summary, the current study shows that early and prolonged intranasal IGF1 treatment is a potent therapeutic strategy to support white matter development and

improve functional outcome in a mouse model of preterm dWMI. Our results imply that IGF1 potently supports maturation of the OL lineage and selectively modulates astrocyte reactivity to boost myelination in the injured preterm brain. Thus, intranasal application of IGF1 may hold great future promise to treat infants after extremely preterm birth to support their brain development and thereby enhance quality of life.

Acknowledgements

This work was funded by Brain Foundation the Netherlands. We would like to thank Roeland Lokhorst, Mirjam Maas, Rebecca Kleisen, and Karima Amarouchi for their technical support.

REFERENCES

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathologica, 134*(3), 331-349. doi:10.1007/s00401-017-1718-6

Back, S. A., & Miller, S. P. (2014). Brain injury in premature neonates: A primary cerebral dysmaturation disorder? *Annals of Neurology, 75*(4), 469-486. doi:10.1002/ana.24132

Beck, K. D., Powell-Braxton, L., Widmer, H. R., Valverde, J., & Hefti, F. (1995). Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. *Neuron, 14*(4), 717-730. doi:10.1016/0896-6273(95)90216-3

Bellini, M. J., Hereñú, C. B., Goya, R. G., & Garcia-Segura, L. M. (2011). Insulin-like growth factor-I gene delivery to astrocytes reduces their inflammatory response to lipopolysaccharide. *J Neuroinflammation, 8*, 21. doi:10.1186/1742-2094-8-21

Brywe, K. G., Mallard, C., Gustavsson, M., Hedtjarn, M., Leverin, A. L., Wang, X., . . . Hagberg, H. (2005). IGF-I neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3beta? *Eur J Neurosci, 21*(6), 1489-1502. doi:10.1111/j.1460-9568.2005.03982.x

Cai, Z., Fan, L. W., Lin, S., Pang, Y., & 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., . . . Guan, J. (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*(6), 739-747. doi:10.1097/01.wcb.0000067720.12805.6f

Chen, Y., Balasubramaniyan, V., Peng, J., Hurlock, E. C., Tallquist, M., Li, J., & Lu, Q. R. (2007). Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc, 2*(5), 1044-1051. doi:10.1038/nprot.2007.149

Chesik, D., De Keyser, J., & Wilczak, N. (2008). Insulin-like growth factor system regulates oligodendroglial cell behavior: therapeutic potential in CNS. *J Mol Neurosci, 35*(1), 81-90. doi:10.1007/s12031-008-9041-2

Craig, A., Ling Luo, N., Beardsley, D. J., Wingate-Pearse, N., Walker, D. W., Hohimer, A. R., & Back, S. A. (2003). Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Exp Neurol, 181*(2), 231-240. doi:10.1016/s0014-4886(03)00032-3

Davila, D., Piriz, J., Trejo, J. L., Nunez, A., & Torres-Aleman, I. (2007). Insulin and insulin-like growth factor I signalling in neurons. *Front Biosci, 12*, 3194-3202. doi:10.2741/2306

Genis, L., Dávila, D., Fernandez, S., Pozo-Rodrigálvarez, A., Martínez-Murillo, R., & Torres-Aleman, I. (2014). Astrocytes require insulin-like growth factor I to protect neurons against oxidative injury. *F1000Res, 3*, 28. doi:10.12688/f1000research.3-28.v2

Gluckman, P., Klempt, N., Guan, J., Mallard, C., Sirimanne, E., Dragunow, M., . . . Nikolics, K. (1992). A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun, 182*(2), 593-599. doi:10.1016/0006-291x(92)91774-k

Grinberg, Y. Y., Dibbern, M. E., Levasseur, V. A., & Kraig, R. P. (2013). Insulin-like growth factor-1 abrogates microglial oxidative stress and TNF-alpha responses to spreading depression. *J Neurochem, 126*(5), 662-672. doi:10.1111/jnc.12267

Guan, J., Bennet, L., George, S., Wu, D., Waldvogel, H. J., Gluckman, P. D., . . . Gunn, A. J. (2001). Insulin-like growth factor-1 reduces postischemic white matter injury in fetal sheep. *J Cereb Blood Flow Metab, 21*(5), 493-502. doi:10.1097/00004647-200105000-00003

Guan, J., Williams, C., Gunning, M., Mallard, C., & Gluckman, P. (1993). The effects of IGF-1 treatment after hypoxic-ischemic brain injury in adult rats. *J Cereb Blood Flow Metab, 13*(4), 609-616. doi:10.1038/jcbfm.1993.79

Hansen-Pupp, I., Hellstrom-Westas, L., Cilio, C. M., Andersson, S., Fellman, V., & Ley, D. (2007). Inflammation at birth and the insulin-like growth factor system in very preterm infants. *Acta Paediatr, 96*(6), 830-836. doi:10.1111/j.1651-2227.2007.00276.x

Hansen-Pupp, I., Hovel, H., Hellstrom, A., Hellstrom-Westas, L., Lofqvist, C., Larsson, E. M., . . . Ley, D. (2011). Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J Clin Endocrinol Metab, 96*(4), 1129-1135. doi:10.1210/jc.2010-2440

Hansen-Pupp, I., Hovel, H., Lofqvist, C., Hellstrom-Westas, L., Fellman, V., Huppi, P. S., . . . Ley, D. (2013). Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatr Res, 74*(5), 564-569. doi:10.1038/pr.2013.135

Hellstrom, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Lofqvist, C., van Marter, L., . . . Smith, L. E. (2016). Insulin-like growth factor 1 has multisystem effects on foetal and preterm infant development. *Acta Paediatr, 105*(6), 576-586. doi:10.1111/apa.13350

Hellström, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Ramenghi, L. A., Löfqvist, C., . . . Hård, A. L. (2016). Role of Insulinlike Growth Factor 1 in Fetal Development and in the Early Postnatal Life of Premature Infants. *Am J Perinatol, 33*(11), 1067-1071. doi:10.1055/s-0036-1586109

Labandeira-Garcia, J. L., Costa-Besada, M. A., Labandeira, C. M., Villar-Cheda, B., & Rodríguez-Perez, A. I. (2017). Insulin-Like Growth Factor-1 and Neuroinflammation. *Frontiers in Aging Neuroscience, 9*, 365-365. doi:10.3389/fnagi.2017.00365

Larroque, B., Ancel, P. Y., Marret, S., Marchand, L., Andre, M., Arnaud, C., . . . Kaminski, M. (2008). Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study. *The Lancet, 371*(9615), 813-820. doi:10.1016/s0140-6736(08)60380-3

Lee, W. H., Clemens, J. A., & Bondy, C. A. (1992). Insulin-like growth factors in the response to cerebral ischemia. *Mol Cell Neurosci, 3*(1), 36-43. doi:10.1016/1044-7431(92)90006-n

Lee, W. H., Wang, G. M., Seaman, L. B., & Vannucci, S. J. (1996). Coordinate IGF-I and IGFBP5 Gene Expression in Perinatal Rat Brain after Hypoxia-Ischemia. *Journal of Cerebral Blood Flow & Metabolism, 16*(2), 227-236. doi:10.1097/00004647-199603000-00007

Ley, D., Hansen-Pupp, I., Niklasson, A., Domellof, M., Friberg, L. E., Borg, J., . . . Hellstrom, A. (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*(1), 68-74. doi:10.1038/pr.2012.146

Lin, S., Fan, L. W., Pang, Y., Rhodes, P. G., Mitchell, H. J., & Cai, Z. (2005). IGF-1 protects oligodendrocyte progenitor cells and improves neurological functions following cerebral hypoxia-ischemia in the neonatal rat. *Brain Res, 1063*(1), 15-26. doi:10.1016/j.brainres.2005.09.042

Lin, S., Fan, L. W., Rhodes, P. G., & Cai, Z. (2009). Intranasal administration of IGF-1 attenuates hypoxic-ischemic brain injury in neonatal rats. *Exp Neurol, 217*(2), 361-370. doi:10.1016/j.expneurol.2009.03.021

Masters, B. A., Werner, H., Roberts, C. T., Jr., LeRoith, D., & Raizada, M. K. (1991). Insulin-like growth factor I (IGF-I) receptors and IGF-I action in oligodendrocytes from rat brains. *Regul Pept, 33*(2), 117-131. doi:10.1016/0167-0115(91)90207-W

McMorris, F. A., Smith, T. M., DeSalvo, S., & Furlanetto, R. W. (1986). Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development. *Proc Natl Acad Sci U S A, 83*(3), 822-826. doi:10.1073/pnas.83.3.822

Ness, J. K., Scaduto, R. C., Jr., & Wood, T. L. (2004). IGF-I prevents glutamate-mediated bax translocation and cytochrome C release in O4+ oligodendrocyte progenitors. *Glia, 46*(2), 183-194. doi:10.1002/glia.10360

Pan, W., & Kastin, A. J. (2000). Interactions of IGF-1 with the blood-brain barrier in vivo and in situ. *Neuroendocrinology, 72*(3), 171-178. doi:10.1159/000054584

Pang, Y., Campbell, L., Zheng, B., Fan, L., Cai, Z., & Rhodes, P. (2010). Lipopolysaccharide-activated microglia induce death of oligodendrocyte progenitor cells and impede their development. *Neuroscience, 166*(2), 464-475. doi:10.1016/j.neuroscience.2009.12.040

Pang, Y., Zheng, B., Fan, L. W., Rhodes, P. G., & Cai, Z. (2007). IGF-1 protects oligodendrocyte progenitors against TNFalpha-induced damage by activation of PI3K/Akt and interruption of the mitochondrial apoptotic pathway. *Glia, 55*(11), 1099-1107. doi:10.1002/glia.20530

Reinhardt, R. R., & Bondy, C. A. (1994). Insulin-like growth factors cross the blood-brain barrier. *Endocrinology, 135*(5), 1753-1761. doi:10.1210/endo.135.5.7525251

Roth, G. A., Spada, V., Hamill, K., & Bornstein, M. B. (1995). Insulin-like growth factor I increases myelination and inhibits demyelination in cultured organotypic nerve tissue. *Brain Res Dev Brain Res, 88*(1), 102-108.

Salmaso, N., Jablonska, B., Scafidi, J., Vaccarino, F. M., & Gallo, V. (2014). Neurobiology of premature brain injury. *Nature Neuroscience, 17*(3), 341-346. doi:10.1038/nn.3604

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods, 9*(7), 671-675. doi:10.1038/nmeth.2089

Sivakumar, V., Ling, E.-A., Lu, J., & Kaur, C. (2010). Role of glutamate and its receptors and insulin-like growth factors in hypoxia induced periventricular white matter injury. *Glia, 58*(5), 507-523. doi:10.1002/glia.20940

Thorne, R. G., Pronk, G. J., Padmanabhan, V., & Frey, W. H., 2nd. (2004). Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. *Neuroscience, 127*(2), 481-496. doi:10.1016/j.neuroscience.2004.05.029

Torres-Aleman, I. (2010). Toward a comprehensive neurobiology of IGF-I. *Dev Neurobiol, 70*(5), 384-396. doi:10.1002/dneu.20778

Vaes, J. E. G., van Kammen, C. M., Trayford, C., van der Toorn, A., Ruhwedel, T., Benders, M., . . . Nijboer, C. H. (2021). Intranasal mesenchymal stem cell therapy to boost myelination after encephalopathy of prematurity. *Glia, 69*(3), 655-680. doi:10.1002/glia.23919

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., & Nijboer, C. H. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Progress in Neurobiology, 136*(1873-5118 (Electronic)), 28-49. doi:10.1016/j.pneurobio.2015.11.002

van Tilborg, E., van Kammen, C. M., de Theije, C. G. M., van Meer, M. P. A., Dijkhuizen, R. M., & Nijboer, C. H. (2017). A quantitative method for microstructural analysis of myelinated axons in the injured rodent brain. *Sci Rep, 7*(1), 16492-16492. doi:10.1038/s41598-017-16797-1

Volpe, J. J. (2009). The encephalopathy of prematurity--brain injury and impaired brain development inextricably intertwined. *Seminars in Pediatric Neurology, 16*(4), 167-178. doi:10.1016/j.spen.2009.09.005

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., & Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *International Journal of Developmental Neuroscience, 29*(4), 423-440. doi:10.1016/j.ijdevneu.2011.02.012

Wilson, H. C., Onischke, C., & Raine, C. S. (2003). Human oligodendrocyte precursor cells in vitro: phenotypic analysis and differential response to growth factors. *Glia, 44*(2), 153-165. doi:10.1002/glia.10280

Wood, T. L., Loladze, V., Altieri, S., Gangoli, N., Levison, S. W., Brywe, K. G., . . . Hagberg, H. (2007). Delayed IGF-1 administration rescues oligodendrocyte progenitors from glutamate-induced cell death and hypoxic-ischemic brain damage. *Dev Neurosci, 29*(4-5), 302-310. doi:10.1159/000105471

Wrigley, S., Arafa, D., & Tropea, D. (2017). Insulin-Like Growth Factor 1: At the Crossroads of Brain Development and Aging. *Front Cell Neurosci, 11*, 14. doi:10.3389/fncel.2017.00014

Ye, P., Carson, J., & D'Ercole, A. J. (1995). In vivo actions of insulin-like growth factor-I (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice. *J Neurosci, 15*(11), 7344-7356.

Ye, P., & D'Ercole, A. J. (1999). Insulin-like growth factor I protects oligodendrocytes from tumor necrosis factor-alpha-induced injury. *Endocrinology, 140*(7), 3063-3072. doi:10.1210/endo.140.7.6754

Zanier, E. R., Fumagalli, S., Perego, C., Pischiutta, F., & De Simoni, M.-G. (2015). Shape descriptors of the "never resting" microglia in three different acute brain injury models in mice. *Intensive care medicine experimental, 3*(1), 39-39. doi:10.1186/s40635-015-0039-0

8

Regenerative therapies to restore interneuron disturbances in experimental models of encephalopathy of prematurity

Josine E.G. Vaes, Chantal M. Kosmeijer*, Marthe Kaal*, Rik van Vliet, Myrna J.V. Brandt, Manon J.N.L. Benders and Cora H. Nijboer

**These authors contributed equally to this work*

Published in Int. J. Mol. Sci., 2020 Dec; 22(1):211

ABSTRACT

Encephalopathy of Prematurity (EoP) is a major cause of morbidity in (extreme) preterm neonates. Though the majority of EoP research has focused on failure of oligodendrocyte maturation as an underlying pathophysiological mechanism, recent pioneer work has identified developmental disturbances in inhibitory interneurons to contribute to EoP. Here we investigated interneuron abnormalities in two experimental models of EoP and explored the potential of two promising treatment strategies, namely intranasal mesenchymal stem cells (MSCs) or insulin-like growth factor I (IGF1), to restore interneuron development. In rats, fetal inflammation and postnatal hypoxia led to a transient increase in total cortical interneuron numbers, with a layer-specific deficit in parvalbumin (PV)+ interneurons. Additionally, a transient excess of total cortical cell density was observed, including excitatory neuron numbers. In the hippocampal cornu ammonis (CA) 1 region, long-term deficits in total interneuron numbers and PV+ subtype were observed. In mice subjected to postnatal hypoxia/ischemia and systemic inflammation, total numbers of cortical interneurons remained unaffected; however, subtype analysis revealed a global, transient reduction in PV+ cells and a long-lasting layer-specific increase in vasoactive intestinal polypeptide (VIP)+ cells. In the dentate gyrus, a long-lasting deficit of somatostatin (SST)+ cells was observed. Both intranasal MSC and IGF1 therapy restored the majority of interneuron abnormalities in EoP mice. In line with the histological findings, EoP mice displayed impaired social behavior, which was partly restored by the therapies. In conclusion, induction of experimental EoP is associated with model-specific disturbances in interneuron development. In addition, intranasal MSCs and IGF1 are promising therapeutic strategies to aid interneuron development after EoP.

INTRODUCTION

Preterm birth is a major cause of neonatal brain injury, leading to significant neurodevelopmental morbidity [1–4]. In recent years, preclinical and clinical imaging studies have identified distinct patterns of white and (subtle) gray matter deficits in preterm-born neonates, collectively known as Encephalopathy of Prematurity (EoP). These deficits are believed to originate from impaired brain development with peri- and postnatal insults interfering with a multitude of developmental processes that occur in the third trimester of pregnancy [5–7].

So far, the majority of research in the EoP field has focused on cerebral white matter injury (WMI), an evident finding by neonatal neuroimaging [8–11]. Preterm WMI is mainly characterized by widespread (diffuse) hypomyelination, which is the result of maturation failure of the oligodendrocyte lineage [6,12]. However, novel insights in the neurodevelopmental events that take place in the third trimester, combined with improvements in neuroimaging, have led to increased recognition of subtle gray matter deficits contributing to EoP [7,13,14]. GABAergic interneurons have been shown to be develop throughout the third trimester of human gestation, continuing for several months after birth [13,15,16]. Disturbances in the number and function of interneurons have been frequently proposed in the underlying pathophysiology of neurodevelopmental disorders, such as attention deficit disorder (ADD), autism spectrum disorder (ASD), and mood disorders. Interestingly these are neurodevelopmental disorders highly prevalent in the preterm population [14,17,18]. Thus, impairments in interneuron development could play a significant role in EoP pathophysiology and associated neurodevelopmental outcome.

Due to their critical role in the postnatal development of (cortical) circuits and strong link with neurodevelopmental disorders, pioneer studies have explored the vulnerability of interneurons following preterm birth. Recent findings in human post-mortem tissue have revealed cortical interneuron deficits after preterm birth [19–22]. Aberrations in interneuron development was confirmed in a few, very recent experimental studies, though a large variety of changes in cortical interneuron density and distribution after different preterm-birth related hits were observed [19,21,23–27]. Apart from the limited amount of preclinical evidence on the role of interneurons in EoP pathophysiology, studies exploring potential therapeutic interventions to restore interneuron deficits induced by EoP are currently lacking. Cell- and growth

factor-based therapies have received an increasing amount of attention in the field of neonatal brain injury, including that of preterm white matter injury [28–30]. One of these emerging treatment options is (intranasal) mesenchymal stem cell (MSC) therapy [28,31]. Administration of MSCs has been shown to boost repair of gray and white matter deficits, dampen neuroinflammation, and improve behavioral outcome in models of term neonatal hypoxia-ischemia and preterm white matter injury [32–35]. MSCs are believed to exert their regenerative properties through secretion of trophic and anti-inflammatory factors, providing a cerebral milieu permissive for repair and development [28]. Insulin-like growth factor 1 (IGF1) is a trophic factor of which levels are often very low in preterm infants compared to fetal in utero levels at a corresponding gestational age. IGF1 has been demonstrated to play an essential role in normal brain development, including neurogenesis, neuronal differentiation, and neuronal survival [36–41]. Moreover, IGF1 treatment was shown to repair white matter injury in models of EoP, boosting maturation and survival of oligodendrocytes [42–45].

In the present study, we aimed to assess subtype-specific changes in the numbers and distribution of cortical and hippocampal interneurons in two double-hit rodent models of EoP, in which two clinically relevant hits, i.e., fetal inflammation plus postnatal hypoxia or postnatal hypoxia/ischemia plus systemic inflammation, were combined. Moreover, we are the first to explore the potential of two promising treatment strategies in the field of neonatal brain injury, i.e., intranasal MSCs and IGF1, to restore interneuron deficits and improve sociability in our EoP mouse model.

RESULTS

Fetal Inflammation and Postnatal Hypoxia in Rats Leads to Transient Disturbances in GABAergic Interneuron Distribution in the Cortex and Hippocampus

To study the total number of GABAergic interneurons in rats subjected to fetal inflammation and postnatal hypoxia (FIPH), brains sections were stained for the general interneuron marker GAD67 (glutamate decarboxylase), an enzyme essential for GABA synthesis. CTIP2, a protein primarily expressed by neurons localized in layers V-VI, was used to discriminate between upper (I-VI) and lower (V-VI) cortical layers [46]. At P15, a significant increase in the number of interneurons per mm^2 in both the upper (I-IV) and lower (V-VI) cortical layers of both hemispheres was observed in FIPH rats compared to controls ($p = 0.014$ and $p = 0.003$, respectively) (Figure 1A–C). To gain more insight in interneuron subtype-specific changes, markers for parvalbumin (PV), somatostatin (SST), and vasoactive intestinal polypeptide (VIP) were used. These markers reportedly represent distinct subpopulations of interneurons, covering ~85% of all cortical interneurons [47–49]. At P15, a reduction in PV+ interneuron density in the upper cortical layers was observed in FIPH animals compared to controls ($p = 0.005$), but not in the lower cortical layers ($p = 0.142$) (Figure 1D–F). The number of SST+ and VIP+ interneurons per mm^2 was not affected by FIPH with no differences in either upper or lower cortical layers at P15 in FIPH rats compared to controls (SST: $p = 0.931$ and $p = 0.363$ VIP: $p = 0.131$ and $p = 0.324$ respectively) (Figure 1G–L). In line with our previous data on transient myelination deficits in this FIPH model [50], the observed (layer-specific) changes in GAD67+ and PV+ interneuron density were restored in adulthood (P69) (GAD67: I-IV $p = 0.746$ and V-VI $p = 0.579$ PV: I-IV $p = 0.254$ and V-VI $p = 0.867$; FIPH compared to control rats) (Figure 1M–P).

The influence of FIPH on hippocampal interneuron density was studied in the CA1 and dentate gyrus (DG) regions as, in line with previous studies [51], we observed the majority of interneurons to reside in these regions. A reduction in GAD67+ and PV+ interneuron density was observed in the CA1 region in FIPH animals compared to controls ($p = 0.041$ and $p = 0.025$ respectively), whilst cell density in the DG was unaffected by FIPH ($p = 0.314$ and $p = 0.860$ respectively) (Figure 2A–E). These interneuron changes in the CA1 region persisted into adulthood, with a reduced number of GAD67+ and PV+ cells per mm^2 in FIPH animals compared to controls at P69 ($p = 0.015$ and $p = 0.008$ respectively) (Figure 2I,K). In line with the findings at P15, no abnormalities in GAD67+ or PV+ cell densities were observed in the dentate gyrus

of adult FIPH rats (p = 0.257 and p = 0.287 respectively) (Figure 2J,L). We did not observe any significant changes the number SST+ cells per mm^2 in both the CA1 and DG of FIPH rats compared to controls at P15 (p = 0.549 and p = 0.344 respectively) (Figure 2F–H). As VIP+ cells were hardly/not present in the hippocampal areas, we did not assess this subtype of interneurons in this region.

To investigate gross alterations in cortical development, DAPI+ and CTIP2+ cell counts were performed. Rats subjected to FIPH showed a significant increase in DAPI+ cells per mm^2 throughout the cortex at P15 (p = 0.023), while we did not observe any changes in CTIP2+ cell density in FIPH animals (p = 0.845) (Figure 2M–N). To further explore which cells contribute to the observed increase in cortical cell density, a NeuN/GAD67 staining was carried out. Aside from the reported increase in GAD67+ cell density at P15, we observed a rise in NeuN+GAD67- cells per mm^2 in FIPH animals, implying an excess of excitatory cortical neurons as well (p = 0.001) (Figure 2O–P). These data, in conjunction with the unchanged CTIP2+ cell density, indicate that the increased neuronal numbers are predominantly observed in layers I-IV of the rat cortex. Analysis of cortical development at P69 revealed normalization of cortical cell density in adult FIPH animals to control levels (p = 0.093) (Figure 2Q).

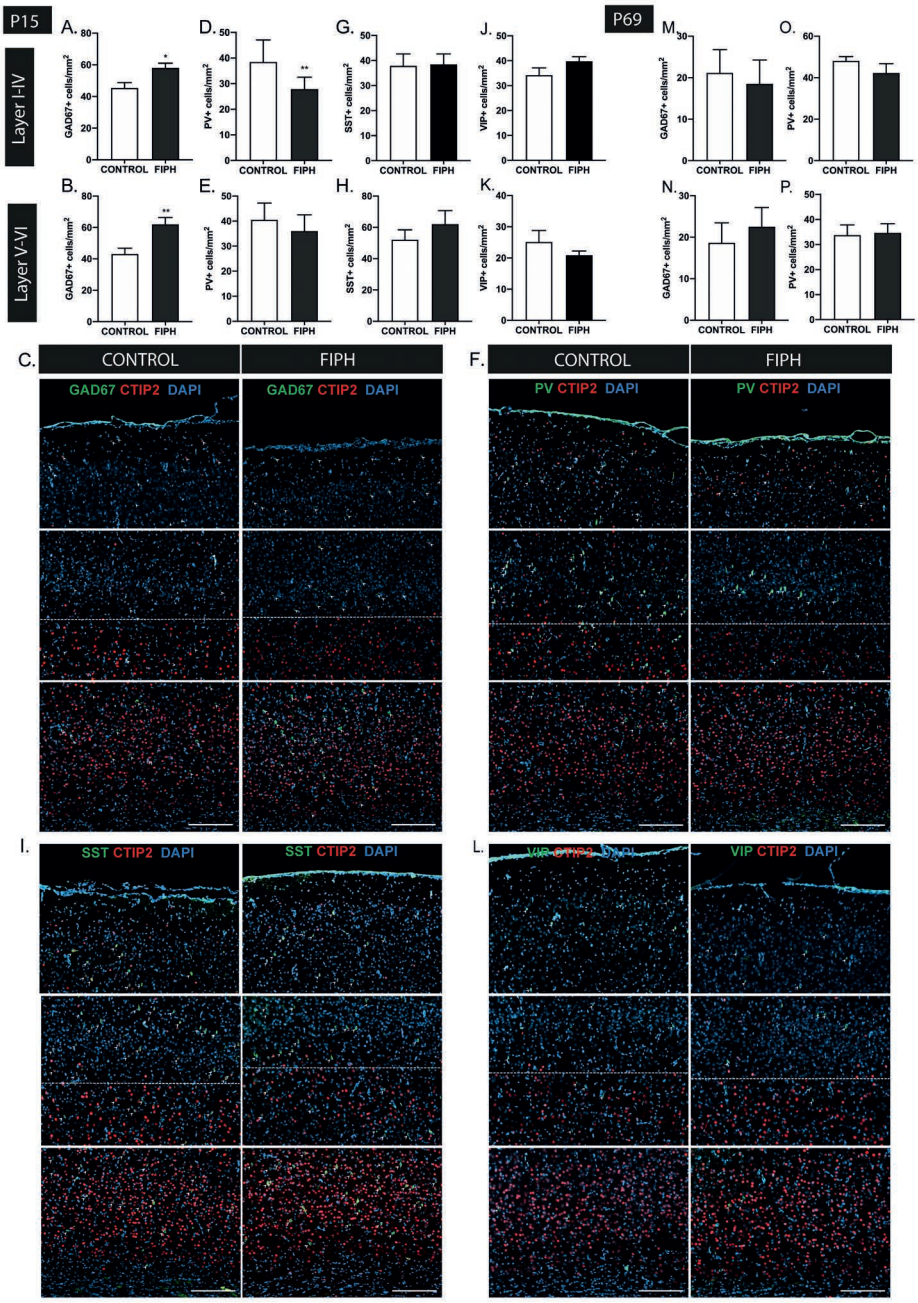


Figure 1. The combination of fetal inflammation and postnatal hypoxia leads to transient disturbances in cortical interneuron density in rats. (**A/B**) Animals exposed to the double-hit model displayed an increase in GAD67+ interneuron density in both the upper (**A**) and lower (**B**) cortical layers at P15 (CONTROL n = 11, fetal inflammation and postnatal hypoxia (FIPH)

n = 10). (**C**) Representative fluorescent images of the P15 cortex of a control rat (left) and FIPH rat (right) stained for the general interneuron marker GAD67 (green) and cortical layer marker CTIP2 (red), counterstained with DAPI (blue). GAD67+ cells are indicated with an arrowhead. Dashed line represents the border between upper (I-IV) and lower (V-VI) cortical layers based on CTIP2 expression. Scale bars: 200 µm. (**D/E**) A significant decrease in PV+ interneurons per mm² was observed in the upper (**D**) but not the lower (**E**) cortical layers in FIPH rats (CONTROL n = 13, FIPH n = 8). (**F**) Representative fluorescent images of the P15 cortex of a control (left) and FIPH animal (right) stained for the interneuron subtype marker PV (green) and cortical layer marker CTIP2 (red), counterstained with DAPI (blue). PV+ cells are indicated with an arrowhead. Dashed line represents the border between upper (I-IV) and lower (V-VI) cortical layers. Scale bars: 200 µm. (**G**/**H**) Induction of FIPH did not alter somatostatin (SST)+ cell density at P15 in the upper (**G**) or lower (**H**) cortical layers (CONTROL n = 9, FIPH n = 9). (**I**) Representative fluorescent images of the P15 cortex of control (left) and FIPH animal (right) stained for the interneuron subtype marker SST (green) and cortical layer marker CTIP2 (red), counterstained with DAPI (blue). SST+ cells are indicated with an arrowhead. Dashed line represents the border between upper (I-IV) and lower (V-VI) cortical layers. Scale bars: 200 µm. (**J**/**K**) The number of vasoactive intestinal polypeptide (VIP)+ interneurons per mm² was not affected in the upper (**J**) or lower (**K**) cortex in our double-hit rat model of Encephalopathy of Prematurity (EoP) (CONTROL n = 9, FIPH n = 8). (**L**) Representative fluorescent images of the P15 cortex of control (left) and FIPH animal (right) stained for the interneuron subtype marker VIP (green) and cortical layer marker CTIP2 (red), counterstained with DAPI (blue). VIP+ cells are indicated with an arrowhead. Dashed line represents the border between upper (I-IV) and lower (V-VI) cortical layers. Scale bars: 200 µm. (**M**/**N**) At P69, the number of GAD67+ interneurons per mm² endogenously recovered in both the upper (**M**) and lower (**N**) cortical regions of FIPH animals (CONTROL n = 6, FIPH n = 6). (**O**/**P**) Quantification of PV+ interneurons at P69 revealed endogenous restoration of cell density in the upper cortex (**O**) in FIPH animals, while parvalbumin (PV)+ cell density in the lower (**P**) cortical layers remained unaffected (CONTROL n = 9, FIPH n = 10). The same group of animals were used for all stainings at P15 and similarly at P69. Animal numbers differ as images with large artefacts or excessive background staining were excluded. *: $p < 0.05$; **: $p < 0.01$ control vs. FIPH animals at the specified time point.

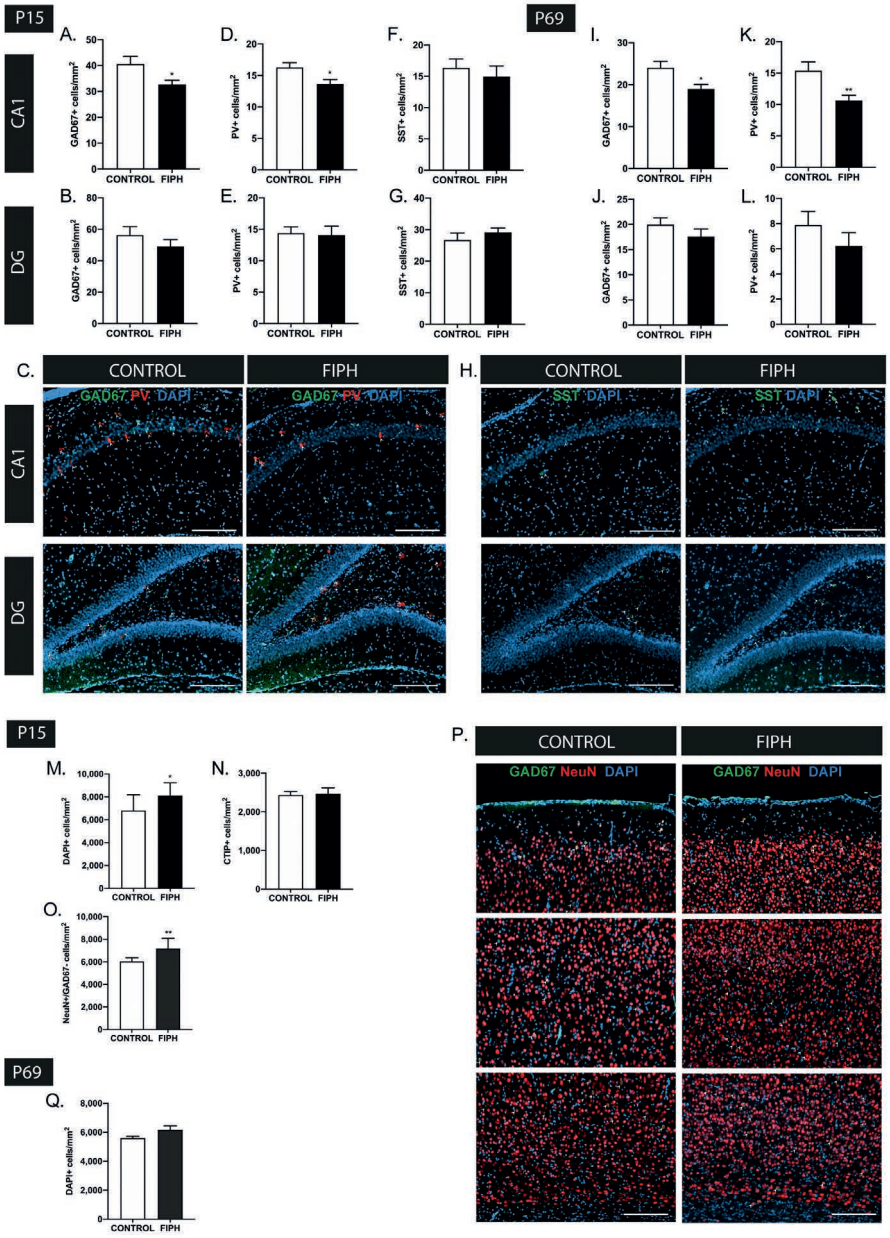


Figure 2. Encephalopathy of Prematurity (EoP) induction is associated with region-specific, persistent changes in hippocampal interneuron density and disruption of cortical development in rats. (**A**/**B**) A significant decrease in GAD67+ cell density was observed in the CA1 region (**A**) of the hippocampus of FIPH animals (n = 8) compared to controls (n = 7) at P15, while GAD67+

cell density in the dentate gyrus (**B**) was not affected. (**C**) Representative fluorescent images of the CA1 region (upper) and dentate gyrus (lower) of the hippocampus of control (left) and FIPH rats (right), stained with interneuron markers GAD67 (green) and PV (red), counterstained with DAPI (blue). GAD67+ cells are indicated with a white arrowhead and PV+ cells are indicated by a red arrowhead. Scale bars: 200 µm. (**D**/**E**) FIPH animals demonstrate a reduction in PV+ interneurons in the CA1 region (**D**), but not the dentate gyrus (**E**) of the hippocampus at P15 (CONTROL n = 8, FIPH n = 7). (**F**/**G**) Induction of FIPH did not impact SST+ interneuron density in the CA1 (**F**) region or dentate gyrus (**G**) of the hippocampus at P15 (CONTROL n = 7, FIPH n = 8). (**H**) Representative fluorescent images of the CA1 region (upper) and dentate gyrus (lower) of the hippocampus of control (left) and FIPH rats (right) stained with interneuron marker SST (green) and counterstained with DAPI (blue). SST+ cells are indicated with a white arrowhead. Scale bars: 200 µm. (**I**/**J**) A reduction in GAD67 cell density was still observed in the CA1 region (**I**) of adult FIPH rats (P69) while the dentate gyrus (**J**) remained unaffected (CONTROL n = 9, FIPH n = 10). (**K**/**L**) Similar to the findings at P15, at P69 FIPH animals showed a reduced amount of PV+ cells per mm^2 in the CA1 region (**K**), but not the dentate gyrus (**L**) of the hippocampus (CONTROL n = 9, FIPH n = 10). (**M**) An increase in DAPI+ cell density was observed in the cortex of FIPH animals at P15 (CONTROL n = 13, FIPH n = 10). (**N**) No changes in CTIP2+ cell density were observed in the cortex of FIPH animals (n = 11), compared to control animals (n = 14). (**O**) A higher quantity of NeuN+GAD67- cells was observed in the cortex of FIPH rats (n = 6) compared to controls (n = 8) at P15, indicative of an excess in excitatory neurons. (**P**) Representative fluorescent images of the cortex of control (left) and FIPH (right) rat, stained for neuronal marker NeuN (red) and interneuron marker GAD67 (green) counterstained with DAPI (blue). White arrowheads indicate double-positive cells. Scale bars: 200 µm. (**Q**) The observed excess in cortical cell density was restored at P69, with no differences in cortical DAPI+ cell numbers between FIPH or control animals (CONTROL n = 10, FIPH n = 9). The same group of animals were used for all stainings at P15 and similarly at P69. Animal numbers differ as images with large artefacts or excessive background staining were excluded. *: $p < 0.05$; **: $p < 0.01$; control vs. FIPH animals at the specified time point.

Intranasal MSC and IGF1 Therapy Restore Subtype-Specific Interneuron Deficits in the Cortex and Hippocampus after Postnatal Hypoxia-Ischemia and Systemic Inflammation in Mice

To investigate the effect of diverse types and timing of insults on interneuron development, we used a second double-hit EoP model in newborn mice combining postnatal hypoxia-ischemia and lipopolysaccharide (HI+LPS) as recently described by our group [32]. Moreover, in this model we assessed the potential of two promising therapies in the field of EoP, namely intranasal MSCs and IGF1, to repair possible EoP-induced interneuron deficits. In contrast to the FIPH rat model, in the mouse model we did not observe any changes in cortical GAD67+ cell density in both hemispheres at 3 weeks (i.e., P26) after injury induction (Layer I-IV: $p > 0.999$ and Layer V-VI $p = 0.648$) (Figure 3A–B). This timepoint was chosen as our earlier study showed EoP-induced myelin deficits at P26 [32]. Interestingly, interneuron subtype stainings revealed a significant decrease in PV+ cells per mm^2 in both the upper ($p = 0.031$) and lower ($p = 0.046$)

cortical layers at P26 (Figure 3C–D). This deficiency in PV+ interneurons throughout the cortex was potently restored following intranasal MSC (layer I-VI $p = 0.005$ and trend in layer V-VI $p = 0.082$) and IGF1 therapy (layer I-VI $p = 0.004$ and layer V-VI $p = 0.014$) (Figure 3C–D). Similar to FIPH rats, SST+ cells density was not affected in P26 mice subjected to HI+LPS compared to sham-controls and both therapies did not show any effect either (layer I-VI $p > 0.999$ and layer V-VI $p = 0.795$) (Figure 3E–F). The decrease in PV+ cells per mm^2 in both cortical layers in HI+LPS mice was accompanied by an increase in VIP+ interneurons in the lower cortical layers ($p = 0.045$) but not in the upper layers ($p = 0.941$) (Figure 3G–H). The increased numbers of VIP+ cells were restored to sham-control level following MSC treatment ($p = 0.007$), while IGF1 treatment did not significantly restore VIP+ cell numbers ($p = 0.491$).

In contrast to the rat FIPH model, we did not observe any hippocampal differences in GAD67+ or PV+ cell density in mice subjected to HI+LPS at P26 (CA1: $p = 0.844$ and $p > 0.999$ DG: $p = 0.923$ and $p > 0.999$ respectively) (Figure 4A–D), nor any effect of the therapies. However, a region-specific reduction in SST+ cells per mm^2 was observed in the DG of HI+LPS mice compared to sham-controls at P26 (CA1: $p = 0.370$ DG: $p = 0.016$) (Figure 4E-F). The reduction in SST+ interneurons in the DG was restored following intranasal MSC therapy ($p = 0.047$), but not after IGF1 therapy ($p = 0.869$) (Figure 4E–F).

In contrast to the observations in the rat FIPH model at adult age, endogenous restoration of myelination in our mouse model, which we earlier determined at P33 [32], was not accompanied by complete resolution of interneuron deficits. Though the reduced cortical PV+ cell density at P26 was restored at P33 (layers I-IV $p = 0.458$ and layers V-VI $p = 0.520$), we observed a reduction in VIP+ interneurons in layers I-VI ($p = 0.006$) and layers V-VI ($p = 0.020$) of HI+LPS mice compared to sham-controls at P33, in contrast to P26 (Figure 5A–D). In addition, the observed deficit in SST+ interneurons observed in the hippocampal DG area of HI+LPS mice at P26, tended to persist at P33 (CA1: $p > 0.999$ DG: $p = 0.08$ (Figure 5E–F).

Contrary to the rat model, we did not observe any gross changes in DAPI+ or CTIP+ cell numbers in the cortices of HI+LPS mice compared to their sham-control littermates at P26 ($p = 0.557$ and $p = 0.807$ respectively) (Figure 5G-H), indicating that cortical density as such was not affected in the HI+LPS mouse model.

To gain more insight in the underlying pathophysiological mechanisms of interneuron deficits in EoP, we measured the expression of Arx, Lhx6, and Sox6, transcription factors reported to play a role in interneuron migration, laminar allocation and maturation [21,24], in our mouse model of EoP. At P6, i.e., 24 h after HI+LPS, we observed a borderline significant increase in Lhx6 expression in the rostral (p = 0.051), but not the caudal section (p = 0.656), of the mouse brain, while Arx and Sox6 expression was not affected (rostral Arx: p = 0.915 Sox6: p = 0.609 caudal Arx: p = 0.633 Sox6: p = 0.905) (Figure 5I–K).

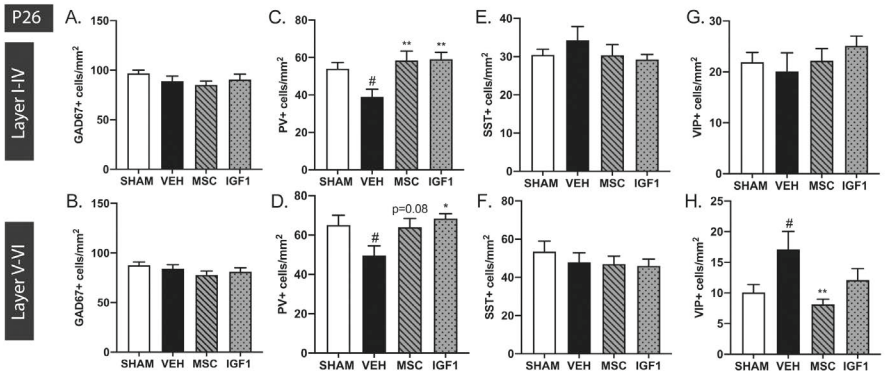


Figure 3. Intranasal mesenchymal stem cells (MSC) or insulin-like growth factor (IGF1) therapy reduces subtype-specific cortical interneuron deficits caused by postnatal hypoxia/ischemia and systemic inflammation in neonatal mice. (**A**/**B**) Injury induction did not lead to changes in GAD67+ cell density in the upper (**A**) or lower (**B**) cortical layers in our validated EoP mouse model at P26 (SHAM n = 12, VEH n = 13, MSC n = 10, IGF1 n = 11). (**C**/**D**) A reduction in PV+ interneuron density was observed in vehicle-treated hypoxia-ischemia and lipopolysaccharide (HI+LPS) animals (n = 13) compared to sham-controls (n = 11) in both the upper (**C**) and lower (**D**) layers of the cortex at P26. Intranasal MSC (n = 10) or IGF1 (n = 10) treatment restored cortical PV+ cell density up to sham-control levels (a trend for MSC treatment-induced recovery of PV+ cells in the lower cortical layers). (**E**/**F**) The number of SST+ interneurons was not affected in the upper (**E**) nor lower (**F**) cortex after HI+LPS (SHAM n = 11, VEH n = 10, MSC n = 10, IGF1 n = 10). (**G**/**H**) Vehicle-treated EoP mice (n = 12) displayed an increase in VIP+ cells per mm² in the lower (**H**) but not the upper (**G**) cortical regions compared to sham-controls (n = 12). Treatment with intranasal MSCs (n = 8) significantly reduced VIP+ cell density. Intranasal IGF1 administration (n = 9) did not significantly affect the amount of VIP+ interneurons after EoP. #: p < 0.05; vehicle-treated HI+LPS animals vs. sham-controls; *: p < 0.05; **: p < 0.01; MSC- or IGF1-treated HI+LPS animals vs. vehicle-treated HI+LPS animals. Nearly significant p values are indicated in d.

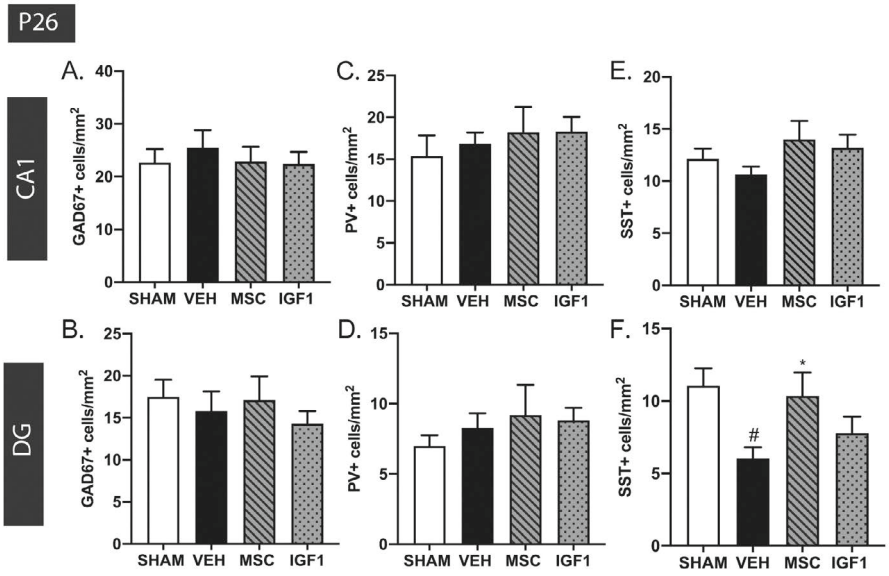


Figure 4. Intranasal administration of MSCs restores SST+ interneuron density deficits in the dentate gyrus. (**A**/**B**) HI+LPS induction did not lead to changes in GAD67+ cell density in the CA1 region (**A**) or dentate gyrus (**B**) of the hippocampus in vehicle-treated HI+LPS mice (SHAM n = 13, VEH n = 14, MSC n = 11, IGF1 n = 10). (**C/D**) HI+LPS induction did not lead to changes in PV+ cells per mm² in hippocampal CA1 (**C**) or dentate gyrus (**D**) (SHAM n = 13, VEH n = 14, MSC n = 11, IGF1 n = 10). (**E/F**) Vehicle-treated HI+LPS animals (n = 10) displayed a strong decrease in SST+ interneuron density in the dentate gyrus (**F**) but not the CA1 region (**E**) compared to sham-controls (n = 7) at P26. Treatment with intranasal MSCs (n = 9) restored SST+ cell density in the dentate gyrus up to sham-control levels. Intranasal IGF1 therapy (n = 11) did not significantly improve SST+ interneuron density in the dentate gyrus of HI+LPS mice. #: $p < 0.05$; vehicle-treated HI+LPS animals vs. sham-controls; *: $p < 0.05$; MSC- treated HI+LPS animals vs. vehicle-treated HI+LPS animals.

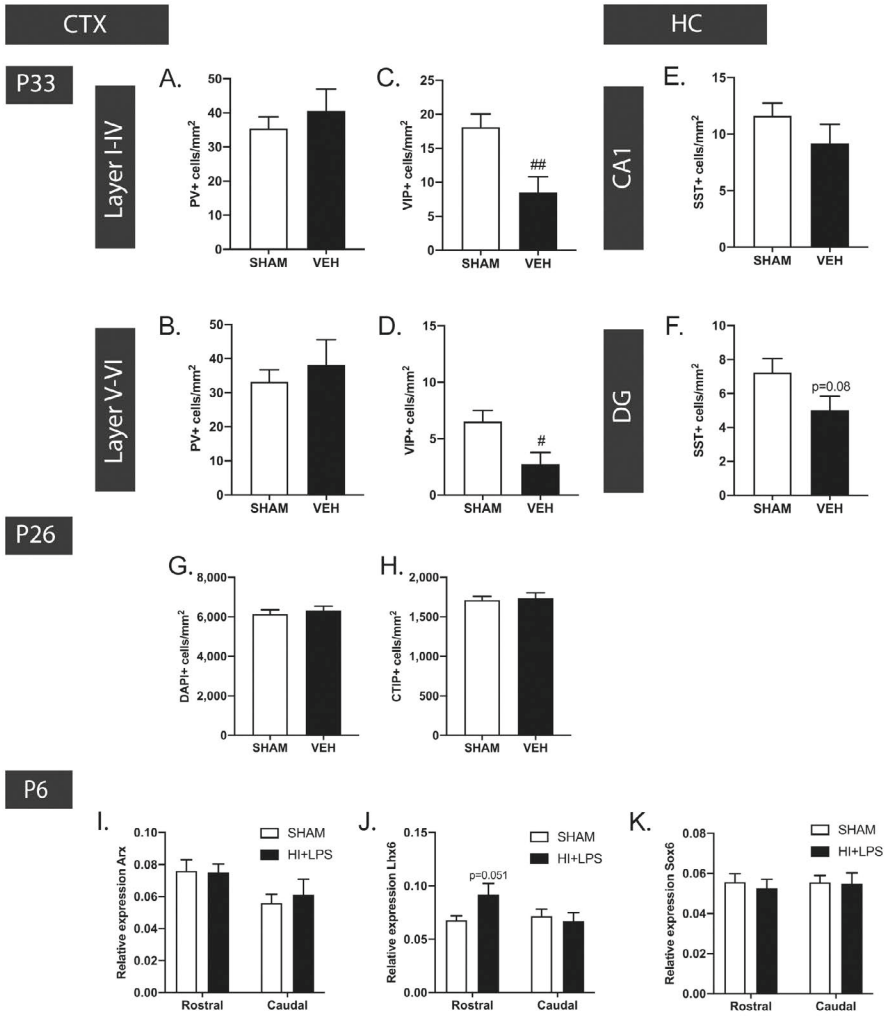


Figure 5. A selection of developmental disturbances in interneurons persist with age. (**A/B**) At P33, the observed changes in PV+ cell density were restored in both the upper (**A**) and lower (**B**) cortex (SHAM n = 12, VEH n = 9). (**C/D**) In contrast to the excess in VIP+ cortical interneurons at P26, a decrease in VIP+ cells per mm² was observed in the upper (**C**) and lower (**D**) layers of the cortex of HI+LPS mice at P33 (SHAM n = 12, VEH n = 8). (**E/F**) The observed SST+ interneuron deficiency in the dentate gyrus (**F**) of HI+LPS mice showed persistence up to P33 (statistical trend), while the hippocampal CA1 region (**E**) remained unaffected (SHAM n = 11, VEH n = 8). (**G/H**) In contrast to the FIPH rat model, cortical cell density, measured by DAPI+ cells/mm² (**G**) and CTIP+ cells/mm² (**H**), was not affected at P26 by HI+LPS (SHAM n = 12, VEH n = 12). (**I–K**) Expression of Lhx6 (**J**) was increased (statistical trend) in the rostral cerebrum of EoP (HI+LPS) animals (n = 4) compared to sham-controls (n = 5) at 24 h after HI+LPS. Induction of EoP did not affect expression of Arx (**I**) and Sox6 (**K**). #: $p < 0.05$; ##: $p < 0.01$ HI+LPS animals vs. sham-controls; nearly significant p values are indicated in **F** and **J.**

Treatment with MSCs or IGF1 Partly Restores Sociability in EoP Mice

The three-chamber task was used to quantify sociability in our EoP mouse model at P26. A deficit in social interaction is a defining feature and early marker of autism-spectrum disorders, a neurodevelopmental disorder with a higher prevalence in the (extreme) preterm infant versus the general population and often associated with interneuron maldevelopment [17,52–57]. Sham-control mice showed a strong preference for the room containing the restrained unknown mouse compared to the chamber containing the object (Figure 6, $p < 0.0001$). Interestingly, vehicle-treated HI+LPS mice failed to show a preference for either the chamber containing the restrained mouse or the chamber with the object ($p > 0.999$) (Figure 6). In other words, when comparing the % of total time spent in the chamber containing the mouse or object between HI+LPS animals and sham-controls, we observed a significant decrease in time spent with the mouse ($p = 0.013$) and significant increase in time spent with the object ($p = 0.004$), indicating reduced sociability after EoP (Figure 6). However, MSC- and IGF1-treated HI+LPS mice displayed a significant preference for the chamber containing the mouse compared to the object ($p = 0.030$ and $p = 0.045$ respectively) (Figure 6). These data imply that HI+LPS in newborn mice leads to a deficit in sociability at P26, which is partly improved by intranasal MSC or IGF1 therapy.

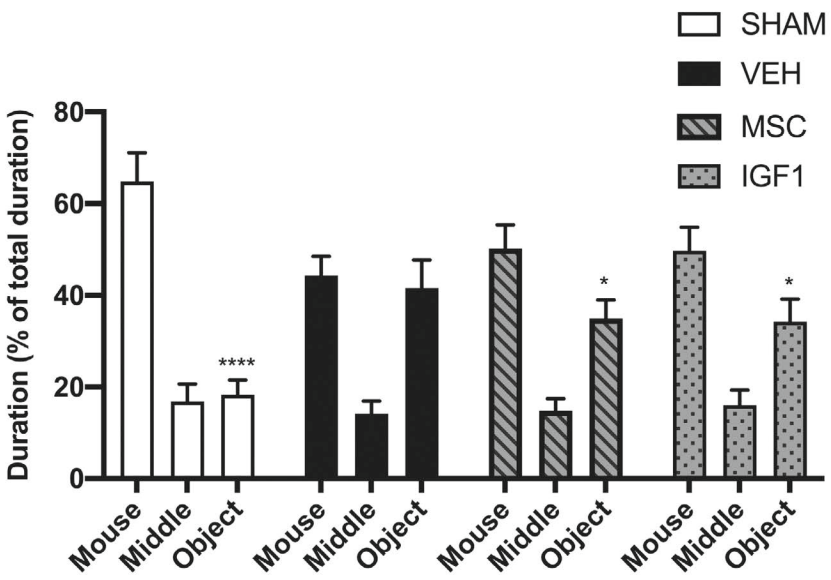


Figure 6. Intranasal treatment with MSCs or IGF1 partially restores sociability in an EoP mouse model. In sharp contrast to sham-operated controls, vehicle-treated EoP mice did not display a preference to spent time in the room containing the novel mouse compared to the novel object. Intranasal MSC or IGF1 partially restored sociability measured using the three chamber test (SHAM n = 6, VEH n = 7, MSC n = 8, IGF1 n = 10). *: $p < 0.05$; ****: $p < 0.0001$ time spent in room with object vs. time spent in room with mouse per experimental group.

DISCUSSION

In the present study, we assessed the distribution and density of interneurons in the cortex and hippocampus in two validated double-hit rodent models of EoP. We show that experimental EoP leads to distinct, model-specific patterns of disturbances in interneuron density and cortical development. Though some of the observed interneuron abnormalities were transient and restored with increasing age, a selection of developmental disturbances in interneurons was shown to persist to ages analogous to puberty or adulthood. Moreover, we demonstrated that EoP leads to a deficit in social behavior in mice, a known hallmark of both interneuron maldevelopment and preterm birth [17,55,58]. Importantly, we are the first to report the effects of two treatment strategies for EoP, i.e., intranasal application of MSCs and IGF1, on restoration of disturbances in interneuron development after EoP: both therapies showed beneficial effects on the majority of anatomical interneuron deficits and partially

recovered impaired social behavior, thereby illustrating their promising potential to aid interneuron development in the preterm brain.

Using GABA as their principal neurotransmitter, interneurons are a heterogeneous population of neurons with distinct anatomical, electrophysiological and molecular features in the over 20 different reported subtypes [49]. Present in a range of brain regions, such as the cortex (~20–30% of all cortical cells) and the hippocampus (~10–15% of the neuronal population), interneurons serve as the principal source of inhibition [51,59]. Hippocampal and cortical interneurons are locally projecting cells that have been implicated in a range of processes including postnatal neuronal circuit maturation, synchronization of cortical rhythms and maintenance of the excitatory/inhibitory balance [16,48,60,61]. The role of interneurons in (postnatal) development of cortical connectivity and their association with neurodevelopmental disorders prevalent in the preterm population has led to a growing interest in interneuron abnormalities in EoP.

Consistent with previous studies that showed susceptibility of interneurons to perinatal insults in animal models and post-mortem human tissue, we observed differences in interneuron density following induction of EoP in both our models [19–21,23–25,62]. However, the previous published studies generally use single-hit (fetal/postnatal inflammation or hypoxia) models, failing to reflect the multifactorial etiology of EoP [19,23–27]. Furthermore, it is good to note that different studies have used different markers of subpopulations of interneurons. In our rats, the combination of fetal inflammation and postnatal hypoxia was associated with a transient increase in total cortical interneuron density. Subtype-specific stainings revealed a transient reduction in PV+ interneurons, restricted to the upper cortical layers. These changes were accompanied by an increase in cell density throughout the cortex with, aside from the observed increase in inhibitory interneurons, a transient excess of excitatory neurons (NeuN+-GAD67- cells). Moreover, we observed a reduction in hippocampal interneurons, specifically PV+ cells, in the CA1 regions of the hippocampus, persisting into adulthood. In contrast, postnatal hypoxia/ischemia and systemic inflammation at P5 in newborn mice led to a reduction of PV+ interneurons in all cortical layers, accompanied by an excess in VIP+ interneurons in the lower cortex. Though the PV+ interneuron population recovered with time, VIP+ cortical interneuron density remained abnormal at the later stage. Unlike the rat model, total cortical cell density was

unaffected by EoP induction in the mouse model. In the hippocampus, a persistent reduction of SST+ interneurons was observed in the dentate gyrus of EoP mice.

Multiple explanations could account for the differences in interneuron deficits between the two EoP models used in this study and to other preclinical models. A multitude of peri- and postnatal events are believed to contribute to EoP [5–7,63]. Diverse patterns of interneuron injury could be the result of differences in timing and the nature of these insults, affecting distinct developmental trajectories. These diverse patterns of injury provide additional insight in the possible mechanisms and timing underlying abnormal interneuron development. First of all, the differential timing of preterm birth-related insults might have affected the generation of specific subpopulations of interneurons, as development of interneuron subpopulations is strictly regulated in time [16]. In this study, interneuron abnormalities were assessed in two distinct EoP models, a rat model incorporating fetal inflammation on E18/19 and postnatal hypoxia on P4, and a mouse model with postnatal hypoxia/ischemia and systemic inflammation on P5. In rodents, the majority of SST+ interneurons are generated during the first half of the neurogenic period in the medial ganglionic eminence (MGE), peaking around E14, whereas PV+ interneurons are produced constantly throughout neurogenesis [16]. This could explain that in experimental models of EoP, often PV+ but not SST+ interneurons are affected [19]. In line with this suggestion, we observed cortical PV+ deficits in both EoP models. However, generation of SST+ and PV+ interneurons destined for the hippocampus have been reported to peak simultaneously, making it unlikely that temporal differences in interneurogenesis account for the model-specific deficits in the hippocampus [51]. Apart from affecting interneurogenesis, preterm birth-related insults could interfere with other developmental interneuron processes, such as tangential migration, laminar allocation, programmed cell death, and maturation of interneurons. Interneurons reportedly migrate towards the cortex between E14.5 and the first postnatal days in rodents, with laminar allocation of cells continuing up to P7 [16]. Therefore, the observed deficits in PV+ interneurons in our FIPH model, limited to the upper cortical layers, might indicate disturbances in tangential migration or impaired laminar allocation after interneurons arrive at the cortical plate after inflammatory hits at E18/19. Impaired migration of interneurons has been reported in other EoP models [21]. Though interneuron progenitors express GAD67 during migration and allocation, it is only after reaching their final location in the hippocampus or cortex that interneurons mature, change morphologically and start expressing their distinctive subtype markers [64,65]. Thus,

the observed increase in GAD67+ cells throughout the cortex of FIPH rats, in absence of an excess of a specific interneuron subtype could possibly reflect a maturational arrest of cortical interneurons. The net absence of GAD67+ abnormalities in our mouse model might be explained by the overshoot of VIP-expressing interneurons, masking the maturation arrest of PV+ cells. Additional evidence for interneuron maturational arrest of interneurons can be found in other preclinical studies [19,23]. In the first postnatal weeks, similar to excitatory neurons, a surplus of interneurons is eliminated via inactivity-dependent programmed cell death, leading to circuit refinement [16]. This process could be affected in our FIPH rat model, with an excess of excitatory neurons and GAD67+ cells in the cortex of injured animals. Interestingly, as the neurogenic peak of the caudal ganglionic eminence (CGE) takes place after that of its medial counterpart, loss of cortical PV+ cells in our mouse model, could possibly induce a compensatory reduction in programmed cell death of CGE-derived cells leading to the observed excessive amounts of VIP+ cells [66]. As the expression of subtype markers in our rat and mouse model was assessed at different ages (P15 vs. P26 respectively) it is possible that the excess of GAD67+ in FIPH is comprised of (immature) CGE-derived cells. Alternatively, the excess of GAD67+ cells in FIPH rats and VIP+ interneurons in EoP mice could be the result of a compensatory proliferative response after suppression of interneuron development or extensive cell loss. Tibrewal et al. [24] demonstrated increased proliferation of interneurons after preterm birth in rabbits. Moreover, Denaxa et al. [67] reported a compensatory increase in the number of interneurons derived from the caudal ganglionic eminence after loss of MGE interneurons. An explorative assessment of the expression of a small selection of transcription factors (Arx, Lhx6, and Sox6), previously shown to regulate interneuron migration and/or maturation, showed a borderline significant increase of Lhx6 expression in the rostral cerebrum of our EoP mice, at 24 h after induction of injury [24,68]. Lhx6 has been shown to regulate migration, laminar sorting and subtype marker expression of MGE-derived interneurons [68]. Lacaille et al. [21] reported similar findings, implying that this increase in Lhx6 reflects a secondary compensatory increase following an initial Lhx6 deficiency. Interestingly, in line with the findings in our EoP mouse model, total interneuron numbers remain unchanged in Lhx6 knockout animals, as CGE-derived interneurons, such as VIP+ cells, compensate for the loss of MGE-derived cells, e.g., PV+ or SST+ interneurons [67,68]. Additional research is needed to determine if the increase in Lhx6 expression is indeed preceded by a decline in mRNA levels. Additionally, future studies should assess the full spectrum of over 20 reported transcription factors that play a role in healthy interneuron devel-

opment to further elucidate the mechanisms underlying interneuron deficits in EoP [59,69]. It is important to note that variations in animal species or region of interest could play a role in the observed disparities between models, as the expression of cortical interneuron markers has been reported to differ between species and cortical regions [70]. Moreover, as mentioned previously, the age at which interneuron deficits have been assessed might play a role in the observed differences. Naturally, it is likely that the observed interneuron abnormalities are the result of a combination of these processes. Further research is warranted to elucidate the exact pathophysiology of interneuron deficits.

Similar to our previous observations in both EoP models regarding transient impairments in the white matter, the majority of interneuron deficits were also restored with age [32,50]. This could imply that in rodent models of EoP interneuron and gross cortical development is delayed rather than irreversibly damaged. Similar observations in interneuron deficits were made in other experimental models and might, to some degree, be explained by the higher regenerative capacity of the rodent central nervous system compared to humans [19,71,72]. In contrast to most cortical observations (except for the persistent cortical VIP+ interneuron aberrations in EoP mice), in both our models hippocampal interneuron abnormalities did persist with age, suggesting a different underlying pathophysiology between regional interneurons. Despite considerable endogenous restoration of interneuron density with progressing age, it is important to note that this does not necessarily equate to proper functioning. Stolp et al. [19] showed that though the absolute number of PV+ cells recovered at P40 in a model of inflammation-induced diffuse WMI, the number of interneurons with perineuronal nets, a morphological marker of functional maturity, was significantly lowered. Similarly, Thion et al. [71] reported impaired synaptic inhibition by interneurons, while cell density had recovered. Thus, even after normalization of interneuron numbers, inhibitory control of the cortex could be permanently affected after EoP. Moreover, even if interneuron numbers and functioning is transiently disturbed, cerebral circuit development could be irreversibly affected [73]. For example, a reduced number of interneurons has been associated with reduced input to subcortical nuclei, leading to persistent volumetric deficits [14]. In line with this hypothesis, we observed volumetric gray matter deficits in our EoP mouse model [32]. Additional studies that include functional outcome parameters, such as morphology, including the formation of perineural nets and electrophysiology of cortical networks,

are needed to determine the long-term effects of preterm birth-related insults on interneuron functionality.

To our knowledge, we are the first to report on the regenerative potential of intranasal MSC and IGF1 therapy to restore aberrant interneuron development in a model of EoP. Aside from our earlier data showing an important role of intranasal MSCs and/or the boosting potential of IGF1 on white matter development [32], here both therapies were shown to restore the majority of interneuron abnormalities after EoP induction in mice. These intranasal therapies therefore seem to target a broad spectrum of processes affected by preterm birth-related insults, thereby aiding in proper development of both the white and gray matter after EoP. Moreover, EoP mice treated with MSCs or IGF1 showed significantly more social interaction, though, after both therapies, their sociability was not completely restored up to sham-control levels. In line with the findings in our mouse model of EoP, we previously demonstrated impaired social behavior in FIPH rats [50]. Thus, the observed deficits in interneuron development in cortex and hippocampus in our EoP models, and the beneficial effects of the therapies on these deficits could partially explain the partial improvement in social behavior. However, multiple brain regions have been implicated to play a role in social functioning, including the prefrontal cortex, basal ganglia, and amygdala and hippocampus [17,74]. Aside from focal lesions, brain injury in other (distant) areas has been shown to impact anatomical structure and functioning of the brain regions involved in social functioning [75]. Furthermore, prior to restoration by regenerative therapies, short-lived aberrant interneuron development, locally or in other connecting brain regions, could possibly still affect long-term (social) functioning. In accordance with this hypothesis, we observed persistent volumetric deficits of the hippocampus, despite early restoration of myelination after intranasal MSC therapy [32]. Moreover, though multiple studies have established an association between social dysfunction and interneuron deficits, we cannot assume 1:1 causality as other underlying mechanisms of EoP might also affect brain regions implicated in social behavior [14]. Though impaired social functioning is a key hallmark of neuropsychiatric disorders that are associated with preterm birth, including ASD, additional studies are needed to determine if our EoP mice exhibit an ASD-like phenotype, including the assessment of other typical ASD behaviors, such as repetitive grooming.

Based on this study, intranasal MSC therapy shows slight superiority in restoration of interneuron abnormalities compared to intranasal IGF1 therapy. This observation might

be explained by the plethora of beneficial (growth) factors secreted by MSCs in the EoP microenvironment compared to one beneficial factor [32]. In our previous study examining the potential of MSCs to restore WMI after EoP, we showed that in vitro exposure of MSCs to the EoP milieu induced distinct secretome changes in MSCs [32]. We observed an upregulation of growth factors associated with interneuron migration and/or maturation, such as hepatocyte growth factor (HGF), nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF) [76–79]. Thus, it is possible that this plethora of MSC-secreted factors underlie the observed regenerative effect of MSCs after EoP. In addition, MSC and IGF1 treatment possibly affect different processes of interneuron development and thereby outcome, due to differences in the timing of administration and/or mechanism of action of the treatments. Though effective, it is currently unclear through which mechanism(s) of action these therapies boost interneuron development. It is possible that the MSCs' secretome or IGF1 directly positively affect interneurons; for example, neuregulins, HGF, GDNF and IGF1 have been implicated in interneuron development [40,77–81]. Moreover, an indirect effect of the treatment, through dampening of neuroinflammation for instance could also contribute to proper interneuron development. For example, astrogliosis and subsequent secretion of BMPs has been shown to halt development of the SST+ interneuron subtype [82]. More insight into the working mechanisms of MSC and IGF1 therapy could be obtained using primary interneuron cultures. Interestingly, restoration of interneuron development could even underlie the earlier observed repair of white matter deficits, as interneurons have been reported to regulate oligodendrocyte maturation by emittance of pro-differentiation cues through transient synaptic input and secreted factors [83,84]. Due to the multifactorial etiology of EoP and distinct clinical course in each patient, it is unlikely that a single animal model represents the entirety of EoP-associated injury. The substantial differences in the pattern of interneuron deficits that were observed in our models and other studies could reflect the range of interneuron deficits that can result from preterm birth. Therefore, the efficacy of MSC or IGF1 therapy to restore interneuron abnormalities should best be confirmed in additional models of EoP.

In summary, induction of EoP led to model-specific alterations in interneuron development, indicating that the vulnerability of interneuron subtypes to preterm birth-related insults is dependent on the specific developmental time-window. Moreover, both intranasal MSCs and IGF1 are promising therapeutic agents to restore atypical interneuron development. This study provides a step forward in understanding the

full spectrum of pathophysiological mechanisms underlying EoP and shows the potential of two regenerative therapies to reduce interneuron maldevelopment in the (extreme) preterm brain.

MATERIALS AND METHODS

Animal Models of Encephalopathy of Prematurity

All animal procedures were carried out according to the Dutch and European guidelines (Directive 86/609, ETS 123, Annex II) and were approved by the Experimental Animal Committee Utrecht (Utrecht University, Utrecht, Netherlands) and the Central Authority for Scientific Procedures on Animals (the Hague, the Netherlands) (project identification code: AVD115002016751, date of approval: 1 December 2016). Animals were kept under standard housing conditions with food and water available ad libitum in a temperature-controlled environment. Both sexes were included in all experiments and randomly assigned to each experimental group.

Rat Model

EoP was induced as described previously [50]. In short, timed-pregnant Wistar rats (Envigo, Horst, the Netherlands) received an intraperitoneal (i.p.) injection of 100 µg/kg lipopolysaccharide (LPS) (Sigma, L2880, Saint Louis, MO, USA), dissolved in sterile 0.9% NaCl, on embryonic day (E) 18 and 19. After birth, postnatal day (P) 4 rat pups were exposed to hypoxia (8% O_2) during 140 min in a temperature-controlled hypoxic chamber. Control dams received 0.9% NaCl i.p. injections on E18 and E19 (1 ml/kg). On P4, control offspring was placed in a normoxic temperature-controlled environment for 140 min. Rats were euthanized by an i.p. overdose of pentobarbital (250 mg/kg) at P15 (i.e., 11 days after EoP induction) and P69 (i.e., 65 days after EoP induction). These time points were based on the previously observed kinetics of dWMI in this model with transient myelination deficits present at P15 and endogenously restored at P69 [50].

Mouse Model

EoP was induced in C57BL/6j mouse pups using a combination of hypoxia-ischemia (HI) and systemic inflammation, as described recently by our group [32], which is an adaptation of the model originally described by from Shen, Plane, and Deng [85]. At P5 HI was induced by permanent unilateral occlusion of the right common carotid artery under isoflurane anesthesia (5–10 min; 5% induction, 3% maintenance with

flow O_2: air 1:1). After a 75-min recovery period, pups were subjected to hypoxia (6% O_2) during 35 min under temperature-controlled conditions (35.8–36.0 °C). Subsequently, animals received an i.p. injection of 1mg/kg LPS (List Biological Laboratories, Campbell, CA, USA), dissolved in sterile 0.9% NaCl. Sham-control littermates underwent isoflurane anesthesia and incision only, without carotid artery occlusion, nor hypoxia nor LPS injection. Mice were euthanized by an i.p. overdose of pentobarbital (250 mg/kg) at P26 (i.e., 21 days after EoP induction) or P33 (i.e., 28 days after EoP induction). Similar to the rat model, we based these time points on our previous study, with evident white matter injury at P26 and endogenous restoration of the observed deficits at P33 [32].

Treatments in the Mouse EoP Model

Mesenchymal Stem Cells

Purchased GIBCO® mouse (C57BL/6) bone marrow-derived MSCs (Invitrogen, S1502-100; Carlsbad, California, USA) were cultured according to the manufacturer's instructions. For all treatments, cells were passaged once (from P2 to P3) prior to administration. Three days after EoP induction (P8), mice received 2 dosages of 2 µl hyaluronidase (12.5 U/µl in total, Sigma-Aldrich, St. Louis, MO, USA) dissolved in H_2O in each nostril (total of 8 µl). Hyaluronidase improves the permeability of the nasal mucosa and is therefore routinely used to facilitate cell delivery [86]. Thirty minutes after hyaluronidase administration, 0.5×10^6 MSCs in dPBS (Thermo Fisher, 14190-169, Waltham, MA, USA) were administered intranasally to the mouse pups, in 2 dosages of 2 µl in each nostril (total of 8 µl). dPBS administration was used as a vehicle treatment. Dose and timing of MSC treatment were based on our earlier study [32].

Insulin-Like Growth Factor 1

Directly following EoP induction, P5 mice received intranasal IGF1 treatment during six consecutive days (i.e., on P5-P10). Mouse pups received a daily dosage of 25 µg recombinant human IGF1 (PeproTech, 100-11, Cranbury, NJ, USA) dissolved in sterile 0.9% NaCl, in 2 nose drops of 2 µl in each nostril (total of 8 µl). Vehicle-treated animals received similar volumes of intranasal 0.9% NaCl.

Arx, Lhx6, and Sox6 Expression in EoP Mice

Sham-control and HI+LPS (untreated) brains were collected at P6, and cerebrum was divided in rostral and caudal sections. Brain sections were snap-frozen in liquid

nitrogen and stored at −80 °C until further processing. Sections were crushed using a mortar and pestle chilled on liquid nitrogen. RNA was isolated using the RNeasy Mini kit (Qiagen, 74104, Hilden, DE) with on-column DNase digestion with the RNase-free DNase set (Qiagen, 79254, Hilden, DE). RNA quantity and quality were assessed by spectrophotometry (NanoDrop 2000, Thermo-scientific, Waltham, MA, USA) at 260 mm and OD 260/280 ratio. Subsequently, cDNA transcription was performed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, 1708840, Hercules, CA, USA). Real-time qPCR was carried out with SYBR Select Master Mix (Thermo Fisher Scientific, 4472908, Waltham, MA, USA) on the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, A28136, Waltham, MA, USA). Primers sequences can be found in Table 1. Mean expression of GAPDH and β-actin were used for data normalization.

Table 1. Overview of primers used in the study.

Symbol	Forward Primer Sequence	Reverse Primer Sequence
Arx	GCACCACGTTCACCAGTTAC	TCTGTCAGGTCCAGCCTCAT
Lhx6	CGTTGAGGAGAAGGTGCTTTGC	GCTTGGGCTGACTGTCCTGTTC
Sox6	ATCTCTCATCCCGGCCTAAGAC	TCCCCAGGCTTCCTCCAATG
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

Three Chamber Social Test in EoP Mice

The three-chamber social test was used to evaluate same-sex sociability. The task, originally described by Nadler et al. [87], was carried out with some adaptations. A rectangular, clear polycarbonate box with three chambers, (all 42.5 cm W × 22.2 cm H; center chamber: 17.8 cm L; side chambers: 19.1 cm L) and dividing walls with retractable doorways, allowing access into each chamber, was used to assess social behavior. Same-sex, unknown (non-litter mates), sham-control mice were used as stimulus mice and were restricted in inverted wire-metal cages that were placed in one of the side-chambers, such that experimental animals had to enter the chamber to initiate social interaction. Testing consisted of two consecutive 10-min stages: 1) habituation in all chambers with empty wire metal cages and 2) the social novelty phase; with the wire cages containing an object or the stimulus sham-control mouse. The location of the stimulus mouse was randomized between mice to avoid side preferences. The experimental set-up was cleaned using a soap solution between all animals to eliminate olfactory stimuli. Investigators were blinded for experimental

groups during assessment and analyses**.** The number of entries and time spent in each of the compartments (center chamber or side chambers with either mouse or object) was measured and calculated as a percentage of total time or chamber entries respectively.

Immunohistochemistry

Following an overdose of pentobarbital, rats or mice were transcardially perfused using PBS followed by 4% PFA in PBS. Brains were post-fixed during 24 h in 4% PFA, followed by a dehydration ethanol series and paraffin embedment. Coronal sections (8 µm) were cut at the level of the hippocampus (–1.8 mm from bregma) in mice and rats, and at the level of the lateral ventricles (bregma) in rats. These brain areas were selected as we observed deficits in the white matter here previously in the respective models [32,50]. For immunofluorescence stainings, sections were deparaffinized using xylene and rehydrated in decreasing concentrations of ethanol. For antigen retrieval, sections were heated to 95 °C in sodium citrate buffer (0.01M, pH 6). After cooling down and three PBS/0.05% Tween20 (PBS-T) washes, non-specific binding was blocked using 5% normal donkey serum in PBS-T. Subsequently, sections were incubated overnight with primary antibodies diluted in PBS (see Table 2 for details and dilutions). The next day, sections were washed three times in PBS and incubated with alexa fluor 594 and 488 conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA; 1:500) for 75 min at room temperature. Sections were counterstained using DAPI (1:5000) and embedded in FluorSave (Merck Millipore, 345789, Burlington, MA, USA).

Table 2. Overview of primary antibodies used in the study.

Antigen	Species (Host)	Company, Product Code	Dilution
Anti-GAD67	Mouse	Sigma, MAB5406	1:200
Anti-PV	Mouse	Sigma, P3088	1:1000
	Rabbit	Novusbio, NB120-11427	1:200
Anti-SST	Mouse	Santa Cruz, SC-74556	1:50
Anti-VIP	Rabbit	ImmunoStar, 20077	1:1000
Anti-CTIP2	Rat	Abcam, ab18465	1:1000
Anti-NeuN	Mouse	Merck Millipore, mab377	1:200
	Rabbit	Abcam, ab177487	1:500

Microscopy and Image Analysis

Investigators were blinded for experimental conditions during image acquisition and analyses**.** Sections were imaged using a Cell Observer microscope with an AxioCam

MRm camera (Zeiss, Oberkochen, Germany). Images of both hemispheres were taken using the 10x objective. In each hemisphere, cortical layers were imaged in two series of two (mouse; four images in total at −1.8mm from bregma) or three (rat; six images in total at bregma) adjacent micrographs (covering the full height of the cortex, layer I-VI) at a fixed distance from the cingulum. In addition, 10x images of the dentate gyrus and CA1 region were obtained in the hippocampal sections. For each animal, the upper (I-IV) and lower (V-VI) cortical layers were delineated based on CTIP2+ staining, a marker primarily expressed by neurons residing in layers V-VI, using AxioVision Rel. 4.8.2 software (Zeiss, Oberkochen, Germany). Subsequently, cells with a clear DAPI+ nucleus and the marker of interest (i.e., either GAD67, PV, SST or VIP) were counted manually using ZEN 2012 software (Zeiss, Oberkochen, Germany) in both the cortex and hippocampus. Areas containing excessively high background staining, non-specific staining, or substantial artefacts were excluded. For the NeuN/CTIP2 staining the total amount of CTIP2+, NeuN+, and DAPI+ cells was counted automatically using the analyze particles plugin for ImageJ v1.47. Cell counts were corrected for the total area per region of interest (upper or lower cortical layers, CA1 or DG) and averaged per animal. Despite unilateral carotid artery occlusion in the mouse model; we did not observe any differences in cortical interneuron density between hemispheres (data not shown). Thus, from here onwards, the reported cortical interneuron density represents the average of both hemispheres in both rats and mice. Due to unilateral hippocampal area loss in our mouse model [32], hippocampal interneurons were counted in the hemisphere contralateral to carotid artery occlusion.

Statistics

Data is presented as mean ± standard error of the mean (SEM). All statistical analyses were carried out using GraphPad Prism 8.3 software. Unpaired t-tests, or in the event of unequal variances non-parametric Mann–Whitney tests, were used for the comparison of two groups. One-way ANOVA with Bonferroni post-hoc tests, or a non-parametric Kruskal–Wallis test with Dunn's post hoc correction in case of unequal variances, was carried out for comparison of multiple groups. p-values < 0.05 were considered statistically significant. Specific sample sizes are mentioned in the figure captions.

Acknowledgments

We would like to thank Caren van Kammen and Diederick Slange for their technical assistance.

REFERENCES

1. Deng, W. Neurobiology of injury to the developing brain. *Nat. Rev. Neurol.* 2010, *6*, 328–336, doi:10.1038/nrneurol.2010.53.
2. Larroque, B.; Ancel, P.Y.; Marret, S.; Marchand, L.; Andre, M.; Arnaud, C.; Pierrat, V.; Roze, J.C.; Messer, J.; Thiriez, G.; et al. Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): A longitudinal cohort study. *Lancet* 2008, *371*, 813–820, doi:10.1016/s0140-6736(08)60380-3.
3. Moster, D.; Lie, R.T.; Markestad, T. Long-term medical and social consequences of preterm birth. *New England Journal of Medicine* 2008, *359*, 262–273, doi:10.1056/NEJMoa0706475.
4. Linsell, L.; Johnson, S.; Wolke, D.; O'Reilly, H.; Morris, J.K.; Kurinczuk, J.J.; Marlow, N. Cognitive trajectories from infancy to early adulthood following birth before 26 weeks of gestation: A prospective, population-based cohort study. *Arch. Dis. Child.* 2018, *103*, 363–370, doi:10.1136/archdischild-2017-313414.
5. Back, S.A.; Miller, S.P. Brain injury in premature neonates: A primary cerebral dysmaturation disorder? *Ann. Neurol.* 2014, *75*, 469–486, doi:10.1002/ana.24132.
6. van Tilborg, E.; Heijnen, C.J.; Benders, M.J.; van Bel, F.; Fleiss, B.; Gressens, P.; Nijboer, C.H. Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Prog. Neurobiol.* 2016, *136*, 28–49, doi:10.1016/j.pneurobio.2015.11.002.
7. Volpe, J.J. Brain injury in premature infants: A complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 2009, *8*, 110–124, doi:10.1016/s1474-4422(08)70294-1.
8. Back, S.A. White matter injury in the preterm infant: Pathology and mechanisms. *Acta Neuropathol.* 2017, *134*, 331–349, doi:10.1007/s00401-017-1718-6.
9. Khwaja, O.; Volpe, J.J. Pathogenesis of cerebral white matter injury of prematurity. *Arch. Dis. Child. Fetal Neonatal Ed.* 2008, *93*, F153–F161, doi:10.1136/adc.2006.108837.
10. de Vries, L.S.; Benders, M.J.N.L.; Groenendaal, F. Imaging the premature brain: Ultrasound or MRI? *Neuroradiology* 2013, *55*, 13–22, doi:10.1007/s00234-013-1233-y.
11. Ment, L.R.; Hirtz, D.; Huppi, P.S. Imaging biomarkers of outcome in the developing preterm brain. *Lancet Neurol.* 2009, *8*, 1042–1055, doi:10.1016/S1474-4422(09)70257-1.
12. Back, S.A.; Luo, N.L.; Borenstein, N.S.; Levine, J.M.; Volpe, J.J.; Kinney, H.C. Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J. Neurosci.* 2001, *21*, 1302–1312, doi:10.1523/JNEUROSCI.21-04-01302.2001.
13. Malik, S.; Vinukonda, G.; Vose, L.R.; Diamond, D.; Bhimavarapu, B.B.R.; Hu, F.; Zia, M.T.; Hevner, R.; Zecevic, N.; Ballabh, P. Neurogenesis continues in the third trimester of pregnancy and is suppressed by premature birth. *J. Neurosci. Off. J. Soc. Neurosci.* 2013, *33*, 411–423, doi:10.1523/JNEUROSCI.4445-12.2013.
14. Fleiss, B.; Gressens, P.; Stolp, H.B. Cortical Gray Matter Injury in Encephalopathy of Prematurity: Link to Neurodevelopmental Disorders. *Front. Neurol.* 2020, *11*, 575–575, doi:10.3389/fneur.2020.00575.
15. Arshad, A.; Vose, L.R.; Vinukonda, G.; Hu, F.; Yoshikawa, K.; Csiszar, A.; Brumberg, J.C.; Ballabh, P. Extended Production of Cortical Interneurons into the Third Trimester of Human Gestation. *Cereb. Cortex* 2016, *26*, 2242–2256, doi:10.1093/cercor/bhv074.
16. Lim, L.; Mi, D.; Llorca, A.; Marín, O. Development and Functional Diversification of Cortical Interneurons. *Neuron* 2018, *100*, 294–313, doi:10.1016/j.neuron.2018.10.009.
17. Marín, O. Interneuron dysfunction in psychiatric disorders. *Nat. Rev. Neurosci.* 2012, *13*, 107–120, doi:10.1038/nrn3155.

18. Marín, O. Developmental timing and critical windows for the treatment of psychiatric disorders. *Nat. Med.* 2016, *22*, 1229–1238, doi:10.1038/nm.4225.
19. Stolp, H.B.; Fleiss, B.; Arai, Y.; Supramaniam, V.; Vontell, R.; Birtles, S.; Yates, A.G.; Baburamani, A.A.; Thornton, C.; Rutherford, M.; et al. Interneuron Development Is Disrupted in Preterm Brains With Diffuse White Matter Injury: Observations in Mouse and Human. *Front. Physiol.* 2019, *10*, 955, doi:10.3389/fphys.2019.00955.
20. Panda, S.; Dohare, P.; Jain, S.; Parikh, N.; Singla, P.; Mehdizadeh, R.; Klebe, D.W.; Kleinman, G.M.; Cheng, B.; Ballabh, P. Estrogen Treatment Reverses Prematurity-Induced Disruption in Cortical Interneuron Population. *J. Neurosci.* 2018, *38*, 7378–7391, doi:10.1523/jneurosci.0478-18.2018.
21. Lacaille, H.; Vacher, C.M.; Bakalar, D.; O'Reilly, J.J.; Salzbank, J.; Penn, A.A. Impaired Interneuron Development in a Novel Model of Neonatal Brain Injury. *eNeuro* 2019, *6*, doi:10.1523/eneuro.0300-18.2019.
22. Robinson, S.; Li, Q.; Dechant, A.; Cohen, M.L. Neonatal loss of gamma-aminobutyric acid pathway expression after human perinatal brain injury. *J. Neurosurg.* 2006, *104*, 396–408, doi:10.3171/ped.2006.104.6.396.
23. Ardalan, M.; Svedin, P.; Baburamani, A.A.; Supramaniam, V.G.; Ek, J.; Hagberg, H.; Mallard, C. Dysmaturation of Somatostatin Interneurons Following Umbilical Cord Occlusion in Preterm Fetal Sheep. *Front. Physiol.* 2019, *10*, 563, doi:10.3389/fphys.2019.00563.
24. Tibrewal, M.; Cheng, B.; Dohare, P.; Hu, F.; Mehdizadeh, R.; Wang, P.; Zheng, D.; Ungvari, Z.; Ballabh, P. Disruption of Interneuron Neurogenesis in Premature Newborns and Reversal with Estrogen Treatment. *J. Neurosci.* 2018, *38*, 1100–1113, doi:10.1523/jneurosci.1875-17.2017.
25. Duchatel, R.J.; Harms, L.R.; Meehan, C.L.; Michie, P.T.; Bigland, M.J.; Smith, D.W.; Jobling, P.; Hodgson, D.M.; Tooney, P.A. Reduced cortical somatostatin gene expression in a rat model of maternal immune activation. *Psychiatry Res.* 2019, *282*, 112621, doi:10.1016/j.psychres.2019.112621.
26. Canetta, S.; Bolkan, S.; Padilla-Coreano, N.; Song, L.J.; Sahn, R.; Harrison, N.L.; Gordon, J.A.; Brown, A.; Kellendonk, C. Maternal immune activation leads to selective functional deficits in offspring parvalbumin interneurons. *Mol. Psychiatry* 2016, *21*, 956–968, doi:10.1038/mp.2015.222.
27. Komitova, M.; Xenos, D.; Salmaso, N.; Tran, K.M.; Brand, T.; Schwartz, M.L.; Ment, L.; Vaccarino, F.M. Hypoxia-induced developmental delays of inhibitory interneurons are reversed by environmental enrichment in the postnatal mouse forebrain. *J. Neurosci.* 2013, *33*, 13375–13387, doi:10.1523/jneurosci.5286-12.2013.
28. Vaes, J.E.G.; Vink, M.A.; de Theije, C.G.M.; Hoebeek, F.E.; Benders, M.; Nijboer, C.H.A. The Potential of Stem Cell Therapy to Repair White Matter Injury in Preterm Infants: Lessons Learned From Experimental Models. *Front. Physiol.* 2019, *10*, 540, doi:10.3389/fphys.2019.00540.
29. van Velthoven, C.T.; Kavelaars, A.; Heijnen, C.J. Mesenchymal stem cells as a treatment for neonatal ischemic brain damage. *Pediatr. Res.* 2012, *71*, 474–481, doi:10.1038/pr.2011.64.
30. Ophelders, D.; Gussenhoven, R.; Klein, L.; Jellema, R.K.; Westerlaken, R.J.J.; Hütten, M.C.; Vermeulen, J.; Wassink, G.; Gunn, A.J.; Wolfs, T. Preterm Brain Injury, Antenatal Triggers, and Therapeutics: Timing Is Key. *Cells* 2020, *9*, 1871, doi:10.3390/cells9081871.
31. Wagenaar, N.; Nijboer, C.H.; van Bel, F. Repair of neonatal brain injury: Bringing stem cell-based therapy into clinical practice. *Dev. Med. Child. Neurol.* 2017, *59*, 997–1003, doi:10.1111/dmcn.13528.

32. Vaes, J.E.G.; van Kammen, C.M.; Trayford, C.; van der Toorn, A.; Ruhwedel, T.; Benders, M.J.N.L.; Dijkhuizen, R.M.; Möbius, W.; van Rijt, S.H.; Nijboer, C.H. Intranasal mesenchymal stem cell therapy to boost myelination after encephalopathy of prematurity. *Glia* 2020, *epub ahead of print,* doi:10.1002/glia.23919.
33. van Velthoven, C.T.; Kavelaars, A.; van Bel, F.; Heijnen, C.J. Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain Behav. Immunity* 2010, *24*, 387–393, doi:10.1016/j.bbi.2009.10.017.
34. Mueller, M.; Oppliger, B.; Joerger-Messerli, M.; Reinhart, U.; Barnea, E.; Paidas, M.; Kramer, B.W.; Surbek, D.V.; Schoeberlein, A. Wharton's Jelly Mesenchymal Stem Cells Protect the Immature Brain in Rats and Modulate Cell Fate. *Stem Cells Dev.* 2017, *26*, 239–248, doi:10.1089/scd.2016.0108.
35. Paton, M.C.B.; Allison, B.J.; Fahey, M.C.; Li, J.; Sutherland, A.E.; Pham, Y.; Nitsos, I.; Bischof, R.J.; Moss, T.J.; Polglase, G.R.; et al. Umbilical cord blood versus mesenchymal stem cells for inflammation-induced preterm brain injury in fetal sheep. *Pediatr. Res.* 2019, 86, 165–173, doi:10.1038/s41390-019-0366-z.
36. Hellstrom, A.; Ley, D.; Hansen-Pupp, I.; Hallberg, B.; Lofqvist, C.; van Marter, L.; van Weissenbruch, M.; Ramenghi, L.A.; Beardsall, K.; Dunger, D.; et al. Insulin-like growth factor 1 has multisystem effects on foetal and preterm infant development. *Acta Paediatr.* 2016, *105*, 576–586, doi:10.1111/apa.13350.
37. Hansen-Pupp, I.; Hovel, H.; Lofqvist, C.; Hellstrom-Westas, L.; Fellman, V.; Huppi, P.S.; Hellstrom, A.; Ley, D. Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatr. Res.* 2013, *74*, 564–569, doi:10.1038/pr.2013.135.
38. Beck, K.D.; Powell-Braxton, L.; Widmer, H.R.; Valverde, J.; Hefti, F. Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. *Neuron* 1995, *14*, 717–730, doi:10.1016/0896-6273(95)90216-3.
39. Hansen-Pupp, I.; Hovel, H.; Hellstrom, A.; Hellstrom-Westas, L.; Lofqvist, C.; Larsson, E.M.; Lazeyras, F.; Fellman, V.; Huppi, P.S.; Ley, D. Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J. Clin. Endocrinol. Metab.* 2011, *96*, 1129–1135, doi:10.1210/jc.2010-2440.
40. Nieto-Estévez, V.; Defterali, Ç.; Vicario-Abejón, C. IGF-I: A Key Growth Factor that Regulates Neurogenesis and Synaptogenesis from Embryonic to Adult Stages of the Brain. *Front. Neurosci.* 2016, *10*, 52, doi:10.3389/fnins.2016.00052.
41. Hellström, A.; Ley, D.; Hansen-Pupp, I.; Hallberg, B.; Ramenghi, L.A.; Löfqvist, C.; Smith, L.E.; Hård, A.L. Role of Insulinlike Growth Factor 1 in Fetal Development and in the Early Postnatal Life of Premature Infants. *Am. J. Perinatol.* 2016, *33*, 1067–1071, doi:10.1055/s-0036-1586109.
42. Cai, Z.; Fan, L.W.; Lin, S.; Pang, Y.; Rhodes, P.G. Intranasal administration of insulin-like growth factor-1 protects against lipopolysaccharide-induced injury in the developing rat brain. *Neuroscience* 2011, *194*, 195–207, doi:10.1016/j.neuroscience.2011.08.003.
43. Lin, S.; Fan, L.W.; Rhodes, P.G.; Cai, Z. Intranasal administration of IGF-1 attenuates hypoxic-ischemic brain injury in neonatal rats. *Exp. Neurol.* 2009, *217*, 361–370, doi:10.1016/j.expneurol.2009.03.021.
44. Guan, J.; Bennet, L.; George, S.; Wu, D.; Waldvogel, H.J.; Gluckman, P.D.; Faull, R.L.; Crosier, P.S.; Gunn, A.J. Insulin-like growth factor-1 reduces postischemic white matter injury in fetal sheep. *J. Cereb. Blood Flow Metab.* 2001, *21*, 493–502, doi:10.1097/00004647-200105000-00003.

45. Cao, Y.; Gunn, A.J.; Bennet, L.; Wu, D.; George, S.; Gluckman, P.D.; Shao, X.M.; Guan, J. 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.* 2003, *23*, 739–747, doi:10.1097/01.wcb.0000067720.12805.6f.
46. Hevner, R.F. Layer-specific markers as probes for neuron type identity in human neocortex and malformations of cortical development. *J. Neuropathol. Exp. Neurol.* 2007, *66*, 101–109, doi:10.1097/nen.0b013e3180301c06.
47. Xu, X.; Roby, K.D.; Callaway, E.M. Immunochemical characterization of inhibitory mouse cortical neurons: Three chemically distinct classes of inhibitory cells. *J. Comp. Neurol.* 2010, *518*, 389–404, doi:10.1002/cne.22229.
48. Tremblay, R.; Lee, S.; Rudy, B. GABAergic Interneurons in the Neocortex: From Cellular Properties to Circuits. *Neuron* 2016, *91*, 260–292, doi:10.1016/j.neuron.2016.06.033.
49. Rudy, B.; Fishell, G.; Lee, S.; Hjerling-Leffler, J. Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. *Dev. Neurobiol.* 2011, *71*, 45–61, doi:10.1002/dneu.20853.
50. van Tilborg, E.; Achterberg, E.J.M.; van Kammen, C.M.; van der Toorn, A.; Groenendaal, F.; Dijkhuizen, R.M.; Heijnen, C.J.; Vanderschuren, L.; Benders, M.; Nijboer, C.H.A. Combined fetal inflammation and postnatal hypoxia causes myelin deficits and autism-like behavior in a rat model of diffuse white matter injury. *Glia* 2018, *66*, 78–93, doi:10.1002/glia.23216.
51. Pelkey, K.A.; Chittajallu, R.; Craig, M.T.; Tricoire, L.; Wester, J.C.; McBain, C.J. Hippocampal GABAergic Inhibitory Interneurons. *Physiol. Rev.* 2017, *97*, 1619–1747, doi:10.1152/physrev.00007.2017.
52. Moy, S.S.; Nadler, J.J.; Perez, A.; Barbaro, R.P.; Johns, J.M.; Magnuson, T.R.; Piven, J.; Crawley, J.N. Sociability and preference for social novelty in five inbred strains: An approach to assess autistic-like behavior in mice. *Genes Brain Behav.* 2004, *3*, 287–302, doi:10.1111/j.1601-1848.2004.00076.x.
53. Silverman, J.L.; Yang, M.; Lord, C.; Crawley, J.N. Behavioural phenotyping assays for mouse models of autism. *Nat. Rev. Neurosci.* 2010, *11*, 490–502, doi:10.1038/nrn2851.
54. Hashemi, E.; Ariza, J.; Rogers, H.; Noctor, S.C.; Martínez-Cerdeño, V. The Number of Parvalbumin-Expressing Interneurons Is Decreased in the Prefrontal Cortex in Autism. *Cereb. Cortex* 2017, *27*, 1931–1943, doi:10.1093/cercor/bhw021.
55. Lunden, J.W.; Durens, M.; Phillips, A.W.; Nestor, M.W. Cortical interneuron function in autism spectrum condition. *Pediatr. Res.* 2019, *85*, 146–154, doi:10.1038/s41390-018-0214-6.
56. Wiebe, S.; Nagpal, A.; Truong, V.T.; Park, J.; Skalecka, A.; He, A.J.; Gamache, K.; Khoutorsky, A.; Gantois, I.; Sonenberg, N. Inhibitory interneurons mediate autism-associated behaviors via 4E-BP2. *Proc. Natl. Acad. Sci. USA* 2019, *116*, 18060, doi:10.1073/pnas.1908126116.
57. Lee, E.; Lee, J.; Kim, E. Excitation/Inhibition Imbalance in Animal Models of Autism Spectrum Disorders. *Biol. Psychiatry* 2017, *81*, 838–847, doi:10.1016/j.biopsych.2016.05.011.
58. Johnson, S.; Marlow, N. Preterm Birth and Childhood Psychiatric Disorders. *Pediatr. Res.* 2011, *69*, 11–18, doi:10.1203/PDR.0b013e318212faa0.
59. Hu, J.S.; Vogt, D.; Sandberg, M.; Rubenstein, J.L. Cortical interneuron development: A tale of time and space. *Development* 2017, *144*, 3867–3878, doi:10.1242/dev.132852.
60. Sohal, V.S.; Rubenstein, J.L.R. Excitation-inhibition balance as a framework for investigating mechanisms in neuropsychiatric disorders. *Mol. Psychiatry* 2019, *24*, 1248–1257, doi:10.1038/s41380-019-0426-0.
61. Kepecs, A.; Fishell, G. Interneuron cell types are fit to function. *Nature* 2014, *505*, 318–326, doi:10.1038/nature12983.

62. Vasistha, N.A.; Pardo-Navarro, M.; Gasthaus, J.; Weijers, D.; Müller, M.K.; García-González, D.; Malwade, S.; Korshunova, I.; Pfisterer, U.; von Engelhardt, J.; et al. Maternal inflammation has a profound effect on cortical interneuron development in a stage and subtype-specific manner. *Mol. Psychiatry* 2020, *25*, 2313–2329, doi:10.1038/s41380-019-0539-5.
63. Volpe, J.J. Dysmaturation of Premature Brain: Importance, Cellular Mechanisms, and Potential Interventions. *Pediatr. Neurol.* 2019, *95*, 42–66, doi:10.1016/j.pediatrneurol.2019.02.016.
64. Laclef, C.; Métin, C. Conserved rules in embryonic development of cortical interneurons. *Semin. Cell Dev. Biol.* 2018, *76*, 86–100, doi:10.1016/j.semcdb.2017.09.017.
65. Wamsley, B.; Fishell, G. Genetic and activity-dependent mechanisms underlying interneuron diversity. *Nat. Rev. Neurosci.* 2017, *18*, 299–309, doi:10.1038/nrn.2017.30.
66. Miyoshi, G.; Fishell, G. GABAergic interneuron lineages selectively sort into specific cortical layers during early postnatal development. *Cereb. Cortex* 2011, *21*, 845–852, doi:10.1093/cercor/bhq155.
67. Denaxa, M.; Neves, G.; Rabinowitz, A.; Kemlo, S.; Liodis, P.; Burrone, J.; Pachnis, V. Modulation of Apoptosis Controls Inhibitory Interneuron Number in the Cortex. *Cell Rep.* 2018, *22*, 1710–1721, doi:10.1016/j.celrep.2018.01.064.
68. Liodis, P.; Denaxa, M.; Grigoriou, M.; Akufo-Addo, C.; Yanagawa, Y.; Pachnis, V. Lhx6 Activity Is Required for the Normal Migration and Specification of Cortical Interneuron Subtypes. *J. Neurosci.* 2007, *27*, 3078, doi:10.1523/JNEUROSCI.3055-06.2007.
69. Kessaris, N.; Magno, L.; Rubin, A.N.; Oliveira, M.G. Genetic programs controlling cortical interneuron fate. *Curr. Opin. Neurobiol.* 2014, *26*, 79–87, doi:10.1016/j.conb.2013.12.012.
70. Gonchar, Y.; Wang, Q.; Burkhalter, A. Multiple distinct subtypes of GABAergic neurons in mouse visual cortex identified by triple immunostaining. *Front. Neuroanat.* 2007, *1*, 3, doi:10.3389/neuro.05.003.2007.
71. Thion, M.S.; Mosser, C.A.; Férézou, I.; Grisel, P.; Baptista, S.; Low, D.; Ginhoux, F.; Garel, S.; Audinat, E. Biphasic Impact of Prenatal Inflammation and Macrophage Depletion on the Wiring of Neocortical Inhibitory Circuits. *Cell Rep.* 2019, *28*, 1119–1126.e1114, doi:10.1016/j.celrep.2019.06.086.
72. Kaplan, H.M.; Mishra, P.; Kohn, J. The overwhelming use of rat models in nerve regeneration research may compromise designs of nerve guidance conduits for humans. *J. Mater. Sci. Mater. Med.* 2015, *26*, 226, doi:10.1007/s10856-015-5558-4.
73. Butt, S.J.; Stacey, J.A.; Teramoto, Y.; Vagnoni, C. A role for GABAergic interneuron diversity in circuit development and plasticity of the neonatal cerebral cortex. *Curr. Opin. Neurobiol.* 2017, *43*, 149–155, doi:10.1016/j.conb.2017.03.011.
74. Sato, W.; Kochiyama, T.; Uono, S.; Yoshimura, S.; Kubota, Y.; Sawada, R.; Sakihama, M.; Toichi, M. Reduced Gray Matter Volume in the Social Brain Network in Adults with Autism Spectrum Disorder. *Front. Hum. Neurosci.* 2017, *11*, 395, doi:10.3389/fnhum.2017.00395.
75. Semple, B.D.; Blomgren, K.; Gimlin, K.; Ferriero, D.M.; Noble-Haeusslein, L.J. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog. Neurobiol.* 2013, *106–107*, 1–16, doi:10.1016/j.pneurobio.2013.04.001.
76. Bae, M.H.; Bissonette, G.B.; Mars, W.M.; Michalopoulos, G.K.; Achim, C.L.; Depireux, D.A.; Powell, E.M. Hepatocyte growth factor (HGF) modulates GABAergic inhibition and seizure susceptibility. *Exp. Neurol.* 2010, *221*, 129–135, doi:10.1016/j.expneurol.2009.10.011.
77. Powell, E.M.; Mars, W.M.; Levitt, P. Hepatocyte Growth Factor/Scatter Factor Is a Motogen for Interneurons Migrating from the Ventral to Dorsal Telencephalon. *Neuron* 2001, *30*, 79–89, doi:10.1016/S0896-6273(01)00264-1.
78. Pozas, E.; Ibáñez, C.F. GDNF and GFRα1 Promote Differentiation and Tangential Migration of Cortical GABAergic Neurons. *Neuron* 2005, *45*, 701–713, doi:10.1016/j.neuron.2005.01.043.

79. Canty, A.J.; Dietze, J.; Harvey, M.; Enomoto, H.; Milbrandt, J.; Ibáñez, C.F. Regionalized Loss of Parvalbumin Interneurons in the Cerebral Cortex of Mice with Deficits in GFRα1 Signaling. *J. Neurosci.* 2009, *29*, 10695, doi:10.1523/JNEUROSCI.2658-09.2009.
80. Mei, L.; Nave, K.-A. Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases. *Neuron* 2014, *83*, 27–49, doi:10.1016/j.neuron.2014.06.007.
81. Hurtado-Chong, A.; Yusta-Boyo, M.J.; Vergaño-Vera, E.; Bulfone, A.; de Pablo, F.; Vicario-Abejón, C. IGF-I promotes neuronal migration and positioning in the olfactory bulb and the exit of neuroblasts from the subventricular zone. *Eur. J. Neurosci.* 2009, *30*, 742–755, doi:10.1111/j.1460-9568.2009.06870.x.
82. Mukhopadhyay, A.; McGuire, T.; Peng, C.Y.; Kessler, J.A. Differential effects of BMP signaling on parvalbumin and somatostatin interneuron differentiation. *Development* 2009, *136*, 2633–2642, doi:10.1242/dev.034439.
83. Benamer, N.; Vidal, M.; Angulo, M.C. The cerebral cortex is a substrate of multiple interactions between GABAergic interneurons and oligodendrocyte lineage cells. *Neurosci. Lett.* 2020, *715*, 134615, doi:10.1016/j.neulet.2019.134615.
84. Zonouzi, M.; Scafidi, J.; Li, P.; McEllin, B.; Edwards, J.; Dupree, J.L.; Harvey, L.; Sun, D.; Hübner, C.A.; Cull-Candy, S.G.; et al. GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury. *Nat. Neurosci.* 2015, *18*, 674–682, doi:10.1038/nn.3990.
85. Shen, Y.; Plane, J.M.; Deng, W. Mouse Models of Periventricular Leukomalacia. *J. Vis. Exp.* 2010, *39*, 1951, doi:10.3791/1951.
86. Danielyan, L.; Schafer, R.; von Ameln-Mayerhofer, A.; Buadze, M.; Geisler, J.; Klopfer, T.; Burkhardt, U.; Proksch, B.; Verleysdonk, S.; Ayturan, M.; et al. Intranasal delivery of cells to the brain. *Eur. J. Cell Biol.* 2009, *88*, 315–324, doi:10.1016/j.ejcb.2009.02.001.
87. Nadler, J.J.; Moy, S.S.; Dold, G.; Simmons, N.; Perez, A.; Young, N.B.; Barbaro, R.P.; Piven, J.; Magnuson, T.R.; Crawley, J.N. Automated apparatus for quantitation of social approach behaviors in mice. *Genes Brain Behav.* 2004, *3*, 303–314, doi:10.1111/j.1601-183X.2004.00071.x.

9

The association between insulin-like growth factor 1 and insulin-like binding protein 3 serum levels and brain development after extreme preterm birth

J.E.G. Vaes, N.E. van der Aa*, F. Groenendaal*, L.M. Baak, F. Lammertink, M.L. Tataranno, J. Dudink, C.H. Nijboer, M.J.N.L Benders

**These authors contributed equally to this work*

Manuscript in preparation

ABSTRACT

Background: Extremely preterm infants are at high risk for developmental brain injury, collectively referred to as 'Encephalopathy of Prematurity'. Insulin-like growth factor (IGF1) plays an essential role in fetal and postnatal brain development. Previous studies have reported low IGF1 and insulin-like growth factor binding protein 3 (IGFBP3) serum levels after extreme preterm birth. In this study, we hypothesized that IGF1 and IGFBP3 serum levels are positively associated with postnatal brain development.

Methods: Infants born at <28 weeks gestational age (GA), with available IGF1/IGFBP3 measurement(s) (at postnatal day 1, 7 or at 30 weeks GA) and magnetic resonance imaging (MRI) scan at term equivalent age were included. IGF1 and IGBP3 levels were related to Kidokoro injury scores, volumetric MRI segmentations and white matter fractional anisotropy (FA).

Results: Forty-eight infants with a median GA of 26.4 weeks (IQR 1.8 weeks) were included. In this cohort, IGF1 levels were not associated with Kidokoro scores, brain volumes or FA values. Borderline significant lower levels of IGFBP3 were observed in infants with a moderate/severe Kidokoro score. A positive association between IGFBP3 levels and FA values in the corpus callosum and right posterior thalamic radiation was found. IGFBP3 levels were positively associated with relative total brain volume, white matter volume and with relative gray matter volume (adjusted for intracranial volume) in the most immature infants (GA <26.4 weeks).

Conclusion: In extremely preterm infants, higher levels of serum IGFBP3, but not IGF1, were associated with a reduction in brain injury severity, and increased regional brain volumes and white matter microstructural integrity at term.

INTRODUCTION

Infants born extremely preterm (i.e. <28 weeks of gestation) are at high risk for Encephalopathy of Prematurity (EoP), an umbrella term used to describe a variety of brain abnormalities, leading to significant neurodevelopmental morbidity (Volpe, 2009a). These abnormalities are thought to be the result of impaired brain development, with peri- and postnatal insults interfering with a multitude of developmental processes that occur in the third trimester of pregnancy (van Tilborg et al., 2016; Volpe, 2009b). Validated biomarkers that predict brain maldevelopment following extreme preterm birth are currently lacking. Early identification of infants at risk for EoP could be essential for future neuroprotective and neuroregenerative therapies aimed to reduce EoP-related neurological morbidity.

Insulin-like growth factor I (IGF1) is a trophic factor with an essential role in fetal and postnatal development of a range of organs, including the brain (Hellstrom et al., 2016). The bioavailability and activity of IGF1 is regulated by insulin-like growth factor binding protein 3 (IGFBP3), its most important carrier protein (Dai et al., 2017; Martin & Baxter, 2011). In addition, IGFBP3 has been shown to modulate differentiation and survival of cells in an IGF-independent manner (Martin & Baxter, 2011; Varma Shrivastav et al., 2020). Blood levels of IGF1 and IGFBP3 have been reported to be very low after extreme preterm birth compared to fetal *in utero* levels at a corresponding gestational age. This persistent IGF1 deficiency is thought to be the result of a combination of events, including disrupted maternal supply and impaired endogenous production of IGF1 due to peri- and postnatal insults (Hansen-Pupp et al., 2011; Hellström et al., 2016). Multiple studies have proposed a relationship between low IGF1 serum levels and impaired postnatal growth, disturbances in development of the retina (retinopathy of prematurity) and lungs (bronchopulmonary dysplasia) of extremely preterm neonates (Hellstrom et al., 2016; Hellstrom et al., 2001). In addition, (pre) clinical studies have shown that IGF1 is an important regulator of neurodevelopmental processes, including maturation of gray- and white matter (Beck et al., 1995; D'Ercole & Ye, 2008; Hansen-Pupp et al., 2011). Interestingly, exogenous IGF1 administration has been shown to improve brain development and behavioural outcome in multiple experimental models of neonatal brain injury (Brywe et al., 2005; Cai et al., 2011; Cao et al., 2003; Guan et al., 2001; Lin et al., 2005; Vaes et al., in preparation).

In this study we aim to investigate the association between IGF1 and IGFBP3 serum levels and brain development assessed using brain MRI in extremely preterm-born infants. Better understanding of the relationship between IGF1/IGFBP3 and brain development in our population of extremely preterm infants is needed to support the potential use of IGF1 and/or IGFBP3 as biomarkers for early identification of infants at risk to develop EoP and corroborate future clinical application of IGF1/IGFBP3 therapy to prevent or restore EoP.

MATERIALS AND METHODS

Study population

For this study, infants with a gestational age (GA) below 28 weeks admitted to the Neonatal Intensive Care Unit of the UMC Utrecht, the Netherlands between July 2017 and January 2020, with at least one routinely obtained IGF1 or IGFBP3 measurement and availability of a routine MRI scan at term equivalent age (TEA), were retrospectively identified. Infants with metabolic or genetic abnormalities were excluded. The patient population was dichotomized based on the median gestation age (26.4 weeks) for some analyses, as previous studies have shown that the most immature infants exhibit the lowest IGF1 levels (Hansen-Pupp et al., 2013; Hellstrom et al., 2016). The ethical Committee of the University Medical Center Utrecht approved this retrospective study and waived the requirement to collect written informed consent for this study analyzing pseudonymized data.

Neonatal clinical and MRI data

Clinical patient data was obtained from the electronic patient files, as well as the neonatal brain MRI scans at TEA performed as standard clinical care. Birth weight z-scores were calculated according to the Dutch Perinatal registry reference data (Hoftiezer et al., 2019). Brain MRI was performed using a 3.0T MR scanner (Philips Healthcare, Best, the Netherlands). The routine scan protocol included conventional T1-weighted and T2- weighted imaging (T1-weighted TR 9.5 ms; TE 4.6 ms; in plane FOV 200 × 200 mm, acquisition matrix 256 × 220 mm; slice thickness 1.2 mm and T2-weighted TR 4847 ms; TE 150 ms; in plane FOV 180 × 180 mm; acquisition matrix 232 × 202 mm; slice thickness 1.2 mm). The DTI protocol comprised of a single-shit spin-echo echo-planar imaging sequence (echo-planar imaging factor 55, TR/TE 5685/70 ms, field of view 180×180 mm, acquisition matrix 128×128 mm, 50 slices with 2mm thickness). The images were obtained in the axial plane with diffusion gradients

applied in 45 directions with a b-value of 800 s/mm2 and a single non-diffusion weighted image.

IGF1 and IGFBP3 measurements

IGF1 and IGFBP3 serum concentrations were measured at three timepoints a) postnatal day 1, b) postnatal day 7 and at c) 30 weeks GA. Blood sampling was omitted in case of critical illness or end-of-life care with withdrawal of intensive care treatment. Serum IGF1 or IGFBP3 values were not measured within 24 hours after transfusion with fresh frozen plasma. All samples were obtained from arterial catheters, or from venous or capillary puncture. After centrifugation, serum samples were stored at -20°C prior to the assays. For samples collected up until November 2017, IGF1 was measured using radioimmuno assay (RIA) on the Immunolite 1000 (Siemens, Munchen, Germany) with a lower limit of detection of 1.54 nmol/L. From December 2017 onwards, IGF1 was measured using chemoluminescentie assay (CLIA) using the Liaison XL (Diasorin, Saluggia, Italy) with a lower limit of detection of 0.31 nmol/L. IGFBP3 concentrations were determined using RIA (Wallac Wizard Gamma Counter, Perkin Elmer, Waltham, Massachusetts, USA), with a lower limit of detection of 0.002 mg/L. Harmonization samples from the IGF1 harmonization program in the Netherlands (SD-NL) were used to correct for inter-assay differences. To correct for the difference in sensitivity between the two assays used to measure IGF1, values at the detection limit of 1.54 nmol/l of the less sensitive RIA method were replaced by the median of all IGF1 values below 1.54 nmol/l measured with the more sensitive CLIA assay. The mean increase in IGF1 and IGFBP3 serum levels was calculated per subject by dividing the absolute increase in IGF1 and IGFBP3 by the number of weeks between postnatal day 7 and 30 weeks of GA.

MRI scoring

To objectively classify the extent of brain injury, neonatal MRI scans were scored according to the MRI scoring system proposed by Kidokoro et al. (2013), by at least two trained neonatologists (M.T, J.D., M.B.). In this method a total score is obtained by assessment severity of brain injury (defined by signal abnormalities) combined with impairment of brain growth (defined with biometrics) in the white matter, cortical gray matter, deep gray matter and cerebellum (Kidokoro et al., 2013). Similar to the original paper, a linear association was found between postmenstrual age (PMA) at scan and transcerebellar diameter ($R=0.486$; $p<0.001$, slope 1.69 mm/week), the slope of the regression analyses was used to correct all measured cerebellar values. We

were unable to detect other significant associations between PMA at scan and the remaining biometric parameters in our population. The sum of the regional scores (ranging between 0-40) was calculated and classified as normal (total score 0-3), mild (total score 4-7) and severe (total score ≥ 12). Presence of significant brain injury was defined as presence of IVH ≥grade III (Papile et al., 1978), white matter injury (focal signal abnormality score ≥2 according to Kidokoro et al. (2013)), cerebellar hemorrhage (signal abnormality score ≥2 according to Kidokoro et al. (2013)), and/or cystic lesions (cystic lesion score ≥3 according to Kidokoro et al. (2013)).

MRI volumetric measurements

To measure regional brain volumes, automatic brain segmentation was performed on T2-weighted images using the protocol described previously (Makropoulos et al., 2014; Makropoulos et al., 2018). Volumes from six brain regions were derived from this segmentation method: namely the cortical gray matter, total white matter, ventricles, cerebellum and deep gray matter. Total brain volume was defined as the sum of all absolute volumes minus the cerebrospinal fluid. Per patient, regional volumes and total brain volume were adjusted for the total intracranial volume, resulting in relative volumes. All segmentations were checked for correctness and rejected when >10% of all segmented images were (partially) incorrect.

DTI analysis

DTI data were analyzed using the diffusion MR toolbox 'ExploreDTI' (Leemans et al., 2009). The diffusion-weighted images were realigned to the b0-image to correct for subject motion and eddy current-induced geometric distortions, in which the diffusion gradients were adjusted with the proper b-matrix rotation (Leemans & Jones, 2009). Next, the diffusion tensor was fitted for each voxel using a nonlinear least squares method. All data were visually inspected in terms of quality of tensor estimation and motion correction.

The tensor was exported for further analyses using the Diffusion Tensor Imaging ToolKit (DTI-TK) (Zhang et al., 2007). DTI-TK allows tensor-based registration and normalization of DTI data and has been shown to result in good registrations, even in the presence of diffuse brain injury (Wang et al., 2011). DTI-TK was used to create a DTI template for the TEA DTI and for subsequent registration of the individual tensor data to the templates. The template was based on the 48 TEA DTI scans in this study. To create the initial template, the tensor images were rotated to the same

orientation by a rigid alignment and averaged using a Log-Euclidean mean. The template was iteratively optimized, initially using rigid, followed by affine alignments and finally non-linear alignments. After finishing the template, the tensor data of all subjects were registered to the corresponding templates using the same rigid, affine and non-linear alignment.

After registration of all data, individual fractional anisotropy (FA) maps were exported for Tract Based Spatial Statistics, part of the FMRIB software library (Smith et al., 2006; Smith et al., 2004). The aligned images were used to create an average FA map. This map was thinned to generate a mean FA skeleton, which represents the center of all white matter tracts common in the aligned FA images. The skeleton was thresholded at 0.15 and individual FA data were projected on the skeleton.

Statistical analysis

Statistical analyses were performed using IBM SPSS 27 (IBM, Armonk, New York, USA). For statistical analyses comparison of two groups was tested using an independent t-test. Multivariable linear regression analyses were performed to assess the relationship between IGF1 and IGFBP3 levels (independent variable) and regional brain volumes (dependent variable). Birth weight (z-score), presence of significant brain injury, GA at birth and PMA at MRI were included in the multivariable model as independent variables. For TBSS, voxelwise cross-subject statistics was performed using Randomise (v2.5) using univariable linear modeling. The effect of the IGF1 and IGFBP3 levels on FA was studied, correcting for birth weight (Z-score), gestational age and post-menstrual age at scan. The results were corrected by controlling for the family-wise error rate following threshold-free cluster enhancement (Smith & Nichols, 2009). For all analyses a p-value <0.05 was considered statistically significant.

RESULTS

Forty-eight extremely preterm born infants, with a median GA of 26.4 weeks (IQR 1.8 weeks), were included for analysis. The clinical characteristics of the included infants are presented in table 1. For the 48 infants included in the analysis, data of at least one IGF1 or IGFBP3 measurement prior to 30 weeks GA was available. More information on the number of available IGF1/IGFBP3 measurements can be found in supplementary table 1.

IGF1 and IGFBP3 serum levels

IGF1 levels were significantly associated with GA at birth on postnatal day 1 (p=0.034, unstandardized B=0.035, 95% CI 0.003 – 0.65, R^2=0.096) and day 7 (p=0.003, unstandardized B=0.081, 95% CI 0.028– 0.134, R^2=0.183). Similarly, a borderline significant association between mean IGF1 levels between postnatal day 7 and 30 weeks GA and GA at birth (p=0.056, unstandardized B=0.043, 95% CI -0.001 – 0.087, R^2=0.082) was observed (supplementary figure 1A-C). Furthermore, a positive association between IGFBP3 levels at postnatal day 7 (p=0.034, unstandardized B=0.007, 95% CI 0.001 – 0.013, R^2=0.105), and a borderline significant association between mean IGFBP3 levels between postnatal day 7 and 30 weeks GA (p=0.062, unstandardized B=0.004, 95% CI 0.00 – 0.009, R^2=0.112) and GA at birth were observed (supplementary figure 1D-E). Birth weight Z-score was only positively associated with IGFBP3 levels on postnatal day 1 (p=0.05, unstandardized B=0.020, 95% CI 0.000 – 0.040, R^2=0.088) (supplementary figure 1F).

Table 1. Clinical characteristics

Gestational age – weeks + days*	26.4 (1.8)
Birth weight – grams*	788 (264)
Birth weight – SDS*	-0.60 (1.64)
Gender – male#	24 (50%)
Sepsis#	22 (45.8%)
Necrotizing enterocolitis#	3 (6.3%)
Bronchopulmonary dysplasia#	13 (27.1%)
Mechanical ventilation >7 days#	24 (49%)
Retinopathy of Prematurity#	
No	22 (45.8%)
Stage I	4 (8.3%)
Stage II	12 (25%)
Stage III	7 (14.6%)
Intraventricular hemorrhage#	
No	29 (60.5%)
Grade I-II	18 (37.6%)
Grade III	0 (0%)
Venous infarct (grade IV)	1 (2.1%)
Cerebellar hemorrhage#	7 (14.6%)
Significant brain injury#$	5 (10.4%)
Postmenstrual age at MRI – weeks*	41.4 (1.28)

*= median (interquartile range)
#= N (%)
$= IVH grade 3 or higher or unilateral porencephaly, >6 punctate white matter lesions or >6 cerebellum hemorrhage.

IGF1 and IGFBP3 serum levels and brain injury

Due to the small number of neonatal MRI scans scored as normal (n=1) or severe injury (n=3), Kidokoro scores were dichotomized as normal/mild (score ≤ 7, n=23) versus moderate/severe (score > 8, n=22). Mean GA at birth or birth weight z-score did not significantly differ between the two groups (p=0.127 and p=0.831 respectively). We were unable to detect any significant differences in IGF1 levels (at all 3 timepoints or in mean IGF1 between postnatal day 7 and 30 weeks GA) between Kidokoro outcome categories (supplementary table 2). However, infants with moderate/severe injury did seem to have at trend towards lower levels of IGFBP3 at postnatal day 7 compared to infants with a normal/mild injury score (trend p=0.067; figure 1A). Similar findings were observed when looking at mean IGFBP3 between timepoint 2 and 3 (trend p=0.073; figure 1B).

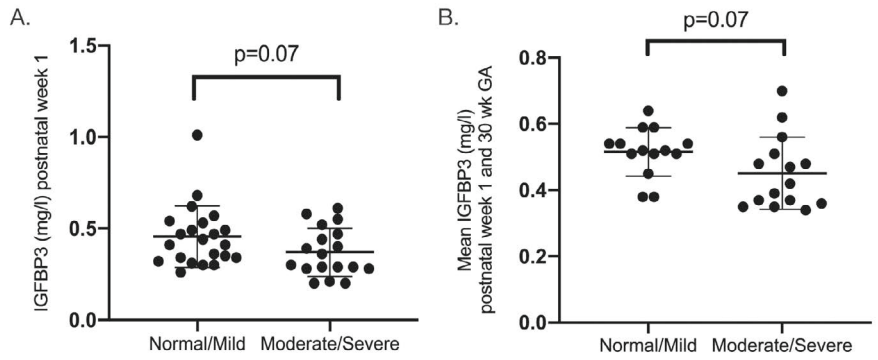


Figure 1. Trend towards lower IGFBP3 serum levels was detected in preterm infants with moderate/severe brain injury according to the Kidokoro scoring system **A**. At postnatal day 7 IGFBP3 serum levels seem to be lower (statistical trend) in children with moderate/severe brain injury (n=18) according to the Kidokoro scoring system compared to infants with a normal/mild (n=22) injury score. **B**. Mean IGFBP3 serum levels between postnatal day 7 and 30 weeks GA seem to be lower (statistical trend) in infants with moderate/severe brain injury (n=14) according to the Kidokoro scoring system compared to preteterm infants scored as normal/mild brain injury (n=15).

IGF1 and IGFBP3 serum levels and relative brain volumes

Univariable linear regression analyses showed a significant negative association between IGF1 levels at postnatal day 7 and relative ventricle volume (adjusted for intracranial volume) (p=0.026, unstandardized B=-0.012, 95% CI -0.022 - -0.001,

R^2=0.115) (supplementary table 3). For IGFBP3, univariable analysis showed a positive association between IGFBP3 levels at postnatal day 1 and relative total brain volume (adjusted for intracranial volume) (p=0.019, unstandardized B= 0.176, 95% CI 0.031 - -0.321, R^2=0.130) (supplementary table 3. In addition, we observed a significant positive association between IGFBP3 levels at postnatal day 1 and relative white matter volume (p=0.003, unstandardized B=0.104, 95% CI 0.037 - -0.172, R^2=0.196) (supplementary table 3). Univariate linear regression analyses did not reveal any significant associations between IGF1 or IGFBP3 and other volumetric outcome parameters (supplementary table 3).

Subsequently, all significant univariable associations were included in a multivariable linear regression model with relative regional brain volumes as dependent variables and IGF1 or IGFBP3 levels, birth weight (z-score), presence of significant brain injury, gestational age at birth and PMA at MRI as independent variables. In this model, the association between relative total brain volume and IGFBP3 serum levels on postnatal day 1 remained significant (p=0.001, unstandardized B=0.227, 95% CI 0.92-0.363, R^2=0.411) (figure 2A). In a similar manner, the model confirmed the association between relative white matter volume and IGFBP3 serum levels on postnatal day 1 (p<0.0001, unstandardized B=0.115, 95% CI 0.55-0.175, R^2=0.50) (figure 2B).

When we dichotomized infants based on the median gestational age (26.4 weeks), multivariable linear regression showed a positive association between IGFBP3 levels on postnatal day 1 and relative total brain volume for infants born before 26.4 weeks (p<0.0001, unstandardized B=0.301, 95% CI 0.164-0.437, R^2=0.72) (figure 3A), but not the older infants (GA ≥26.4 weeks) (p=0.715, unstandardized B=-0.018, 95% CI -0.352-0.316, R^2=0.195). Similarly, we observed a significant positive association between IGFBP3 levels on postnatal day 1 and relative white matter volume for infants born before 26.4 weeks (p=0.001, unstandardized B=0.140, 95% CI 0.062-0.218, R^2=0.63) (figure 3B), but not the infants born ≥26.4 weeks (p=0.534, unstandardized B=0.037, 95% CI -0.09-0.165, R^2=0.50). In addition, a significant positive association between IGFBP3 levels on postnatal day 1 and relative gray matter volume was observed for infants born <26.4 weeks (p=0.042, unstandardized B=0.105, 95% CI 0.004-0.207, R^2=0.49) (figure 3C), but not infants born ≥26.4 weeks (p=0.497, unstandardized B=-0.037, 95% CI -0.211-0.137, R^2=0.28). For other volumetric outcome data no significant associations were observed after dichotomizing data by gestational age at birth (data not shown).

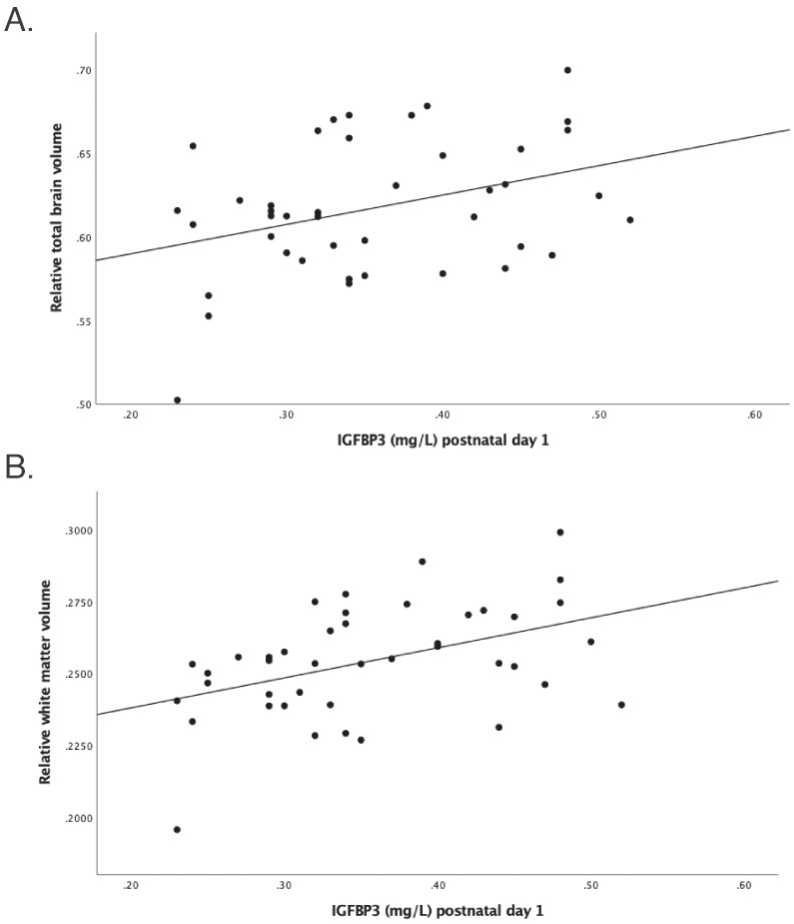


Figure 2. Association between IGFBP3 serum levels at postnatal day 1 and relative brain matter volume at term equivalent age. Association between IGFBP3 serum levels at postnatal day 1 and **A.** relative total brain volume (p=0.001), **B**. relative white matter volume at term equivalent age (p<0.0001).

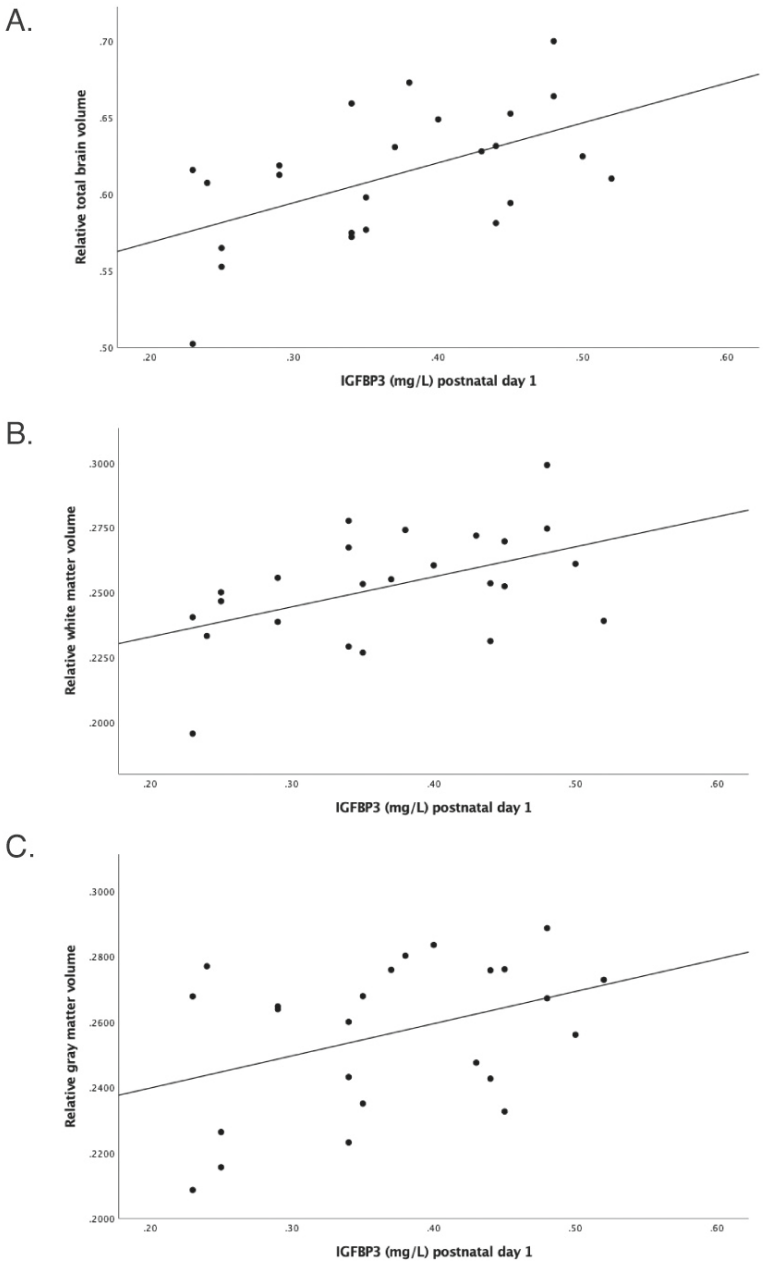


Figure 3. Association between IGFBP3 serum levels at postnatal day 1 and relative brain volumes at term equivalent age for infants born <26.4 weeks. Association between IGFBP3 serum levels at postnatal day 1 and **A.** relative total brain volume at term equivalent age (p<0.0001), **B**. relative white matter volume at term equivalent age (p=0.001) and **C**. relative gray matter volume at term equivalent age (p=0.042).

IGF1 and IGFBP3 serum levels and white matter microstructure

TBSS analyses did not show any significant association between IGF1 levels (at all 3 timepoints nor in mean IGF1 between postnatal day 7 and 30 weeks GA) and white matter FA-values (corrected for PMA at scan and GA at birth) throughout the brain (data not shown). For IGFBP3 a significant association was found between levels at postnatal week 1 and FA-values in the corpus callosum and right posterior thalamic radiation, corrected for PMA at scan and GA at birth (figure 4A-B & 5).

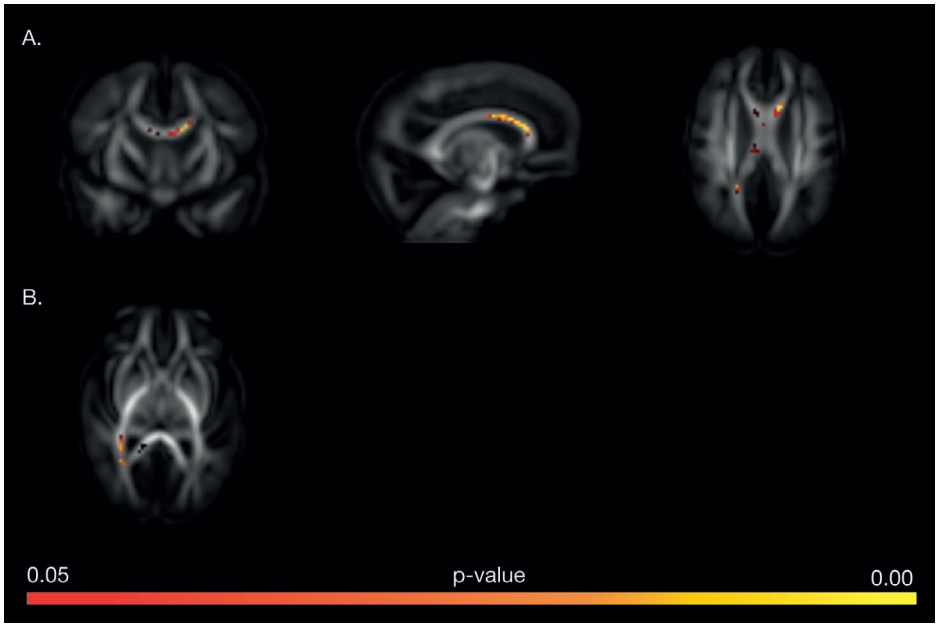


Figure 4. Association between IGBP3 serum levels at postnatal day 7 and FA values. IGFBP3 levels at postnatal day 7 are associated with FA values in **A.** the corpus callosum (coronal, sagittal and axial view respectively) and **B.** right posterior thalamic radiation (axial view). All analyses were corrected for PMA at scan and GA at birth.

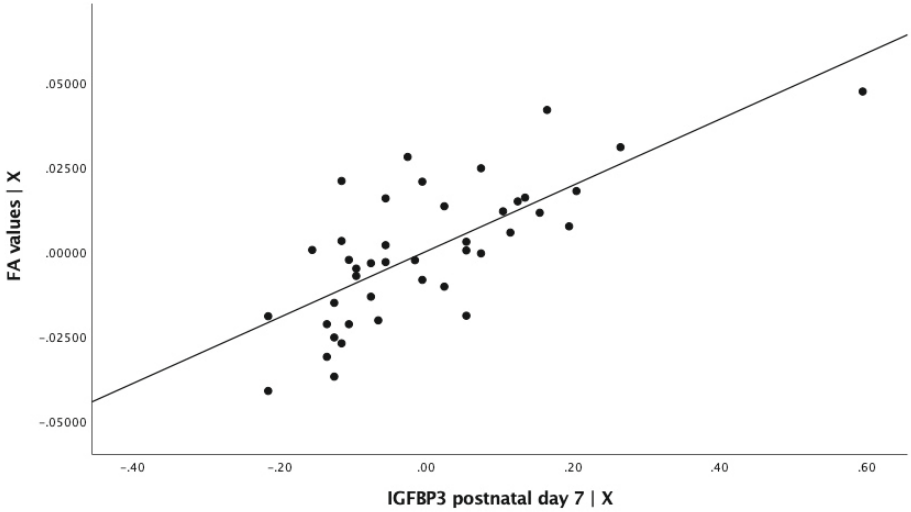


Figure 5. The unstandardized residuals for FA values of regions which showed a significant association with IGBP3 on day 7 in the TBSS analyses are plotted. Corrected for PMA at scan and GA at birth.

DISCUSSION

In this study we explored the relationship between IGF1 and IGFBP3 serum levels and brain development in extremely preterm infants. To our knowledge we are the first to combine the Kidokoro scoring system, MR segmentation and DTI as outcome parameters to study the relationship between the IGF system and postnatal brain development in extremely preterm infants in one study. We report borderline significantly lower IGFBP3 levels at postnatal day 7 and lower mean IGFBP3 up to 30 weeks of gestation in infants with moderate/severe brain injury compared to infants with no/mild brain injury at TEA. Moreover, a positive association between IGFBP3 serum levels on the first postnatal day and total brain volume, as well as, white matter volume at TEA was found. Explorative subgroup analyses showed that the most immature infants (i.e. GA below 26.4) likely account for the observed associations between IGFBP3 and (regional) brain volumes. Moreover, a positive relationship between IGFBP3 serum levels at postnatal day 1 and relative gray matter volume at TEA was observed in this subpopulation. We observed an association between IGFBP3 levels at postnatal day 7 and higher FA values at term in important white matter tracts. We were unable to

detect any significant associations between IGF1 levels and brain development at TEA in this cohort of extremely preterm born infants.

Over the years, a substantial amount of evidence supporting an important role of the IGF system in the regulation of fetal and postnatal brain development has been published (Vaes et al., 2021). IGF1, a polypeptide produced in virtually every type of tissue, has been shown to boost proliferation, maturation and survival of multiple cell types of the central nervous system (CNS) (Joseph D'Ercole & Ye, 2008; Zeger et al., 2007). The pleiotropic effects of IGF1 are mediated by activation of the broadly expressed IGF receptor (IGFR), leading to downstream activation of the PI3kinase-Akt (PI3k/Akt) pathway, mitogen-activated protein kinase (MAPK) activation and mammalian target of rapamycin (mTOR) activation (O'Kusky & Ye, 2012; Palacios et al., 2005; Vaes et al., 2021). Among the cells that express the IGFR are cells of the oligodendrocyte lineage (Zeger et al., 2007). This cell lineage is responsible for CNS myelination, starting around 32 weeks of gestation (Back et al., 2001). Immature oligodendrocytes are particularly vulnerable for peri- and postnatal insults associated with (extreme) preterm birth, leading to white matter maldevelopment (Volpe, 2009a). Multiple *in vivo* studies have proposed IGF1 administration as a promising treatment strategy to repair the injured neonatal brain, with significant improvement of histological and behavioral outcome after hypoxic-ischemic and/or inflammatory insults (Brywe et al., 2005; Cai et al., 2011; Cao et al., 2003; Lin et al., 2009).

Previous clinical studies have demonstrated an association between decreased postnatal IGF1 and IGFBP3 levels and lower regional brain volumes at term age in extremely- and very preterm infants (Hansen-Pupp et al., 2011; Hansen-Pupp et al., 2013). In their pioneer study Hansen-Pupp et al. (2011) reported an association between mean serum concentration of IGFBP3 between birth and 35 weeks of gestation and larger total brain, unmyelinated white matter and cerebellar volumes. Likewise, we report a relationship between higher levels of IGFBP3 and greater relative total brain volume and white matter volume at TEA. In addition, a positive association between IGFBP3 and relative gray matter volume was found, but as expected only in the most immature infants. However, unlike previous studies, we were unable to detect associations between IGF1 serum levels and regional brain volumes (Hansen-Pupp et al., 2011; Hansen-Pupp et al., 2013). Univariable analyses revealed a significant association between higher IGF1 levels at postnatal day 7 and smaller ventricles (adjusted for intracranial volume), however this association was lost when corrected for GA at birth, GA at scan, BW z-score and presence of significant brain injury. Direct comparison

of IGF1 concentrations in our study population and values reported by other studies shows that though mean IGF1 concentrations in the first postnatal week seem similar (+/- 11-18 µg/L in our study compared to +/- 15 µg/L in Hansen-Pupp et al. (2011); Hansen-Pupp et al. (2013); Hellstrom et al. (2016)), mean levels of IGF1 found at 30 weeks of gestation are higher in our population (+/- 30 µg/L compared to previously reported +/- 20 µg/L) (Hansen-Pupp et al., 2011; Hansen-Pupp et al., 2013; Hellstrom et al., 2016). Though a small difference, it is possible that certain threshold concentrations of IGF1 are essential for proper brain development, similar to observations made in the pathophysiology of retinopathy of prematurity (ROP) (Hellstrom et al., 2016; Hellstrom et al., 2001). In addition, the observed differences in IGF1 levels between these studies indicate that IGF1 levels around 30 weeks of gestation might play a more pivotal role in brain development compared to the first postnatal week, which might be associated with the onset of myelination. This is supported by a study by Hansen-Pupp et al. (2011), in which the relationship between low IGF1 levels and decreased brain volume was most prominent at a gestational age of 30 weeks and onwards. Therefore, it is possible that assessment of IGF1 levels beyond a GA of 30 weeks could reveal a relationship between IGF1 and brain development in our study population. Exploration of the differences between these studies and our study, for example in population characteristics (e.g. GA at birth) or standard clinical care, could potentially contribute to a better understanding of the complex interplay of factors regulating the IGF system.

IGFBP3, the most abundant IGF binding protein in the brain, modulates IGF1 bioavailability by facilitating transport in the plasma, increasing the half-life and regulating the clearance of IGF1 (Dai et al., 2017). In addition, IGFBP3 is believed to play a role in regulation of IGF1 activity, both potentiating and inhibiting the effect of IGF1 (Dai et al., 2017; Martin & Baxter, 2011; Russo et al., 2005). Aside from IGF1-dependent functions, IGFBP3 has been shown to interact with the epidermal growth factor (EGF) receptor and transforming growth factor-β (TGFβ) receptor (Martin & Baxter, 2011; Poreba & Durzynska, 2020; Varma Shrivastav et al., 2020). Both EGFR and TGFβR activation have been reported to induce OL lineage proliferation and differentiation (Vaes et al., 2021). Other IGF1-independent functions of IGFBP3, including modulation of cell survival, have been described in literature (Dai et al., 2017; Firth & Baxter, 2002; Martin & Baxter, 2011; Varma Shrivastav et al., 2020). In this study we observed an association between IGFBP3 serum levels in the first postnatal week and brain development at TEA defined by three outcome parameters. Though exact mechanisms behind the relationship between IGFBP3 serum levels and brain development are still

unclear, IGFBP3 levels in the first postnatal week could potentially have value in the prediction of preterm brain injury. Our study suggests that IGFBP3 predominantly plays a supporting role in early brain development (first postnatal week). This implies that IGF1-independent actions of IGFBP3, for example through EGF or TGFβ receptor activation, could mainly be responsible for the observed positive association between IGFBP3 and early postnatal brain development. It is however important to note that it is unknown to which extent serum levels of IGF1 and IGFBP3 in preterm infants directly correspond with cerebral levels (Hansen-Pupp et al., 2007). The potential biomarker function of IGFBP3 is likely most applicable in the most immature infants, as the relationship between IGFBP3 and regional brain volumes seemed more pronounced in this population. Early identification of infants at risk for preterm brain injury could contribute in the translation of promising treatment strategies, including intranasal administration of mesenchymal stem cells (Vaes et al., 2019). Additional (larger) studies are needed for further validation of IGFBP3 as a biomarker for preterm brain injury.

Studies identifying low serum levels of IGF1 and IGFBP3 following extreme preterm birth have suggested exogenous IGF1 (and IGFBP3) supplementation as a potential treatment option for preterm brain injury (Hansen-Pupp et al., 2011; Hellstrom et al., 2016). The effects of continuous intravenous IGF1/IGFBP3 administration after extreme preterm birth are currently being investigated in a randomized control trial, with neurodevelopmental outcome as a secondary outcome measure (Clinicaltrials.gov: NCT03253263). Based on our current findings, early administration of IGFBP3 might benefit development of the cerebral white- and gray matter following extreme preterm birth. In this study, we were unable to detect a relationship between IGF1 serum levels and brain development at TEA in our population. These results imply that a certain population of extremely preterm infants would potentially not be in need of normalization of endogenous IGF1 levels through exogenous administration. However, these infants could still possibly benefit from IGF1 administration. A recent study performed by our group using a mouse model of EoP implies the need for supraphysiological IGF1 levels to effectively restore the injured white matter (Vaes et al., in preparation). In addition, though many studies have demonstrated a robust relationship between regional brain volumes or DTI at TEA and cognitive and/or motor outcome of preterm born children at a later age (Cheong et al., 2013; Keunen et al., 2017; Keunen et al., 2016; Lind et al., 2011; Moeskops et al., 2017; Peterson et al., 2003; Ullman et al., 2015), it is possible that MRI descriptors used in the current study fail to notice subtle alterations in the cerebral micro-organization, for example in interneuron distribution (Vaes et al.,

2020). Therefore, future studies using other neurodevelopment outcome parameters, such as fMRI or clinical evaluation of neurodevelopment, are needed to further explore the relationship between IGF1 serum levels and neurodevelopment in our population.

This study has limitations. Brain development is influenced by a numerous amount of intricately intertwined factors. Therefore, multiple confounding factors could have potentially influenced brain development independently of the actions of IGF1 or IGFBP3. As a result of the small number of infants included in this study, we were able to correct for a limited number of potential confounders. Moreover, due to the small sample size we were unable to perform subgroup analyses based on etiology of prematurity (e.g. preeclampsia, placental abruption, cervical incompetence or preterm premature rupture of membranes). Hence, associations between IGF1/IGFBP3 and outcome parameters that might exclusively emerge in a subgroup with a certain etiology of extreme preterm birth may have been averaged out in our heterogenous population. Larger studies are needed to explore the relationship between IGF1/IGFBP3 levels, etiology of preterm birth and brain development. In addition, in our study population IGF1 and IGFBP3 serum levels were obtained in combination with clinically relevant blood draws. Therefore, a number of children that were not in need of routine blood sampling due to good clinical condition, or in contrast in case of critical illness or end-of-life care, were not included in the later timepoints (either at postnatal day 7 or 30 weeks GA), which might have led to a selection bias.

In conclusion, here we show that higher levels of serum IGFBP3 in the first postnatal week are associated with a reduction in the incidence of brain injury, increased regional brain volumes and white matter microstructural integrity at term equivalent age. Future studies are needed to further investigate the potential biomarker function of IGFBP3. In addition, an association between IGF1 serum levels and imaging outcome at TEA was not observed. IGF1 administration has been proposed as a potent therapeutic strategy to aid in brain development after extreme preterm birth. In this study we propose that restoration of IGFBP3 levels might be of equal importance in supporting early brain development in EoP, due to its pleiotropic IGF-dependent and -independent effects. Additional multicenter studies are needed to identify the patients that might benefit from IGF1/IGFBP3 supplementation as well as determine the most optimal timing of treatment.

Acknowledgements

This work was funded by Brain Foundation the Netherlands.

REFERENCES

Back, S. A., Luo, N. L., Borenstein, N. S., Levine, J. M., Volpe, J. J., & Kinney, H. C. (2001). Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci, 21*(1529-2401 (Electronic)), 1302-1312. doi:10.1523/JNEUROSCI.21-04-01302.2001

Beck, K. D., Powell-Braxton, L., Widmer, H. R., Valverde, J., & Hefti, F. (1995). Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. *Neuron, 14*(4), 717-730. doi:10.1016/0896-6273(95)90216-3

Brywe, K. G., Mallard, C., Gustavsson, M., Hedtjarn, M., Leverin, A. L., Wang, X., . . . Hagberg, H. (2005). IGF-I neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3beta? *Eur J Neurosci, 21*(6), 1489-1502. doi:10.1111/j.1460-9568.2005.03982.x

Cai, Z., Fan, L.-W., Lin, S., Pang, Y., & Rhodes, P. G. (2011). Intranasal Administration of IGF-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., . . . Guan, J. (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*(6), 739-747. doi:10.1097/01.wcb.0000067720.12805.6f

Cheong, J. L., Anderson, P. J., Roberts, G., Burnett, A. C., Lee, K. J., Thompson, D. K., . . . Doyle, L. W. (2013). Contribution of brain size to IQ and educational underperformance in extremely preterm adolescents. *PLoS One, 8*(10), e77475. doi:10.1371/journal.pone.0077475

D'Ercole, a. J., & Ye, P. (2008). Minireview: Expanding the mind: Insulin-like growth factor I and brain development. *Endocrinology, 149*(12), 5958-5962. doi:10.1210/en.2008-0920

Dai, H., Goto, Y.-i., & Itoh, M. (2017). Insulin-Like Growth Factor Binding Protein-3 Deficiency Leads to Behavior Impairment with Monoaminergic and Synaptic Dysfunction. *Am J Pathol, 187*(2), 390-400. doi:10.1016/j.ajpath.2016.10.011

Firth, S. M., & Baxter, R. C. (2002). Cellular Actions of the Insulin-Like Growth Factor Binding Proteins. *Endocrine Reviews, 23*(6), 824-854. doi:10.1210/er.2001-0033

Guan, J., Bennet, L., George, S., Wu, D., Waldvogel, H. J., Gluckman, P. D., . . . Gunn, A. J. (2001). Insulin-like growth factor-1 reduces postischemic white matter injury in fetal sheep. *J Cereb Blood Flow Metab, 21*(5), 493-502. doi:10.1097/00004647-200105000-00003

Hansen-Pupp, I., Hellstrom-Westas, L., Cilio, C. M., Andersson, S., Fellman, V., & Ley, D. (2007). Inflammation at birth and the insulin-like growth factor system in very preterm infants. *Acta Paediatr, 96*(6), 830-836. doi:10.1111/j.1651-2227.2007.00276.x

Hansen-Pupp, I., Hovel, H., Hellstrom, A., Hellstrom-Westas, L., Lofqvist, C., Larsson, E. M., . . . Ley, D. (2011). Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J Clin Endocrinol Metab, 96*(4), 1129-1135. doi:10.1210/jc.2010-2440

Hansen-Pupp, I., Hovel, H., Lofqvist, C., Hellstrom-Westas, L., Fellman, V., Huppi, P. S., . . . Ley, D. (2013). Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatr Res, 74*(5), 564-569. doi:10.1038/pr.2013.135

Hellstrom, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Lofqvist, C., van Marter, L., . . . Smith, L. E. (2016). Insulin-like growth factor 1 has multisystem effects on foetal and preterm infant development. *Acta Paediatr, 105*(6), 576-586. doi:10.1111/apa.13350

Hellström, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Ramenghi, L. A., Löfqvist, C., . . . Hård, A. L. (2016). Role of Insulinlike Growth Factor 1 in Fetal Development and in the Early Postnatal Life of Premature Infants. *Am J Perinatol, 33*(11), 1067-1071. doi:10.1055/s-0036-1586109

Hellstrom, A., Perruzzi, C., Ju, M., Engstrom, E., Hard, A. L., Liu, J. L., . . . Smith, L. E. (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*(10), 5804-5808. doi:10.1073/pnas.101113998

Hoftiezer, L., Hof, M. H. P., Dijs-Elsinga, J., Hogeveen, M., Hukkelhoven, C., & van Lingen, R. A. (2019). From population reference to national standard: new and improved birthweight charts. *Am J Obstet Gynecol, 220*(4), 383.e381-383.e317. doi:10.1016/j.ajog.2018.12.023

Joseph D'Ercole, A., & Ye, P. (2008). Expanding the mind: insulin-like growth factor I and brain development. *Endocrinology, 149*(12), 5958-5962. doi:10.1210/en.2008-0920

Keunen, K., Benders, M. J., Leemans, A., Fieret-Van Stam, P. C., Scholtens, L. H., Viergever, M. A., . . . van den Heuvel, M. P. (2017). White matter maturation in the neonatal brain is predictive of school age cognitive capacities in children born very preterm. *Developmental Medicine & Child Neurology, 59*(9), 939-946. doi:10.1111/dmcn.13487

Keunen, K., Išgum, I., van Kooij, B. J. M., Anbeek, P., van Haastert, I. C., Koopman-Esseboom, C., . . . Benders, M. J. N. L. (2016). Brain Volumes at Term-Equivalent Age in Preterm Infants: Imaging Biomarkers for Neurodevelopmental Outcome through Early School Age. *J Pediatr, 172*, 88-95. doi:10.1016/j.jpeds.2015.12.023

Kidokoro, H., Neil, J. J., & Inder, T. E. (2013). New MR imaging assessment tool to define brain abnormalities in very preterm infants at term. *American Journal of Neuroradiology, 34*(11), 2208-2214. doi:10.3174/ajnr.A3521

Leemans, A., Jeurissen, B., Sijbers, J., & Jones, D. K. (2009). ExploreDTI: A graphical toolbox for processing, analyzing, and visualizing diffusion MR data. *17th Annual Meeting of Intl Soc Mag Reson Med, 17*.

Leemans, A., & Jones, D. K. (2009). The B-matrix must be rotated when correcting for subject motion in DTI data. *Magn Reson Med, 61*(6), 1336-1349. doi:10.1002/mrm.21890

Lin, S., Fan, L. W., Pang, Y., Rhodes, P. G., Mitchell, H. J., & Cai, Z. (2005). IGF-1 protects oligodendrocyte progenitor cells and improves neurological functions following cerebral hypoxia-ischemia in the neonatal rat. *Brain Res, 1063*(1), 15-26. doi:10.1016/j.brainres.2005.09.042

Lin, S., Fan, L. W., Rhodes, P. G., & Cai, Z. (2009). Intranasal administration of IGF-1 attenuates hypoxic-ischemic brain injury in neonatal rats. *Exp Neurol, 217*(2), 361-370. doi:10.1016/j.expneurol.2009.03.021

Lind, A., Parkkola, R., Lehtonen, L., Munck, P., Maunu, J., Lapinleimu, H., . . . Group, P. S. (2011). Associations between regional brain volumes at term-equivalent age and development at 2 years of age in preterm children. *Pediatric Radiology, 41*(8), 953-961. doi:10.1007/s00247-011-2071-x

Makropoulos, A., Gousias, I. S., Ledig, C., Aljabar, P., Serag, A., Hajnal, J. V., . . . Rueckert, D. (2014). Automatic whole brain MRI segmentation of the developing neonatal brain. *IEEE Trans Med Imaging, 33*(9), 1818-1831. doi:10.1109/tmi.2014.2322280

Makropoulos, A., Robinson, E. C., Schuh, A., Wright, R., Fitzgibbon, S., Bozek, J., . . . Rueckert, D. (2018). The developing human connectome project: A minimal processing pipeline for neonatal cortical surface reconstruction. *NeuroImage, 173*, 88-112. doi:10.1016/j.neuroimage.2018.01.054

Martin, J. L., & Baxter, R. C. (2011). Signalling pathways of insulin-like growth factors (IGFs) and IGF binding protein-3. *Growth Factors, 29*(6), 235-244. doi:10.3109/08977194.2011.614237

Moeskops, P., Išgum, I., Keunen, K., Claessens, N. H. P., van Haastert, I. C., Groenendaal, F., . . . Benders, M. J. N. L. (2017). Prediction of cognitive and motor outcome of preterm infants based on automatic quantitative descriptors from neonatal MR brain images. *Sci Rep, 7*(1), 2163. doi:10.1038/s41598-017-02307-w

O'Kusky, J., & Ye, P. (2012). Neurodevelopmental effects of insulin-like growth factor signaling. *Front Neuroendocrinol, 33*(3), 230-251. doi:10.1016/j.yfrne.2012.06.002

Palacios, N., Sanchez-Franco, F., Fernandez, M., Sanchez, I., & Cacicedo, L. (2005). Intracellular events mediating insulin-like growth factor I-induced oligodendrocyte development: modulation by cyclic AMP. *J Neurochem, 95*(4), 1091-1107. doi:10.1111/j.1471-4159.2005.03419.x

Papile, L. A., Burstein, J., Burstein, R., & 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*(4), 529-534. doi:10.1016/s0022-3476(78)80282-0

Peterson, B. S., Anderson, A. W., Ehrenkranz, R., Staib, L. H., Tageldin, M., Colson, E., . . . Ment, L. R. (2003). Regional Brain Volumes and Their Later Neurodevelopmental Correlates in Term and Preterm Infants. *Pediatrics, 111*(5), 939. doi:10.1542/peds.111.5.939

Poreba, E., & Durzynska, J. (2020). Nuclear localization and actions of the insulin-like growth factor 1 (IGF-1) system components: Transcriptional regulation and DNA damage response. *Mutation Research/Reviews in Mutation Research, 784*, 108307. doi:10.1016/j.mrrev.2020.108307

Russo, V. C., Gluckman, P. D., Feldman, E. L., & Werther, G. A. (2005). The Insulin-Like Growth Factor System and Its Pleiotropic Functions in Brain. *Endocrine Reviews, 26*(7), 916-943. doi:10.1210/er.2004-0024

Smith, S. M., Jenkinson, M., Johansen-Berg, H., Rueckert, D., Nichols, T. E., Mackay, C. E., . . . Behrens, T. E. (2006). Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data. *Neuroimage, 31*(4), 1487-1505. doi:S1053-8119(06)00138-8 [pii]
10.1016/j.neuroimage.2006.02.024

Smith, S. M., Jenkinson, M., Woolrich, M. W., Beckmann, C. F., Behrens, T. E., Johansen-Berg, H., . . . Matthews, P. M. (2004). Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage, 23 Suppl 1*, S208-219. doi:10.1016/j.neuroimage.2004.07.051

Smith, S. M., & Nichols, T. E. (2009). Threshold-free cluster enhancement: addressing problems of smoothing, threshold dependence and localisation in cluster inference. *Neuroimage, 44*(1), 83-98. doi:10.1016/j.neuroimage.2008.03.061

Ullman, H., Spencer-Smith, M., Thompson, D. K., Doyle, L. W., Inder, T. E., Anderson, P. J., & Klingberg, T. (2015). Neonatal MRI is associated with future cognition and academic achievement in preterm children. *Brain, 138*(11), 3251-3262. doi:10.1093/brain/awv244

Vaes, J. E. G., Baak, L. M., van Kammen, C. M., Groenendaal, F., Benders, M. J. N. L., & Nijboer, C. H. A. (in preparation). *Insulin-like growth factor 1 therapy to restore hypomyelination in Encephalopathy of Prematurity*.

Vaes, J. E. G., Brandt, M. J. V., Wanders, N., Benders, M. J. N. L., de Theije, C. G. M., Gressens, P., & Nijboer, C. H. (2021). The impact of trophic and immunomodulatory factors on oligodendrocyte maturation: Potential treatments for encephalopathy of prematurity. *Glia, 69*(6), 1311-1340. doi:10.1002/glia.23939

Vaes, J. E. G., Kosmeijer, C. M., Kaal, M., van Vliet, R., Brandt, M. J. V., Benders, M., & Nijboer, C. H. (2020). Regenerative Therapies to Restore Interneuron Disturbances in Experimental Models of Encephalopathy of Prematurity. *Int J Mol Sci, 22*(1). doi:10.3390/ijms22010211

Vaes, J. E. G., Vink, M. A., de Theije, C. G. M., Hoebeek, F. E., Benders, M., & Nijboer, C. H. A. (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

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., & Nijboer, C. H. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Progress in Neurobiology, 136*(1873-5118 (Electronic)), 28-49. doi:10.1016/j.pneurobio.2015.11.002

Varma Shrivastav, S., Bhardwaj, A., Pathak, K. A., & Shrivastav, A. (2020). Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3): Unraveling the Role in Mediating IGF-Independent Effects Within the Cell. *Frontiers in Cell and Developmental Biology, 8*(286). doi:10.3389/fcell.2020.00286

Volpe, J. J. (2009a). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurology, 8*(1), 110-124. doi:10.1016/s1474-4422(08)70294-1

Volpe, J. J. (2009b). The encephalopathy of prematurity--brain injury and impaired brain development inextricably intertwined. *Seminars in Pediatric Neurology, 16*(4), 167-178. doi:10.1016/j.spen.2009.09.005

Wang, Y., Gupta, A., Liu, Z., Zhang, H., Escolar, M. L., Gilmore, J. H., . . . Styner, M. (2011). DTI registration in atlas based fiber analysis of infantile Krabbe disease. *Neuroimage, 55*(4), 1577-1586. doi:10.1016/j.neuroimage.2011.01.038

Zeger, M., Popken, G., Zhang, J., Xuan, S., Lu, Q. R., Schwab, M. H., . . . Ye, P. (2007). Insulin-like growth factor type 1 receptor signaling in the cells of oligodendrocyte lineage is required for normal in vivo oligodendrocyte development and myelination. *Glia, 55*(4), 400-411. doi:10.1002/glia.20469

Zhang, H., Avants, B. B., Yushkevich, P. A., Woo, J. H., Wang, S., McCluskey, L. F., . . . Gee, J. C. (2007). High-dimensional spatial normalization of diffusion tensor images improves the detection of white matter differences: an example study using amyotrophic lateral sclerosis. *IEEE Trans Med Imaging, 26*(11), 1585-1597. doi:10.1109/TMI.2007.906784

SUPPLEMENTARY DATA

Table S1. Overview IGF1/IGFBP3 measurements

		n	median (range)
1) Postnatal day 1	IGF1 (nmol/l)	47	1.40 (0.31-4.10)
	IGFBP3 (mg/l)	44	0.34 (0.23-0.52)
2) Postnatal day 7	IGF1 (nmol/l)	45	2.40 (0.70-6.80)
	IGFBP3 (mg/l)	43	0.36 (0.20-1.01)
3) GA 30 weeks	IGF1 (nmol/l)	36	3.90 (1.54-6.40)
	IGFBP3 (mg/l)	33	0.51 (0.37-0.87)

Table S2. Postnatal IGF1/IGFBP3 levels and Kidokoro score

		Normal/Mild		Moderate/Severe		p-value
		n	mean (SD)	n	mean (SD)	
1) Postnatal day 1	IGF1 (nmol/l)	22	1.64 (0.97)	22	1.71 (0.75)	0.647
	IGFBP3 (mg/l)	20	0.36 (0.08)	21	0.37 (0.09)	0.754
2) Postnatal day 7	IGF1 (nmol/l)	23	2.80 (1.63)	19	2.37 (1.26)	0.478
	IGFBP3 (mg/l)	22	0.46 (0.17)	18	0.37 (0.13)	*0.066*
3) GA 30 weeks	IGF1 (nmol/l)	15	4.10 (1.26)	18	4.06 (1.45)	0.941
	IGFBP3 (mg/l)	14	0.56 (0.13)	16	0.54 (0.13)	0.686
Mean (postnatal day 7 – 30 weeks GA)	IGF1	23	3.15 (1.28)	19	3.24 (1.03)	0.789
	IGFBP3	14	0.51 (0.72)	15	0.44 (0.11)	*0.073*

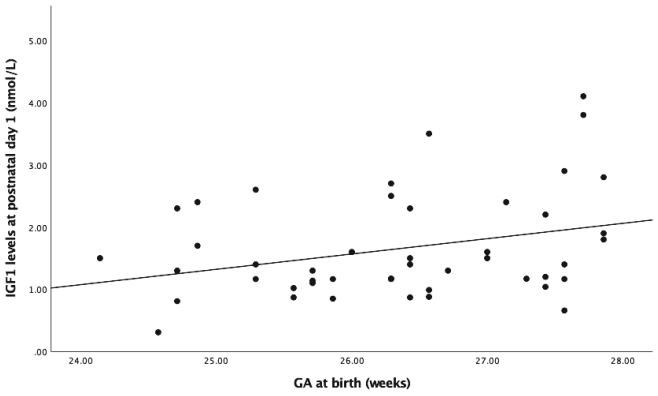
Table S3. Univariate association between postnatal IGF1/IGFBP3 and relative regional brain volumes

	Rel. total brain	Rel. gray matter	Rel. white matter	Rel. ventricle	Rel. deep gray matter	Rel. cerebellum
IGF1	p-value (95% CI)					
PND 1	0.805 (-0.012 – 0.016)	0.895 (-0.008 – 0.007)	0.379 (-0.004 – 0.10)	0.273 (-0.028 – 0.008)	0.716 (-0.033 – 0.004)	0.392 (-0.029 – 0.012)
PND 7	0.785 (-0.010 – 0.007)	0.857 (-0.005 – 0.004)	0.941 (-0.004 – 0.004)	**0.026*** (-0.022 - -0.001)	0.375 (-0.001 – 0.003)	0.054 (-0.023 – 0.000)
30 wks GA	0.791 (-0.013 – 0.010)	0.921 (-0.006 – 0.005)	0.843 (-0.006 – 0.005)	0.601 (-0.018 – 0.011)	0.447 (-0.002 – 0.004)	0.546 (-0.020 – 0.011)
Mean IGF1	0.664 (-0.013 – 0.008)	0.863 (-0.006 – 0.005)	0.833 (-0.006 – 0.005)	0.081 (-0.026 – 0.002)	0.232 (-0.001 – 0.004)	0.064 (-0.30 – 0.001)
IGFBP3						
PND 1	**0.019*** (0.031 – 0.321)	0.199 (-0.028 – 0.133)	**0.003**** (0.037 – 0.172)	0.526 (-0.136 – 0.262)	0.158 (-0.011 – 0.067)	0.954 (-0.233 – 0.220)
PND 7	0.053 (-0.001 – 0.140)	0.070 (-0.003 – 0.074)	0.245 (-0.015 – 0.056)	0.103 (-0.172 – 0.017)	0.173 (-0.006 – 0.030)	0.211 (-0.139 – 0.033)
30 wks GA	0.598 (-0.091 – 0.156)	0.534 (-0.042 – 0.080)	0.988 (-0.062 – 0.063)	0.485 (-0.219 – 0.095)	0.266 (-0.014 – 0.047)	0.360 (-0.248 – 0.093)
Mean IGFBP3	0.265 (-0.065 – 0.226)	0.351 (-0.041 – 0.111)	0.838 (-0.066 – 0.081)	0.220 (-0.307 – 0.074)	0.203 (-0.012 – 0.053)	0.540 (-0.188 – 0.101)

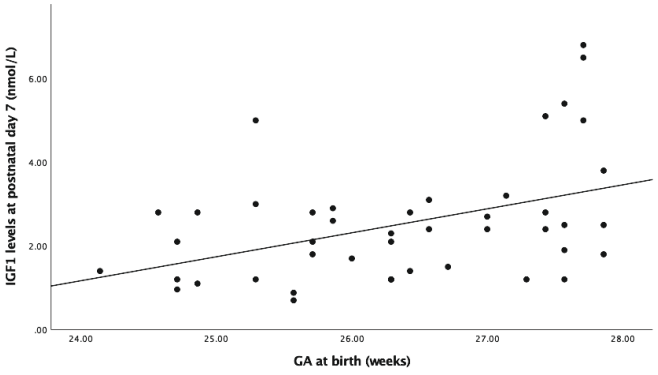
** indicates p-value below 0.05, ** indicates p-value below 0.01*

IGF1

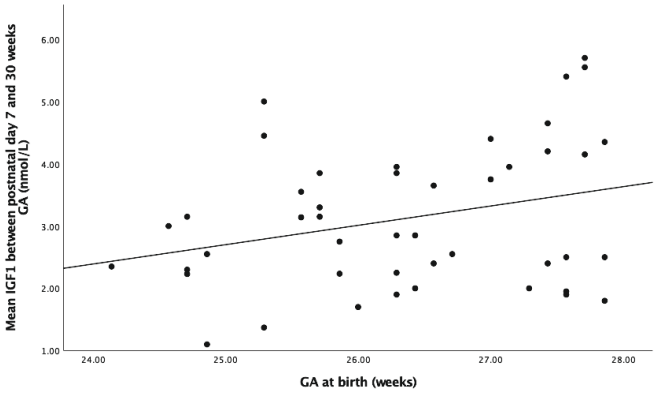
A.


IGF1 levels at postnatal day 1 (nmol/L)
5.00
4.00
3.00
2.00
1.00
.00
24.00
25.00
26.00
27.00
28.00
GA at birth (weeks)

B.


IGF1 levels at postnatal day 7 (nmol/L)
6.00
4.00
2.00
.00
24.00
25.00
26.00
27.00
28.00
GA at birth (weeks)

C.


Mean IGF1 between postnatal day 7 and 30 weeks GA (nmol/L)
6.00
5.00
4.00
3.00
2.00
1.00
24.00
25.00
26.00
27.00
28.00
GA at birth (weeks)

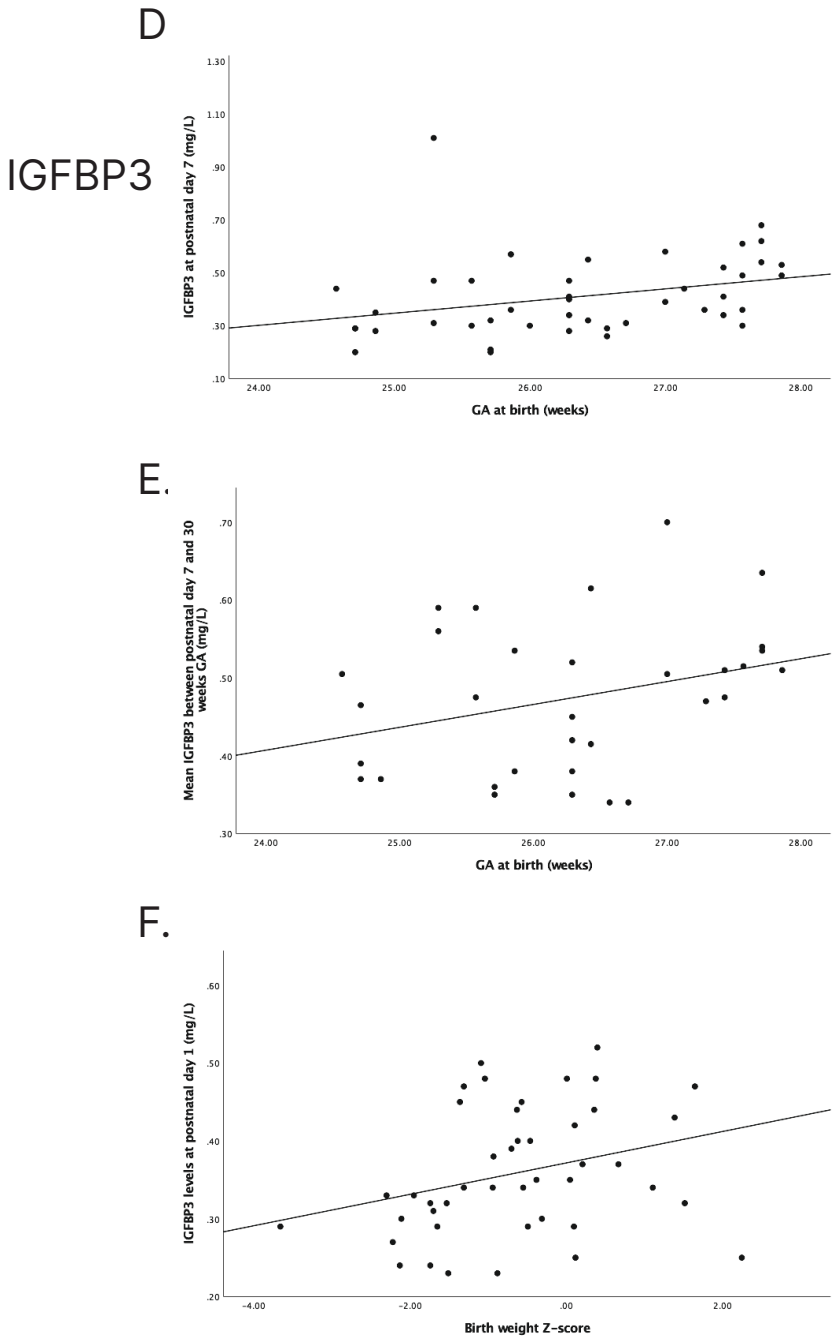


Figure S1. IGF1 and IGFBP3 blood levels are associated with GA at birth (A-E) and birth weight Z-score (F).

10

Summary & Discussion

Encephalopathy of Prematurity (EoP), a term used to describe a spectrum of pathological changes in the developing brain, is a major cause of neurodevelopmental morbidity after (extreme) preterm birth. Clinical treatment options to improve brain development during EoP are still lacking. Therefore, preclinical studies exploring promising novel therapies in clinically-relevant experimental models are urgently needed. This thesis includes multiple translational studies that 1) investigate the cellular and molecular mechanisms that underlie EoP and 2) explore the potential of novel treatment options to combat EoP. In this final chapter, the most important findings presented in this thesis are summarized, followed by a discussion that includes the potential implications of our research and our recommendations for the next steps towards clinical application of regenerative therapies for EoP.

SUMMARY

In **chapter 2**, we provided a comprehensive overview of the current standard of care and latest developments in the prevention and/or reduction of types of brain injury often observed in EoP, namely periventricular-intraventricular hemorrhage (PIVH) and white matter injury (WMI). In **chapters 3** and **4**, we summarized the latest findings regarding EoP pathophysiology. In short, (extreme) preterm birth exposes the immature brain to perinatal insults, including (neuro)inflammation and fluctuations in cerebral oxygenation and perfusion that can interfere with neurodevelopmental processes that take place in the third trimester of human gestation. Immature cells of the oligodendrocyte (OL) lineage are particularly vulnerable to these damaging insults, leading to an OL maturational arrest and subsequently diffuse WMI, characterized by global hypomyelination of the brain. Aside from white matter maldevelopment, the presence of subtle cerebral gray matter deficits in EoP has gained more attention in recent years. Particularly gamma-aminobutyric acid (GABA)-ergic interneurons are believed to be a vulnerable neuronal subpopulation in EoP. In **chapter 3**, we reviewed comprehensively the therapeutic potential of mesenchymal stem cells (MSCs) to restore brain injury in a wide range of neurological disorders. Subsequently, we used this existing knowledge to propose the next steps towards translation of MSC therapy for the preterm population. In **chapter 4**, we integrated the current body of knowledge from experimental and clinical studies, on the role of trophic and immunomodulatory factors in healthy brain development and brain injury repair, in order to identify potential therapeutic targets for EoP.

In **chapter 5** and **6,** we focused on the therapeutic potential of intranasal MSC therapy to repair the injured white matter in EoP. We introduced a novel double-hit mouse model of EoP (**chapter 5**). In this model, the combination of postnatal hypoxia-ischemia and systemic inflammation caused a transient pattern of EoP that mimics the clinical situation, including myelination deficits, impaired OL differentiation, neuroinflammation, behavioral impairments and global volumetric deficits of gray and white matter structures. Intranasal delivery of MSCs, that were shown to secrete several beneficial factors *in situ* (e.g. epidermal growth factor (EGF), leukemia inhibitory factor (LIF) and interleukin (IL) 11), restored OL maturation and myelination, dampened neuroinflammation and improved functional outcome (**chapter 5**). Interestingly, delay of cell administration (i.e. starting at 6 days after EoP induction) significantly reduced histological and functional improvement after MSC treatment in the mouse model. In a follow-up study described in **chapter 6** we explored the mechanisms underlying the limited treatment window of intranasal MSC administration and optimized the regenerative potential by means of genetic engineering of MSCs to transiently hypersecrete trophic and anti-inflammatory factors. We showed that reduced migration and regenerative capacity of intranasally-administered MSCs likely underlies the observed loss in therapeutic efficacy after delay of cell administration. Moreover, we reported that delayed intranasal administration of insulin-like growth factor 1 (IGF1)-, LIF- or IL11- hypersecreting MSCs improved myelination and functional outcome in EoP mice, thus prolonging the window for effective MSC treatment in EoP.

In **chapter 7** the novel double-hit mouse model was used to study the therapeutic potential of intranasal IGF1 therapy for EoP. Similar to the human situation, induction of EoP in mice led to a transient reduction in both systemic and cerebral levels of IGF1 and insulin-like growth factor binding protein 3 (IGFBP3). Intranasally administered IGF1 rapidly diffused throughout the brain and potently stimulated OL maturation, thereby improving myelination and functional outcome. In addition, IGF1 administration dampened astrocyte activation, but not microglia activity after EoP. Using primary cell cultures, we showed *in vitro* that this is likely the result of differential expression of the IGF1 receptor on cells of the central nervous system.

In **chapter 8** we shift our focus from myelination failure in EoP pathophysiology to disturbances in a new player in the field of EoP: the interneurons. In this chapter we studied the distribution and density of different subtypes of cortical and hippocampal interneurons in two validated double-hit rodent models of EoP. We showed that

induction of experimental EoP was associated with model-specific disturbances in interneuron density and cortical development. In mice, aberrant interneuron development was accompanied by impaired social behavior. Additionally, we explored the potential of two promising therapies, intranasal IGF1 or MSCs, to restore interneuron development. Both therapies were shown to restore the majority of interneuron abnormalities and to improve social behavior in EoP mice.

In **chapter 9** the relationship between postnatal IGF1 and IGFBP3 blood levels and neurodevelopmental outcome was explored in extreme prematurely born infants (gestational age (GA) <28 weeks). The brain magnetic resonance imaging (MRI) based Kidokoro scoring system, MRI brain segmentation and diffusion tensor imaging (DTI) were used to assess postnatal brain development at term-equivalent age. In our cohort of extreme preterm infants, higher levels of blood IGFBP3, but not of IGF1, were associated with a reduction in brain injury severity, increased regional volumes of the cerebral white- and gray matter and white matter microstructural integrity at term. Future studies are needed to further investigate the potential role of IGFBP3 as a biomarker for EoP.

DISCUSSION

EoP pathophysiology: should we focus only on the white matter?

Until a few years ago, it was thought that brain damage following (extreme) preterm birth primarily consisted of cerebral white matter injury and was only accompanied by cortical and/or deep gray matter injury in severe cases of (focal) white matter necrosis, such as cystic periventricular leukomalacia (cPVL). Advances in supportive care in the neonatal intensive care unit (NICU) have led to a decline in WMI severity after preterm birth, with the majority of infants now suffering from the diffuse form of WMI instead of (c)PVL (Back & Miller, 2014; van Haastert et al., 2011; van Tilborg et al., 2016). Interestingly, although this shift has led to a reduction of cerebral palsy (CP), it is not accompanied by a notable improvement in cognitive or neurodevelopmental outcome, implying that there is more to EoP than only WMI (Anderson & Burggren, 2014; Linsell et al., 2018). Novel insights in neurodevelopmental processes that take place in the third trimester of pregnancy, in combination with post-mortem studies, have revealed that EoP is a global dysmaturation disorder, involving cerebral white and (subtle) gray matter deficits as well as cerebellar maldevelopment (Barron & Kim, 2020; Fleiss et al., 2020; Malik et al., 2013; Spoto et al., 2021; Volpe, 2009, 2021).

The oligodendrocyte lineage

Though not the sole pathological substrate of neurological morbidity after preterm birth, impaired development of the OL lineage is believed to play a central role in EoP pathophysiology (Volpe et al., 2011) (summarized in **chapters 3** and **4**). The majority of OLs present in the brain of (extreme) preterm infants are OL precursor cells (OPCs) and pre-myelinating OLs (pre-OLs) (Volpe et al., 2011). A lack of particular anti-oxidant enzymes like superoxide dismutase (SOD), in combination with profuse expression of glutamate and cytokine receptors predispose these immature OLs to cell injury by respectively oxidative stress, excitotoxicity and inflammation induced by preterm birth-related insults (Volpe et al., 2011). Human post-mortem and experimental animal studies show that diffuse hypomyelination (diffuse WMI) in EoP is the result of OL lineage maturation arrest (Billiards et al., 2008; Buser et al., 2012). The mechanisms behind this maturation arrest of the OL lineage are still to be explored. Some studies propose arrested maturation of the existing pool of OPCs and pre-OLs, while other studies report an initial wave of pre-OL cell death and subsequent failure of differentiation of new proliferating OLs (Buser et al., 2012; Robinson et al., 2006; Segovia et al., 2008; Verney et al., 2012). In our novel mouse model of EoP, introduced in **chapter 5**, an initial increase in the number of proliferating OLs was followed by a strong reduction in the number of mature OLs. In line with observations in our rat model of fetal inflammation and postnatal hypoxia (van Tilborg et al., 2018a), we did not find clear evidence of OL cell death within 3 days after EoP induction. Thus, in both our models of EoP myelination failure is likely the result of a primary arrest in OL maturation, without extensive loss of (immature) OLs. The discrepancy between studies on the role OL cell death in the pathogenesis of EoP might reflect the heterogeneity of the disorder, with differences in the timing, nature and severity of damaging insults after preterm birth. Nevertheless, with- or without initial OL cell death, novel treatment options should focus on promoting differentiation of the OL lineage in order to boost myelination after OL maturation arrest in EoP.

Impaired gray matter development in EoP

Recently it has become more and more clear that major developmental changes take place in the gray matter during the time window analogous to (extreme) preterm birth, including migration and maturation of (inter)neurons, axonal development and synaptogenesis (Fleiss et al., 2020; Volpe, 2019). Human neuroimaging studies have indeed confirmed persistent reductions in volume of both cortical and deep gray matter structures after preterm birth, including thalamic volumes (Ball et al., 2012; Nosarti

et al., 2002; Nosarti et al., 2008; Zhang et al., 2015). In addition, reduced cerebellar volumes have been observed after preterm birth (Keunen et al., 2012; Matthews et al., 2018). Apart from volumetric changes, a decrease in cortical surface area and gyrification has been reported in preterm born children in the neonatal period and at school age (Engelhardt et al., 2015; Kersbergen et al., 2016). The exact cellular substrates of these deficits are still unclear, though overt neuronal cell death has not been reported in combination with diffuse WMI (Dean et al., 2013; Fleiss et al., 2020; Volpe, 2019). In line with these findings, we did not observe acute neuronal loss or axonal damage in our EoP mouse model (**chapter 5**). In contrast, at an older postnatal age hypoxia-ischemia was shown to induce acute gray matter loss and is therefore used to model term hypoxic ischemia encephalopathy (HIE) (van Velthoven et al., 2010). This differential response can mainly be explained by the developmental stage of the rodent brain at different ages and the depth and duration of the hypoxic hit (Semple et al., 2013). Instead, it is proposed that the observed macroscopic changes in EoP are the result of dysmaturation of the gray matter, either through a direct effect of preterm-birth related insults on the development of (inter)neurons, axons and synapses (primary dysmaturation) and/or as an indirect consequence of OL maldevelopment (secondary dysmaturation) (Volpe, 2019). GABAergic interneurons have been shown to develop throughout the third trimester of human gestation, continuing for several months after birth (Lim et al., 2018). Disturbances in the number and distribution of interneurons following (extreme) preterm birth have been reported in multiple post-mortem studies, often irrespective of the presence of WMI (Lacaille et al., 2019; Panda et al., 2018; Robinson et al., 2006; Stolp et al., 2019). Interneuron maldevelopment after EoP has recently been confirmed in a handful of experimental studies (Ardalan et al., 2019; Canetta et al., 2016; Tibrewal et al., 2018). In **chapter 8**, we reported distinct deficits in cortical and hippocampal interneuron density and cortical development in two clinically-relevant rodent models of EoP. Based on the heterogeneity of interneuron abnormalities observed in our models and those of other groups, one could speculate that distinct developmental trajectories (i.e. neurogenesis, migration or maturation) are affected based on the timing and nature of preterm birth-related insults. Apart from playing a role in maturation of neural circuitry, interneuron signaling has been directly linked to OL lineage development, as interneurons are known to promote maturation of OLs through transient synaptic input and secreted factors (Benamer et al., 2020; Voronova et al., 2017; Zonouzi et al., 2015). Thus, disturbances in interneuron development might contribute to white matter injury after extreme preterm birth. Aside from a direct interaction, interneurons and

OLs have been reported to share signaling cascades during development, including neuregulin and bone morphogenetic protein (BMP) signaling (Mei & Nave, 2014; Mukhopadhyay et al., 2009; Reid et al., 2012). In addition, both developing interneurons and OLs have been shown to express glutamate receptor subtypes that are known to mediate excitotoxicity (Desfeux et al., 2010; Kinney & Volpe, 2012). Therefore, it is likely that overlapping pathophysiological mechanisms play a role in both OL and interneuron maldevelopment in EoP. In addition to interneuron maldevelopment, disturbances in axonal development, dendritic arborization and synaptogenesis have been reported after induction of (severe) preterm brain injury (Balakrishnan et al., 2013; Dean et al., 2013; Li et al., 2014; McClendon et al., 2014; McClendon et al., 2019). However, it is still unclear to what extent these neurodevelopmental processes are affected in milder forms of EoP. Moreover, preterm birth-related insults have recently been shown to negatively affect maturation of cerebellar cells, including Purkinje neurons, in a direct and/or indirect manner (i.e. as a result of impaired input from the injured cerebrum) (Barron & Kim, 2020; Spoto et al., 2021; Volpe, 2021). Future studies, in multiple *in vivo* EoP models, are needed to further elucidate the complex pathophysiological mechanisms underlying cerebellar maldevelopment after preterm birth. Many of the gray matter disturbances mentioned in this paragraph, including interneuron deficits, Purkinje cell maldevelopment and impaired dendritic arborization, have been associated with neurodevelopmental disorders, like autism spectrum disorder (ASD), with a high prevalence in the preterm population (Fleiss et al., 2020; Marín, 2012; Volpe, 2021). Additional preclinical and clinical studies that include functional outcome parameters (including electroencephalogram (EEG) and functional (f) MRI) are needed to determine the relationship between the observed structural and cellular alterations in the developing gray matter and long-term functional outcome. In addition, novel imaging techniques might allow more detailed examination of gray matter microstructure, including axonal development and dendritic arborization in the human preterm brain (Genç et al., 2018; Veraart et al., 2020). To conclude, a vast amount of evidence supports an important role of (subtle) gray matter deficits in EoP pathophysiology. Therefore, gray matter development should be considered as a key target for potential therapeutic interventions in order to reduce preterm birth-related neurological morbidity. More insight in the complex pathophysiology of EoP, including the (cellular) interactions between white- and gray matter development, is needed to determine the best strategy to target both primary- and secondary dysmaturation of the gray matter.

Microglia and astrocytes: key players in EoP pathophysiology

Neuroinflammation, driven by activation of microglia and astrocytes, is considered a key etiological hallmark of EoP (Bennet et al., 2018; Delahaye-Duriez et al., 2021; Favrais et al., 2011; van Tilborg et al., 2016). Acute inflammation of the amnion and chorion is often a trigger of preterm birth, with histological signs of chorioamnionitis in more than 40% of spontaneous preterm deliveries at <32 weeks of gestation (Maisonneuve et al., 2020). In addition, postnatal conditions can induce or worsen inflammation, including neonatal sepsis, necrotizing enterocolitis and mechanical ventilation, which causes pulmonary and systemic inflammation (Bose et al., 2013; Hagberg et al., 2015).

Under physiological circumstances both microglia and astrocytes are known to support OL differentiation and myelination through the release of growth factors and other beneficial proteins, including IGF1 (Hagberg et al., 2015; Hagemeyer et al., 2017; Miron et al., 2013; Traiffort et al., 2020; Wlodarczyk et al., 2017). Activation of microglia and astrocytes occurs via systemic inflammatory mediators, like circulating cytokines, reaching the brain parenchyma by means of a leaky blood-brain-barrier (BBB) (Delahaye-Duriez et al., 2021). Activated, pro-inflammatory microglia and astrocytes have been observed in multiple experimental models of EoP (Favrais et al., 2011; Gussenhoven et al., 2018; van Tilborg et al., 2018a), including our novel double-hit mouse model, in which we showed persistent microgliosis and astrocyte reactivity (up to 3 weeks after injury induction) in mice subjected to postnatal hypoxia-ischemia and systemic inflammation (**chapter 5**). Activated microglia and astrocytes can negatively affect development of OLs and interneurons via the release of damaging compounds, including glutamate, reactive oxygen and nitrogen species (respectively ROS and RNS) and pro-inflammatory cytokines (Fleiss et al., 2021; Hagberg et al., 2015; Shiow et al., 2017; Stolp et al., 2019; Vasistha et al., 2020). A direct negative effect of neuroinflammation on the development of the OL lineage is supported by our findings presented in **chapter 5**. Here we show that inflammatory medium obtained from activated primary microglia induced an OL maturational arrest and subsequent hypomyelination *in vitro*. Moreover, we observed *dampening* of IGF1 secretion by microglia and astrocytes after exposure to lipopolysaccharide (LPS) *in vitro,* indicating that loss of trophic support by these glial cells could contribute to impaired neurodevelopment (**chapter 7**). Recent work has shown that perinatal inflammation can incite long-term (epigenetic) changes in microglia, persisting for months to years after the initial insult. This 'tertiary' phase of microglia activation is hypothesized to predispose patients

for future injury at adult age, including cognitive decline associated with Alzheimer's disease (Desplats et al., 2020; Fleiss et al., 2021). Prevention of this 'tertiary' phase of microgliosis might reduce the risk of neurodegenerative disease at a later age in patients born (extremely) preterm. Whether chronic activation of astrocytes occurs and whether this plays a similar role on the long-term is currently understudied.

The contribution of microglia and astrocytes in EoP pathophysiology and their supportive properties in normal development make these cells a compelling target for future therapies. Interestingly, cell-specific delivery (e.g. using nanoparticles) of immunomodulatory proteins, aimed to prevent or suppress microglia and astrocyte activation, was shown to reduce white matter deficits and improve functional outcome in experimental models of perinatal brain injury (Fleiss et al., 2021; Kannan et al., 2012; Lei et al., 2017; Van Steenwinckel et al., 2019). Therapeutic interventions that selectively dampen neuroinflammation likely indirectly contribute to an intracerebral milieu more permissive for brain development, including maturation of OLs and (inter) neurons, and could therefore be a viable strategy in the prevention or repair of EoP. In **chapter 4** we provide an overview of immunomodulatory factors that could potentially be used in treatment of neuroinflammation following preterm birth, including LIF, transforming growth factor b (TGF-b) and the neurotrophin family. Additional strategies to target microglia activation have been recently reviewed by Delahaye-Duriez et al. (2021). Apart from activation of microglia and astrocytes, recruitment and infiltration of peripheral immune cells has been reported in models of preterm brain injury and might play an important role in EoP pathophysiology (Herz et al., 2021; Jellema et al., 2013a; Winerdal et al., 2012). Disruption of BBB integrity, as a consequence of systemic inflammation, likely facilitates the influx of peripheral immune cells into the immature central nervous system (CNS) and restoration of the BBB could therefore be an interesting target for neuroprotective strategies (Gussenhoven et al., 2019; Moretti et al., 2015; Yap & Perlman, 2020).

Preclinical models: can we model the complex human neuropathology of EoP?

Translational research, aimed to unravel underlying pathophysiology or evaluate novel treatment options, relies on experimental models that mimic pathological hallmarks of disease as closely as possible, ensuring optimal relevance for the human condition. Clinically relevant animal models of EoP should include some key translational aspects: 1) induction of injury at a developmental brain stage comparable to the human preterm neonate, 2) incorporation of (multiple) etiological factors relevant for preterm

birth (i.e. perinatal inflammation and fluctuations in cerebral oxygen supply) and 3) a pattern of injury that resembles pathological hallmarks of human EoP, including diffuse WMI.

Single- versus multiple hits

Several rodent models for EoP haven been proposed (reviewed in van Tilborg et al. (2016)). For example, daily systemic injections with IL1b, a pro-inflammatory cytokine, from postnatal day (P)1-5 in mice led to myelination deficits persisting up to P30 (Favrais et al., 2011). Moreover, chronic hypoxia (P3-P11) in neonatal mice was shown to induce long-lasting hypomyelination, in absence of cystic lesions (Scafidi et al., 2014). These studies incorporate a single-factor hit, though based on current knowledge it is believed that EoP is generally the result of multiple hits, working synergistically (Kaindl et al., 2009; Rezaie & Dean, 2002; Zhao et al., 2013). For example, microglia are reportedly primed by a first hit (e.g. chorioamnionitis), becoming more responsive to subsequent insults (Fleiss et al., 2021). In **chapter 5**, we investigated the effects of combined postnatal hypoxia-ischemia and systemic inflammation in P5 mice on postnatal brain development. In line with other studies, we focused on the most apparent pathological hallmark of EoP: diffuse WMI. Whereas relatively mild postnatal hypoxia-ischemia or systemic inflammation individually did not lead to myelination deficits, the combination of both clinically-relevant insults induced myelination failure up to three weeks after injury induction. Similarly, van Tilborg et al. (2018a) observed myelination deficits up to P30 after combined fetal inflammation and postnatal hypoxia in rats. Thus, both single- and multiple hit models have been shown to induce myelination failure, capturing a key pathophysiological hallmark of human EoP. Though it can be argued that a combination of perinatal insults mimics the complex clinical course of preterm infants more faithfully, animal models using a single insult allow for more detailed examination of causal mechanisms after EoP induction. Hence, these differential experimental approaches might answer different questions, albeit ultimately all contributing to increased understanding of EoP pathophysiology.

White versus gray matter abnormalities

In line with the hypothesis of EoP as a global dysmaturation disorder (Volpe, 2009; Volpe, 2019), we observed a reduction in both gray- and white matter volumes on postnatal MRI and a reduction in hippocampus size on histological staining after EoP induction (**chapter 5**). Moreover, in **chapter 8** we showed that the combination of postnatal hypoxia-ischemia and systemic inflammation caused a specific pattern

of interneuron deficits in the cortex and hippocampus of EoP mice. Interestingly, a different pattern of interneuron abnormalities was observed in the rat model, combining fetal inflammation and postnatal hypoxia. Other models of EoP have also been associated with aberrant interneuron development (Lacaille et al., 2019; Stolp et al., 2019). The usage of animal models that mimic multiple neuropathological hallmarks of the EoP spectrum, instead of solely WMI, facilitates the search for shared cellular and molecular mechanisms that underlie dysmaturation of both the white- and gray matter after (extreme) preterm birth. More insight in these common pathophysiological pathways paves the way for therapeutic interventions that positively impact multiple cellular aspects of EoP.

Loss of growth factor support

Clinical studies have identified a reduction in plasma IGF1 in the first postnatal weeks following (extreme) preterm birth, when compared to *in utero* fetal levels at a corresponding gestational age (Hansen-Pupp et al., 2007; Hellström et al., 2016). The relevance of impaired IGF1 production in the postnatal period is supported by a vast quantity of preclinical studies that demonstrate an essential role of IGF1 in normal brain development, including maturation of cells implicated in EoP pathophysiology, such as OLs and interneurons (D'Ercole & Ye, 2008; Hurtado-Chong et al., 2009; Masters et al., 1991; Nieto-Estévez et al., 2016; Wilson et al., 2003). Moreover, this relative IGF1 deficiency has been associated with lower regional brain volumes at term-equivalent age (TEA) and subnormal neurodevelopmental at the age of two (Hansen-Pupp et al., 2011a; Hansen-Pupp et al., 2013). It is unclear whether this inadequate endogenous production of IGF1 is also observed in experimental models of EoP. In **chapter 7** we show that postnatal hypoxia-ischemia and systemic inflammation in neonatal mice indeed leads to a transient reduction of IGF1 and its binding protein IGFBP3 in the plasma and brain. Perinatal insults, particularly hypoxia-ischemia, have been directly linked to reduced local secretion of IGF1 in other *in vitro* and *in vivo* models of (term) neonatal brain injury (Lee et al., 1992; Lee et al., 1996; Sivakumar et al., 2010). Though the exact mechanisms behind the transient decrease in endogenous IGF1/IGFBP3 production in EoP are still unclear, our findings imply that there are similarities between rodent and human etiology. Though we have focused on the regulation of endogenous IGF1 secretion, it is very likely that the secretion of other trophic factors is affected by preterm birth-related insults in a similar manner. Additional studies, in our double-hit mouse model and other models of EoP, are needed to explore the role of IGF1/IGFBP3 deficiency in the pathophysiology of EoP.

Functional deficits after EoP

A key advantage of rodent models is the opportunity for extensive behavioral testing. In **chapter 5** and **8**, we demonstrated that combined postnatal hypoxia-ischemia and systemic inflammation transiently induced clinically relevant behavioral impairments, including deficits in motor functioning, cognition and sociability. Other EoP models have also reported functional deficits, postnatal inflammation was associated with cognitive deficits (Favrais et al., 2011), maternal inflammation with reduced social interaction (Vasistha et al., 2020) and fetal inflammation combined with postnatal hypoxia led to impaired motor skills, anxiety-like behavior, repetitive behavior, and reduced social play (van Tilborg et al., 2018a). Several clinical studies have found an association between preterm birth and motor and cognitive impairments as well as a higher prevalence in neurodevelopmental disorders, including ASD, characterized by reduced sociability (Johnson et al., 2009; Johnson & Marlow, 2011; Linsell et al., 2018). Thus, experimental induction of EoP leads to functional impairments that overlap with clinical findings in the human preterm neonate. The exact underlying mechanisms of impaired behavior on different domains in models of EoP and the link to the deficits in white and gray matter structures is currently understudied.

Long-term consequences of EoP: what you see is what you get?

In our model the majority of the observed anatomical, microstructural and functional deficits were restored with age (**chapter 5** and **8**). In line with our findings, endogenous recovery of myelination and interneuron deficits with age has been reported in other rodent models of EoP (Brehmer et al., 2012; Stolp et al., 2019; Thion et al., 2019; van Tilborg et al., 2018a; van Tilborg et al., 2016). This could imply that induction of experimental EoP leads to a developmental delay in the immature rodent brain, rather than inducing irreversible long-lasting changes. Such findings are in contrast with clinical observations in extremely preterm infants, in whom changes in structural and diffusion-weighted MRI persist into adulthood (Allin et al., 2011; Eikenes et al., 2011; Nosarti et al., 2008). The transient nature of brain injury in the majority of EoP animal models does currently not allow for long-term follow-up of therapeutic interventions, an important challenge in translational research. The apparent discrepancy between experimental EoP and observations in the clinic might be, to some degree, be explained by differences in species, with a higher regenerative capacity of the rodent CNS compared to that of humans (Kaplan et al., 2015). In addition, a higher turnover rate of the OL lineage in rodents compared to humans might play a role in the endogenous recovery of myelination deficits after experimental induction of EoP

(van Tilborg et al., 2018b). Interestingly, some gray matter deficits (including vasoactive-intestinal peptide (VIP)$^+$ cortical interneuron aberrations, reduced hippocampal size and hippocampal interneuron abnormalities) did persist into adulthood, implying a different underlying pathophysiology between brain regions and cell types (**chapter 5** and **8**). Despite considerable endogenous repair of the observed (cellular) deficits with progression of age, it is important to consider that this does not necessarily indicate proper functioning. In a mouse model of postnatal inflammation, the absolute number of interneurons was restored at P40, however, the number of cells with perineural nets, a morphological hallmark of functional maturity was significantly lowered (Stolp et al., 2019). Similarly, in a model of maternal inflammation and fetal macrophage depletion synaptic inhibition by interneurons was impaired, while cell density had recovered (Thion et al., 2019). Some studies do report long-lasting alternations in white matter integrity on DTI or electron microscopy (EM) and subsequent behavioral functioning following experimental induction of EoP. Schmitz et al. (2011) showed that though oligodendrocyte numbers recovered after postnatal hyperoxia in neonatal rats, subtle white matter deficiencies persisted up to P60 on DTI. Similarly, Chahboune et al. (2009) demonstrated subtle anisotropy differences on DTI in certain white matter regions, in conjunction with spatial memory deficits, that persisted into adulthood after chronic sublethal hypoxia. Scafidi et al. (2014) observed a reduction in myelin sheath thickness using EM and impaired motor functioning in adult mice that were subjected to chronic hypoxia shortly after birth (P3-P11). Moreover, even if white matter and interneuron development are only transiently disturbed, development of cerebral circuitry and other linked brain regions could be irreversibly affected (Butt et al., 2017; Fleiss et al., 2020; Volpe, 2009). Therefore, future studies that include additional outcome measures, such as electrophysiology of cortical networks, are needed to determine the long-term effects of EoP induction in the rodent brain.

Beyond the rodent

Aside from a difference in longevity of brain abnormalities, other dissimilarities between rodents and humans may limit translation. For example, though there are many similarities between white matter development in rodents and humans, differences in OL receptor expression or pro-differentiation cues have been proposed (Filipovic & Zecevic, 2008; van Tilborg et al., 2018b; Wilson et al., 2003). Similarly, variations between species in the proliferation, migration and differentiation of interneurons have been described (Gonchar et al., 2007; Kelsom & Lu, 2013). Larger animal species, including piglets, sheep and baboons, share major (anatomical) similarities with

the developing human brain and the usage of such models is strongly advised prior to clinical translation (Inder et al., 2005; Jantzie & Robinson, 2015; Ophelders et al., 2016). In addition, usage of human tissue, for example fetal tissue or human induced pluripotent stem cell (iPSC)-derived OLs, could facilitate optimal translationability (van Tilborg et al., 2018b). Moreover, iPSC-derived brain organoids could provide a unique opportunity to investigate basic mechanisms of disease and novel therapeutic strategies for EoP in a complex structure that recapitulates important stages in human brain development (Kim et al., 2019; Madhavan et al., 2018; Ormel et al., 2018).

Modelling EoP: is there an ideal model?

In conclusion, animal models of EoP are able to mimic clinically relevant patterns of developmental brain injury, with regard to anatomical, microstructural and functional outcome. The patterns of injury after induction of experimental EoP are highly dependent on the specific maturational stages of vulnerable cell types, including oligodendrocytes and interneurons. For example, in **chapter 8** we show that the pattern of interneuron deficits is model-specific, with selective vulnerability of interneuron subtypes dependent on the timing on injury induction. This reflects the human situation, as the multifactorial etiology of EoP and distinct clinical course in each patient likely accounts for the heterogeneity of neurodevelopmental abnormalities observed in (extreme) preterm infants. Thus, we propose that an 'ideal' model that recapitulates the full spectrum of EoP might not exist, and that the underlying mechanisms of disease or efficacy of potential therapeutic agents should best be evaluated in multiple models of EoP.

Regenerative therapies for EoP: cells or cargo?

Mesenchymal stem cells (MSCs)

An extensive body of evidence supports the regenerative potential of MSC therapy in a myriad of CNS pathologies (reviewed in **chapter 3**). In addition, previous studies by our group have shown that MSC therapy is safe. MSCs do not engraft after administration, limiting the risk of tumor formation (Donega et al., 2015; van Velthoven et al., 2011). In **chapter 5**, we explored the therapeutic potential of intranasal MSC administration for the most prevalent pattern of injury in EoP: diffuse WMI. Intranasal administration of MSCs potently restored OL maturation and myelination, dampened neuroinflammation and improved functional outcome in our double-hit EoP mouse model. These results are in line with several other studies in the EoP field (**chapter 3**). Mueller et al. (2017) observed a reduction in myelin loss and astrocyte reactivity

after intracranial MSC treatment of rats subjected to postnatal systemic inflammation and hypoxia-ischemia. Similarly, intranasal administration of MSCs in a rat model of EoP, induced by postnatal hypoxia and systemic inflammation, led to an increase in myelin production and decrease of gliosis in EoP animals (Oppliger et al., 2016). The therapeutic potential of MSC therapy for diffuse WMI has been confirmed in a handful preterm sheep models. Intravenous administration of MSCs was shown to promote myelination and dampen the (neuro)inflammatory response in lambs subjected to intra-uterine inflammation or hypoxia-ischemia (Jellema et al., 2013b; Li et al., 2016; Li et al., 2018; Paton et al., 2019; Paton et al., 2018). Apart from restoring diffuse WMI, MSC treatment has been shown to reduce (neuro)inflammation and boost white matter development in preclinical models of severe preterm brain injury, including IVH and cPVL (Ahn et al., 2013; Chen et al., 2010; Morioka et al., 2017; Mukai et al., 2017).

Previous studies, in the field of adult stroke, multiple sclerosis and a range of neonatal brain pathologies, revealed MSCs to exhibit anti-inflammatory, immunomodulatory and trophic properties (**chapter 3**). MSCs have been shown to exert their regenerative potential through adaptation of their secretome *in situ*, contributing to a cerebral environment permissive for endogenous repair through the release of trophic and anti-inflammatory factors (Kassis et al., 2011; Paton et al., 2017; van Velthoven et al., 2010). In line with this hypothesis, we observed increased expression of growth factors and anti-inflammatory factors in MSCs exposed to the EoP milieu *ex vivo* (**chapter 5**). In addition, using primary pre-OL and microglia cultures we demonstrate that the MSC secretome (via non-contact coculture) can simultaneously target multiple pathophysiological hallmarks of EoP, promoting OL differentiation under pro-inflammatory conditions and attenuating microglia activation (**chapter 5**). In addition to the secretion of trophic factors, MSCs are reported to release microvesicles and exosomes, containing different cargos, including mitochondria, messenger RNA (mRNA), regulatory microRNA (miRNA), cytokines and other proteins (Cunningham et al., 2018; Liang et al., 2014; Lin et al., 2015; Xiao et al., 2018). Some *in vitro* studies report a superior effect of MSCs on OL differentiation in direct contact co-cultures compared to non-contact cocultures (Oppliger et al., 2017; Zhang et al., 2016). Multiple studies have shown that MSCs are capable to support cells through reciprocal transport via gap junctions or nanotubes and production of extracellular matrix proteins (Mahrouf-Yorgov et al., 2017; Oppliger et al., 2017; Paliwal et al., 2018; Zhang et al., 2016). A particular mechanism of interest is the potential of MSCs to donate mitochondria to damaged cells after oxidative stress (Nair et al., 2021), as accumulation of ROS in immature

OLs has been shown to inhibit maturation (van Tilborg et al., 2016). Future studies are needed to explore the role and contribution of the above-mentioned cell-cell and/or secretome-driven properties of MSCs in the repair of EoP, especially on the different target cells (i.e. OLs, interneurons, microglia and astrocytes) directly and on the intricate cellular interplay.

As mentioned previously, EoP is associated with maldevelopment of both the white and gray matter. In **chapter 8** we reveal that intranasal MSC therapy is a potent strategy to restore atypical interneuron development after experimental induction of EoP. Though effective in restoring the majority of interneuron deficits, the exact mechanism of action remains unknown. Similar to OL differentiation, interneuron development could be positively affected by MSC-secreted factors (e.g. neuregulins, hepatocyte growth factor (HGF), glial cell line-derived neurotrophic factor (GDNF) and IGF1) in a direct manner. Moreover, reduction of neuroinflammation could indirectly contribute to restoration of interneuron abnormalities. BMP4, a inhibitory factor secreted by reactive astrocytes (Molina-Gonzalez & Miron, 2019), has been reported to regulate interneuron subtype development (Mukhopadhyay et al., 2009). Primary cell cultures are needed to determine the effects of MSCs on individual cell types, including interneurons.

Thus, an increasing amount of preclinical evidence supports the therapeutic potential of MSCs to restore brain development in EoP. However, methodological differences between preclinical studies complicate future clinical translation (**chapter 3**). One of the challenges in adopting a uniform treatment protocol is the selection of the most optimal source of MSCs. MSCs can be harvested from a variety of sources, including adipose tissue, bone marrow and the umbilical cord (blood and wharton's jelly), with each distinct cell characteristics (Berebichez-Fridman & Montero-Olvera, 2018). The therapeutic potential of MSCs is believed to decline with age, favoring the use of umbilical cord-derived cells (Kalaszczynska & Ferdyn, 2015; Park et al., 2018; Scruggs et al., 2013). Moreover, cell harvest from the umbilical cord is non-invasive, in contrast to other sources. Wharton's jelly derived MSCs might show the highest potential, with a larger, stable yield of cells after harvest and lower immunogenicity compared to cord blood-derived MSCs (El Omar et al., 2014; Zeddou et al., 2010). Back-to-back comparison of umbilical cord MSCs harvested after preterm versus term birth demonstrated an apparent superior capacity of 'term' MSCs to restore white matter injury after preterm birth (Li et al., 2017; Oppliger et al., 2017). In addition to a

potentially inferior therapeutic efficacy, limitations in time and logistics, as well as a presumably high variability in cellular properties due to the patient's clinical condition (for example in birth asphyxia) could complicate the use of autologous umbilical cord-derived MSCs after preterm birth. Thus, allogenic Wharton's jelly derived MSCs obtained from healthy term-born infants should perhaps be the stem cell of choice in the treatment of EoP. A promising cell-free alternative to MSC administration might be the use of extracellular vesicles (EVs), which contain a variety of components as cargo, including proteins, mRNA or miRNA. Clinical applicability of EV therapy might be superior to live cell administration, with fewer concerns on production, storage and safety. However, more studies are needed to evaluate the therapeutic potential of EV treatment compared to live cell administration (Vaes et al., 2019). In sum, additional preclinical studies are required to reach consensus on the most promising cell source, as at this time few studies offer a back-to-back comparison of the efficacy of multiple cell origins. The use of an off-the-shelf standardized (cellular) product will reduce variability in therapeutic efficacy between patients and thereby improve in clinical applicability.

Insulin-like growth factor 1

As described in **chapter 4**, a vast number of experimental studies has provided evidence for an essential role of IGF1 in normal fetal and postnatal brain development, and in brain repair following injurious events. IGF1 binds to its Type I IGF receptor (IGFR), which is broadly expressed by cells of the CNS, including the OL lineage. In **chapter 7** we studied the potential of IGF1 as a treatment to improve diffuse WMI, a key feature of EoP. We show that intranasal IGF1 treatment promotes OL maturation, myelination and improved functional outcome in our double-hit mouse model of EoP. Interestingly, intranasal IGF1 treatment reduced astrocyte reactivity, but not microgliosis. These findings are in line with other studies in the field of neonatal brain injury (**chapter 4**). For example, intranasal IGF1 treatment was reported to reduce OL loss and improve myelination in rat model of severe preterm WMI (Cai et al., 2011). Similarly, Lin et al. (2009) demonstrated a significant improvement in myelination and functional outcome after intranasal IGF1 administration in a rat model of *near-term* HIE. In addition to restoring diffuse WMI, in **chapter 8** we show that intranasal IGF1 treatment restores the majority of interneuron deficits in our double-hit mouse model of EoP.

More insight in the underlying supportive mechanisms of IGF1 was obtained using primary glial cultures. Our *in vitro* data show that IGF1 promotes differentiation of

OL lineage cells after inflammation-induced maturation arrest. These findings are in line with multiple *in vitro* studies that show a direct effect of IGF1 on OL lineage cells in normal development and disease (McMorris et al., 1986; Ness, 2002; Pang et al., 2007; Wilson et al., 2003). In contrast, similar to our *in vivo* findings IGF1 treatment failed to dampen microglia activation *in vitro*. Western blot analyses demonstrated activation of the IGF1 receptor in OLs and astrocytes, but not in microglia. Based on these findings we propose that IGF1 promotes myelination by directly acting on OLs, though its neuroregenerative effects could also be partially medicated by a direct effect of IGF1 on astrocytes, but not by dampening of microglia activation. These findings indicate that restoration of brain development in EoP can be achieved without dampening microglia activation, implying that the OL lineage might be the key therapeutic target to improve neurodevelopmental outcome after preterm birth. The underlying mechanism of action of IGF1 on the repair of interneuron deficits is still unknown, though IGF1 has been implicated to directly affect interneuron development (Dai et al., 2017; Hurtado-Chong et al., 2009; Nieto-Estévez et al., 2016). Additional insights into the working mechanism of IGF1 therapy could be obtained by using primary interneuron cultures.

The therapeutic potential of IGF1 to promote brain development in EoP is supported by observations in clinical studies (**chapter 4**). Extremely preterm infants are shown to suffer from a relative IGF1 deficiency, with a reduction in plasma IGF1 in the first postnatal weeks when compared to *in utero* levels at a similar post-menstrual age (Hansen-Pupp et al., 2007; Hellström et al., 2016). Similar to IGF1, blood levels of IGFBP3 have been reported to be low after (extreme) preterm birth (Hellström et al., 2016). These low postnatal levels of IGF1 and IGFBP3, during a time window critical for brain development, have been associated with volumetric deficits in multiple brain regions and a poorer neurodevelopmental outcome (Hansen-Pupp et al., 2011a; Hansen-Pupp et al., 2013). In **chapter 9**, we aimed to confirm the relationship between postnatal IGF1 and IGFBP3 blood levels and brain development at TEA in a cohort of extreme preterm infants. Similar to a pioneer study performed in Sweden, we observed a positive association between blood IGFBP3 levels and regional brain volumes (Hansen-Pupp et al., 2011a). Moreover, higher blood IGFBP3 levels were associated with a reduction in brain injury severity and were positively associated with white matter microstructural integrity in the corpus callosum and right posterior thalamic. However, in contrast to previous reports, we were unable to detect a relationship between postnatal IGF1 blood levels and brain development at TEA

(**chapter 9**). This discrepancy could be the result of differences in study methodology, population characteristics or standard of care (see discussion **chapter 9**). In addition, these results could imply that not all preterm infants are in need of normalization of endogenous IGF1 levels through exogenous administration. However, we propose that if this is the case these infants could still benefit from supraphysiological IGF1 therapy. In **chapter 7** we show that though the drop in endogenous IGF1 levels after experimental EoP induction was recovered after 3 days, shortening of our treatment protocol to match this timeline did not lead to histological or functional recovery. These data imply that instead of restoring IGF1 to physiological levels, supraphysiological levels of IGF1 might be needed to effectively repair the injured preterm brain. Additional preclinical dosing studies are needed to substantiate this hypothesis. In pioneer clinical feasibility studies (intravenous) infusion of IGF1/IGFBP3 after (extreme) preterm birth was not associated with any safety concerns (Hansen-Pupp et al., 2017). Recently, the first multicenter, randomized, controlled trial on the effect of postnatal IGF1/IGFBP3 treatment after extreme preterm birth (250 µg/kg daily via continuous intravenous infusion; from ≤ 24 hours after birth until 29 weeks + 6 days postmenstrual age (PMA)) reported a lower prevalence of severe IVH in preterm infants receiving treatment, as assessed by cranial ultrasound (CUS) (Horsch et al., 2020). The prevalence of PVL and diffuse WMI on CUS did not differ between the treatment and control groups. However, this study was not powered to assess reduction of preterm brain injury. A larger clinical trial is currently underway (NCT03253263). As proposed by the authors, future studies should consider the use of MRI (at TEA) as an outcome parameter to increase sensitivity for subtle patterns brain injury, using segmentation analyses and DTI.

Based on our findings in **chapter 9**, early postnatal IGFBP3 levels could potentially serve as an interesting therapeutic target and/or biomarker for EoP. However, the relationship between low blood levels of IGFBP3 and neurological outcome is still unclear. IGFBP3, the most important binding protein of IGF1 in the CNS, plays an essential role in modulation of IGF1 bioavailability and activity (Dai et al., 2017; Martin & Baxter, 2011). In addition, multiple IGF1-independent actions have described, including activation of the EGFR and TGFβR, both reported to induce OL lineage maturation (Martin & Baxter, 2011; Varma Shrivastav et al., 2020). Clinical studies in preterm infants use a recombinant human protein complex of IGF1 and IGFBP3, in order to counteract the low endogenous levels of binding protein after preterm birth and thereby optimize the biological actions of IGF1 (Holgersen et al., 2020; Ley et al., 2019; Ley et al., 2013).

Interestingly, in our preclinical study addition of IGFBP3 to the IGF1 complex did not lead to superior effects on white matter recovery (**chapter 7**). This discrepancy could potentially be explained by the mode of administration, with intranasal IGF1 rapidly diffusion throughout the brain after administration (Thorne et al., 2004). The therapeutic efficacy of IGFBP3 monotherapy and its potential effects on the bioavailability of endogenously produced IGF1 or beneficial IGF1-independent actions have not yet been studied in the field of preterm brain injury. A potential biomarker function of IGFBP3 is likely most applicable in the most immature infants, as the relationship between IGFBP3 and regional brain volumes seemed more pronounced in this population (GA<26.4 weeks) (**chapter 9**). Additional (larger) studies are needed for further validation of IGFBP3 as a biomarker for preterm brain injury.

Other trophic or immunomodulatory factors

As summarized in **chapter 4**, a wide range of growth factors and cytokines have been shown to impact OL lineage differentiation and microglia/astrocyte activation during healthy brain development and after injury. Candidates for monotherapy after EoP preferably impact multiple pathophysiological processes, including differentiation of the OL lineage, development of interneurons and polarization of microglia/astrocytes. An overview of the most promising therapeutic candidates can be found in table 1 of **chapter 4**. Neuregulin (NRG) 1 might be particularly interesting in the treatment of EoP, as this factor has been implicated to play a role in myelination and interneuron development as well as modulation of neuroinflammation (Mei & Nave, 2014; Raabe et al., 1997; Xu et al., 2017). GDNF has also been shown to play a pivotal role in interneuron development and was shown to promote OL maturation and myelination in a rat model of PVL (Li et al., 2015; Pozas & Ibáñez, 2005). However, it should be noted that sustained exposure to GDNF has been associated with prolongation of neuroinflammation (Duarte Azevedo et al., 2020).

Other promising candidates for monotherapy after EoP include IL11, LIF and C-X-C motif chemokine ligand (CXCL)12, as these all-round factors were shown to boost OL maturation while simultaneously dampening neuroinflammation in experimental studies (**chapter 4**). The therapeutic potential of IL11, CXCL12 and LIF for EoP was confirmed in our *in vitro* studies using primary pre-OL cultures. Addition of these factors promoted OL differentiation and myelination under inflammatory conditions (**chapter 5**). Similarly, EGF and granulocyte colony-stimulating factor (GCSF) were shown to boost OL maturation and myelin production *in vitro*. However, in some

preclinical studies these factors were associated with exacerbation of WMI (**chapter 4**). Interestingly, the majority of the factors proposed in **chapter 4** (including GDNF, IL11, CXCL12 and LIF) were identified in the secretome of MSCs exposed to the EoP milieu (**chapter 5**). Thus, these factors likely play an essential role in the observed regenerative properties of MSCs after EoP. It should be noted that for the majority of factors proposed in **chapter 4** evidence is still inconclusive, and thus future (experimental) studies are urgently needed to further explore the therapeutic potential and safety of these factors in EoP.

Intranasal application: the optimal route for CNS delivery?

The most optimal route of administration to target the CNS in the preterm infant is still under debate. Though earlier experimental studies often rely on intracerebral injections of MSCs or IGF1, ensuring direct and targeted delivery, it is an invasive procedure not easily applicable in human patients. More recently, focus has shifted to intranasal administration of cells or substances as a non-invasive, safe and rapid method of local administration. Both cells and proteins have been shown to respectively migrate or diffuse towards the CNS (Danielyan et al., 2009; Thorne et al., 2004). A very similar therapeutic efficacy of MSC therapy after intranasal versus intracerebral administration was reported in mouse model of HIE (van Velthoven et al., 2012). The effectivity of intranasal administration to target the immature brain after EoP was confirmed in **chapter 5** and **7**. In **chapter 5** we show that intranasally applied MSCs are dispersedly distributed throughout the brain after EoP, with minimal loss of cells to peripheral organs. In contrast, in uninjured animals the majority of cells were found in the periphery, suggesting that MSCs only favor CNS migration after injury. Homing of MSCs to sites of injury is regulated by chemokine gradients (Ullah et al., 2019). In addition, intranasal application of IGF1 led to rapid diffusion of the protein throughout the injured brain (**chapter 7**). Additional information on the proposed pathways underlying transport of MSCs or IGF1 after intranasal application can be found elsewhere (Danielyan et al., 2009; Thorne et al., 2004). In line with rodent studies, rapid migration of intranasally administered MSCs to the injured brain was recently confirmed by our group in a baboon model of neonatal HIE (Wagenaar, 2019). Additional studies in large animals are needed to confirm efficient delivery of cells or proteins to the CNS after intranasal administration in EoP. A clinical trial studying the safety and feasibility of intranasal allogenic bone marrow derived MSC administration after perinatal arterial ischemia stroke is currently taking place in our center (PASSIoN; NCT03356821).

Another commonly used mode of administration is the intravenous route. However, intravenous administration has been shown to lead to significantly lower cell numbers or protein concentrations in the CNS when compared to intranasal administration as cells or proteins will get trapped or taken up by peripheral organs (Danielyan et al., 2009; Thorne et al., 2004). Options to improve CNS delivery of factor-based therapies after intravenous administration, including the use of focused ultrasound or nanoparticles, are summarized in **chapter 4**. Moreover, nanoparticle-based drug delivery could be a promising strategy to facilitate cell-specific delivery of proteins to the CNS in a controlled manner (Nag & Delehanty, 2019; Rittchen et al., 2015). In addition, CNS delivery of proteins through intravenous administration might be complicated by a short half-life. For IGF1, continuous infusion was deemed necessary to reach therapeutic blood levels (Hansen-Pupp et al., 2017). However, continuous intravenous infusion of IGF1 during the first weeks of life could be a substantial clinical burden, as prolonged intravenous access has been associated with complications such as late-onset sepsis and thrombosis (Advani et al., 2011; Jumani et al., 2013; Shah et al., 2015). Intranasal administration of IGF1 may offer a less invasive, safe, rapid and direct route to target the CNS in extreme preterm infants. Though delivery of cells or proteins to the brain is impaired after peripheral administration, one could speculate that "loss" of cells or proteins in the periphery could benefit the development of multiple organ systems, including the lungs and gut, and target peripheral inflammation. For example, entrapment of MSCs in the spleen and liver has been reported to reduce T-cell activation, and thereby contribute to inactivation peripheral immune responses that harm CNS development (Jellema et al., 2013b). However, diffuse loss of cells or protein in the periphery may also cause unwanted side-effects.

Timing of treatment: earlier is better?

The optimal timing of treatment of EoP patients is still unclear, even though one could speculate that treatment with MSCs or trophic factors during a time window analogous to healthy white- and gray matter development could prove to be most effective. Interestingly, we observed a limited treatment window for both MSCs (**chapter 5**) (most optimal: P8) and IGF1 therapy (most optimal: P5-P10) (**chapter 7**). Similar observations have been made after neonatal stroke or IVH, with a superior therapeutic efficacy of MSCs after early versus late treatment (**chapter 4**) (Kim et al., 2012; Park et al., 2016). Collectively, these data might imply a limited regenerative capacity in the later stages of EoP pathophysiology.

In **chapter 6**, we explored the underlying mechanisms of reduced efficacy of intranasal MSC treatment after delayed administration. Using primary cultures, we show that MSCs have a limited capacity to boost myelination under prolonged pro-inflammatory conditions after delayed administration *in vitro*. *In vivo* tracing experiments revealed partial impaired migration of MSCS after delayed intranasal administration. We successfully modified MSCs to transiently overexpress a range of growth factors or anti-inflammatory cytokines. Interestingly, IGF1-, LIF- and IL11-overexpressing MSCs significantly improved myelination and functional deficits after delayed intranasal administration, prolonging the treatment window for effective MSC treatment after EoP. The exact mechanisms underlying the superior treatment efficacy of modified MSCs after postponed treatment remain unknown. It is possible that modified MSCs have superior regenerative properties, either as a result of overexpressed trophic factor as such or a superior MSC secretome evoked by autocrine mechanisms, to compensate for the smaller proportion of MSCs that reach the lesion site after delayed administration. Moreover, modification of MSCs could lead to improved migration, with an upregulation of receptors essential for MSC migration (De Becker & Riet, 2016; Ullah et al., 2019). Future preclinical studies are needed to determine 1) which chemoattractants play a role in the migration of MSCs after EoP and 2) the effect of selective overexpression of trophic factors on chemokine receptor expression by MSCs. Other MSC optimization strategies, including *in vitro* preconditioning of MSCs before *in vivo* administration, are discussed in **chapter 3**. Pioneer clinical studies, using modified human MSCs in adult stroke, did not report safety concerns (Steinberg et al., 2016). Moreover, IGF1 protein monotherapy was deemed safe in a recent clinical trial (Hansen-Pupp et al., 2017), and could therefore be the most promising target for MSC modification. Additional preclinical studies assessing long-term outcome and conventional clinical safety studies are needed to confirm safety of modified MSCs in preterm neonates.

Irrespective of the treatment of choice, validated biomarkers that reliably predict EoP would aid in the selection of patients that could benefit from early regenerative therapies. The multifactorial etiology of EoP, with multiple perinatal and postnatal insults potentially interfering with postnatal brain development make it difficult to pinpoint the exact timeframe in which injury develops (Rezaie & Dean, 2002; Zhao et al., 2013). At this time, EoP diagnosis is largely based on MRI at TEA, when white matter development is advancing (de Vries et al., 2013). As an alternative to patient selection via biomarkers, standard treatment of *all* (extreme) preterm infants

with MSCs or IGF1 could be considered after careful evaluation of safety concerns. However, this strategy is likely associated with high costs. In recent years, identification of biomarkers to predict preterm brain injury has received increasing attention. Multiple blood biomarkers, including S100B, glial fibrillary acidic protein (GFAP), interleukins and metabolites as well as non-invasive monitoring, including EEG and near infrared spectroscopy (NIRS) have been shown to predict development of IVH, PHVD and PVL (Douglas-Escobar & Weiss, 2012; Jin et al., 2015; Stewart et al., 2013; Yue et al., 2021). However, to our knowledge, biomarkers for early identification of more subtle patterns of EoP-associated injury, including diffuse WMI, are still lacking. Based on our findings described in **chapter 9**, we propose IGFBP3 blood levels, measured in the first postnatal week, as a potential biomarker for CNS maldevelopment following extreme preterm birth. Aside from blood biomarkers, identification of predisposing genetic profiles could aid in the selection of high-risk patients for whom early regenerative therapy could be essential. Pioneer studies have identified multiple single nucleotide polymorphisms (SNPs) that are associated with differential susceptibility to preterm birth-related insults, in particular to (neuro)inflammation (Boardman et al., 2014; Harding et al., 2007; Hoffmann et al., 2010; Kapitanović Vidak et al., 2012; Krishnan et al., 2017; Oldenburg et al., 2020; Varner et al., 2020). Future multicenter studies, with a larger sample size, are needed to identify and validate early predictors of EoP.

Regenerative therapies for EoP: good, better, best

Based on the results presented in **chapters 5, 6, 7** and **8** we conclude that intranasal MSC or IGF1 therapy both seem to target a broad spectrum of processes affected by preterm birth-related insults, thereby aiding in proper development of both the white and gray matter after EoP. In addition, a broad range of potential therapeutic targets for EoP are discussed in **chapter 4**. Based on our findings, one could propose that MSC therapy shows slight superiority over IGF1 treatment, with a more pronounced effect on repair of both diffuse WMI and interneuron deficits, as well as dampening of neuroinflammation. This observation might be explained by the plethora of beneficial (growth) factors continuously (at least days-long) secreted by MSCs in the EoP microenvironment in tailor-made way compared to one beneficial factor alone. In addition, as proposed in **chapter 6**, modification of MSCs could prolong the therapeutic window in EoP, improving future clinical translationability. Moreover, MSC administration could be the less invasive option, as a single dose was shown to restore brain development in EoP mice (**chapter 5**), while prolonged intranasal administration of

IGF1 was needed to achieve a similar therapeutic effect (**chapter 7**). Our experimental IGF1 treatment during 6 consecutive days (P5-P10) likely corresponds to multiple weeks of treatment in the human preterm neonate, as white matter development at P9/10 equals TEA in humans (Semple et al., 2013). However, cell-free options (either EVs or trophic factors) might be the more clinically desirable option, as these alternatives might come with fewer concerns of production, storage and safety. Aside from monotherapy, administration of a cocktail of multiple promising factors, aimed at OL differentiation, interneuron development and reduction of neuroinflammation could potentially be a viable treatment option. The contents of such a cocktail of factors could be tailor-made to the individual patient's disease course, for example by administration of factors with strong anti-inflammatory properties during episodes of inflammation. Similar effects might be accomplished by genetic modification of MSCs, as this technique could induce (transient) overexpression of specific beneficial factors in a controlled manner. In addition to MSCs, nutritional interventions have been proposed to modulate cerebral growth factor and cytokine concentrations (Hortensius et al., 2019; Keunen et al., 2015). Interestingly, dietary intake has been suggested to modulate endogenous IGF1 production, however the correlation between nutrition and IGF1 concentration in the first postnatal weeks following extreme preterm birth is still under discussion (Hansen-Pupp et al., 2011b; Yumani et al., 2015; Yumani et al., 2020). Though it would be an option to select one most optimal treatment strategy for EoP based on strengths and limitations, a combination of cell-based, cell-free and nutritional therapies might prove to even further benefit the injured preterm brain.

Concluding remarks

To conclude, in this thesis we propose a novel multiple-hit mouse model of EoP to study the cellular and molecular mechanisms that underlie EoP. We show that experimental induction of EoP is associated with global dysmaturation of the immature brain, impacting both white- and gray matter development. We and others have observed distinct patterns of injury (e.g. interneuron abnormalities) after EoP induction, most likely the result of differences in the timing and nature of experimental hits. This heterogeneity implies that promising therapeutic options should be evaluated in multiple models of EoP, reflecting the distinct patterns of developmental disturbances elicited by each patient's unique clinical course. Our clinically-relevant model enabled detailed investigation of novel regenerative therapies to restore brain development after EoP. We show that both early intranasal MSC and growth factor treatment, in particular IGF1, show great promise to support brain development after EoP. However,

there are still challenges to overcome for optimal translation from bench-to-incubator. Biomarkers to select infants at risk for EoP in an early stage are urgently needed. Alternatively, in absence of major side-effects, promising therapies (e.g. IGF1) could potentially be included in standard clinical care. Future preclinical and clinical studies should aim to improve methodological uniformity, including the use of standardized (cell) products and determination of the most optimal route of delivery and timing of treatment. Interesting recommendations to overcome heterogeneity in preclinical and clinical trials in the field of neonatal brain injury were recently published by Passera et al. (2021). By close collaborations in the EoP field (e.g. the PREMSTEM project), we can refine these treatment strategies, and thereby improve neurodevelopmental outcome and quality of life after (extreme) preterm birth in the future.

REFERENCES

Advani, S., Reich, N. G., Sengupta, A., Gosey, L., & Milstone, A. M. (2011). Central line-associated bloodstream infection in hospitalized children with peripherally inserted central venous catheters: extending risk analyses outside the intensive care unit. *Clin Infect Dis, 52*(9), 1108-1115. doi:10.1093/cid/cir145

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Yoo, H. S., Lee, J. H., . . . Park, W. S. (2013). Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. *Stroke, 44*(2), 497-504. doi:10.1161/strokeaha.112.679092

Allin, M. P., Kontis, D., Walshe, M., Wyatt, J., Barker, G. J., Kanaan, R. A., . . . Nosarti, C. (2011). White matter and cognition in adults who were born preterm. *PLoS One, 6*(10), e24525. doi:10.1371/journal.pone.0024525

Anderson, A., & Burggren, A. (2014). Cognitive and neurodevelopmental benefits of extended formula-feeding in infants: Re: Deoni et al. 2013. *NeuroImage, 100*, 706-709. doi:10.1016/j.neuroimage.2014.05.011

Ardalan, M., Svedin, P., Baburamani, A. A., Supramaniam, V. G., Ek, J., Hagberg, H., & 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

Back, S. A., & Miller, S. P. (2014). Brain injury in premature neonates: A primary cerebral dysmaturation disorder? *Annals of Neurology, 75*(4), 469-486. doi:10.1002/ana.24132

Balakrishnan, B., Dai, H., Janisse, J., Romero, R., & Kannan, S. (2013). Maternal endotoxin exposure results in abnormal neuronal architecture in the newborn rabbit. *Dev Neurosci, 35*(5), 396-405. doi:10.1159/000353156

Ball, G., Boardman, J. P., Rueckert, D., Aljabar, P., Arichi, T., Merchant, N., . . . Counsell, S. J. (2012). The effect of preterm birth on thalamic and cortical development. *Cereb Cortex, 22*(5), 1016-1024. doi:10.1093/cercor/bhr176

Barron, T., & Kim, J. H. (2020). Preterm Birth Impedes Structural and Functional Development of Cerebellar Purkinje Cells in the Developing Baboon Cerebellum. *Brain Sci, 10*(12). doi:10.3390/brainsci10120897

Benamer, N., Vidal, M., & Angulo, M. C. (2020). The cerebral cortex is a substrate of multiple interactions between GABAergic interneurons and oligodendrocyte lineage cells. *Neurosci Lett, 715*, 134615. doi:10.1016/j.neulet.2019.134615

Bennet, L., Dhillon, S., Lear, C. A., van den Heuij, L., King, V., Dean, J. M., . . . Gunn, A. J. (2018). Chronic inflammation and impaired development of the preterm brain. *Journal of Reproductive Immunology, 125*, 45-55. doi:10.1016/j.jri.2017.11.003

Berebichez-Fridman, R., & Montero-Olvera, P. R. (2018). Sources and Clinical Applications of Mesenchymal Stem Cells: State-of-the-art review. *Sultan Qaboos University medical journal, 18*(3), e264-e277. doi:10.18295/squmj.2018.18.03.002

Billiards, S. S., Haynes, R. L., Folkerth, R. D., Borenstein, N. S., Trachtenberg, F. L., Rowitch, D. H., . . . Kinney, H. C. (2008). Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia. *Brain Pathology, 18*(2), 153-163. doi:10.1111/j.1750-3639.2007.00107.x

Boardman, J. P., Walley, A., Ball, G., Takousis, P., Krishnan, M. L., Hughes-Carre, L., . . . Edwards, A. D. (2014). Common genetic variants and risk of brain injury after preterm birth. *Pediatrics, 133*(6), e1655-1663. doi:10.1542/peds.2013-3011

Bose, C. L., Laughon, M. M., Allred, E. N., O'Shea, T. M., Van Marter, L. J., Ehrenkranz, R. A., . . . Leviton, A. (2013). Systemic inflammation associated with mechanical ventilation among extremely preterm infants. *Cytokine, 61*(1), 315-322. doi:10.1016/j.cyto.2012.10.014

Brehmer, F., Bendix, I., Prager, S., van de Looij, Y., Reinboth, B. S., Zimmermanns, J., . . . Gerstner, B. (2012). Interaction of Inflammation and Hyperoxia in a Rat Model of Neonatal White Matter Damage. *PLoS One, 7*(11), 1-13. doi:10.1371/journal.pone.0049023

Buser, J. R., Maire, J., Riddle, A., Gong, X., Nguyen, T., Nelson, K., . . . Back, S. A. (2012). Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. *Annals of Neurology, 71*(1), 93-109. doi:10.1002/ana.22627

Butt, S. J., Stacey, J. A., Teramoto, Y., & 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

Cai, Z., Fan, L.-W., Lin, S., Pang, Y., & Rhodes, P. G. (2011). Intranasal Administration of IGF-1 Protects against Lipopolysaccharide-induced Injury in the Developing Rat Brain. *Neuroscience, 194*, 195-207. doi:10.1016/j.neuroscience.2011.08.003

Canetta, S., Bolkan, S., Padilla-Coreano, N., Song, L. J., Sahn, R., Harrison, N. L., . . . Kellendonk, C. (2016). Maternal immune activation leads to selective functional deficits in offspring parvalbumin interneurons. *Mol Psychiatry, 21*(7), 956-968. doi:10.1038/mp.2015.222

Chahboune, H., Ment, L. R., Stewart, W. B., Rothman, D. L., Vaccarino, F. M., Hyder, F., & Schwartz, M. L. (2009). Hypoxic injury during neonatal development in murine brain: correlation between in vivo DTI findings and behavioral assessment. *Cerebral Cortex, 19*(12), 2891-2901. doi:10.1093/cercor/bhp068

Chen, A., Siow, B., Blamire, A. M., Lako, M., & Clowry, G. J. (2010). Transplantation of magnetically labeled mesenchymal stem cells in a model of perinatal brain injury. *Stem Cell Res, 5*(3), 255-266. doi:10.1016/j.scr.2010.08.004

Cunningham, C. J., Redondo-Castro, E., & Allan, S. M. (2018). The therapeutic potential of the mesenchymal stem cell secretome in ischaemic stroke. *Journal of Cerebral Blood Flow and Metabolism, 38*(8), 1276-1292. doi:10.1177/0271678×18776802

D'Ercole, a. J., & Ye, P. (2008). Minireview: Expanding the mind: Insulin-like growth factor I and brain development. *Endocrinology, 149*(12), 5958-5962. doi:10.1210/en.2008-0920

Dai, H., Goto, Y.-i., & Itoh, M. (2017). Insulin-Like Growth Factor Binding Protein-3 Deficiency Leads to Behavior Impairment with Monoaminergic and Synaptic Dysfunction. *Am J Pathol, 187*(2), 390-400. doi:10.1016/j.ajpath.2016.10.011

Danielyan, L., Schafer, R., von Ameln-Mayerhofer, A., Buadze, M., Geisler, J., Klopfer, T., . . . Frey, W. H., 2nd. (2009). Intranasal delivery of cells to the brain. *European Journal of Cell Biology, 88*(6), 315-324. doi:10.1016/j.ejcb.2009.02.001

De Becker, A., & Riet, I. V. (2016). Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy? *World J Stem Cells, 8*(3), 73-87. doi:10.4252/wjsc.v8.i3.73

de Vries, L. S., Benders, M. J. N. L., & Groenendaal, F. (2013). Imaging the premature brain: ultrasound or MRI? *Neuroradiology, 55*(2), 13-22. doi:10.1007/s00234-013-1233-y

Dean, J. M., McClendon, E., Hansen, K., Azimi-Zonooz, A., Chen, K., Riddle, A., . . . Back, S. A. (2013). Prenatal cerebral ischemia disrupts MRI-defined cortical microstructure through disturbances in neuronal arborization. *Sci Transl Med, 5*(168), 168ra167. doi:10.1126/scitranslmed.3004669

Delahaye-Duriez, A., Dufour, A., Bokobza, C., Gressens, P., & Van Steenwinckel, J. (2021). Targeting Microglial Disturbances to Protect the Brain From Neurodevelopmental Disorders Associated With Prematurity. *J Neuropathol Exp Neurol, 80*(7), 634-648. doi:10.1093/jnen/nlab049

Desfeux, A., El Ghazi, F., Jégou, S., Legros, H., Marret, S., Laudenbach, V., & Gonzalez, B. J. (2010). Dual Effect of Glutamate on GABAergic Interneuron Survival during Cerebral Cortex Development in Mice Neonates. *Cerebral Cortex, 20*(5), 1092-1108. doi:10.1093/cercor/bhp181

Desplats, P., Gutierrez, A. M., Antonelli, M. C., & Frasch, M. G. (2020). Microglial memory of early life stress and inflammation: Susceptibility to neurodegeneration in adulthood. *Neuroscience & Biobehavioral Reviews, 117*, 232-242. doi:10.1016/j.neubiorev.2019.10.013

Donega, V., Nijboer, C. H., van Velthoven, C. T., Youssef, S. A., de Bruin, A., van Bel, F., . . . Heijnen, C. J. (2015). Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse. *Pediatric Research, 78*(5), 520-526. doi:10.1038/pr.2015.145

Douglas-Escobar, M., & Weiss, M. D. (2012). Biomarkers of brain injury in the premature infant. *Front Neurol, 3*, 185. doi:10.3389/fneur.2012.00185

Duarte Azevedo, M., Sander, S., & Tenenbaum, L. (2020). GDNF, A Neuron-Derived Factor Upregulated in Glial Cells during Disease. *Journal of Clinical Medicine, 9*(2), 456-456. doi:10.3390/jcm9020456

Eikenes, L., Løhaugen, G. C., Brubakk, A. M., Skranes, J., & Håberg, A. K. (2011). Young adults born preterm with very low birth weight demonstrate widespread white matter alterations on brain DTI. *NeuroImage, 54*(3), 1774-1785. doi:10.1016/j.neuroimage.2010.10.037

El Omar, R., Beroud, J., Stoltz, J. F., Menu, P., Velot, E., & Decot, V. (2014). Umbilical cord mesenchymal stem cells: the new gold standard for mesenchymal stem cell-based therapies? *Tissue Eng Part B Rev, 20*(5), 523-544. doi:10.1089/ten.TEB.2013.0664

Engelhardt, E., Inder, T. E., Alexopoulos, D., Dierker, D. L., Hill, J., Van Essen, D., & Neil, J. J. (2015). Regional impairments of cortical folding in premature infants. *Ann Neurol, 77*(1), 154-162. doi:10.1002/ana.24313

Favrais, G., van de Looij, Y., Fleiss, B., Ramanantsoa, N., Bonnin, P., Stoltenburg-Didinger, G., . . . Gressens, P. (2011). Systemic inflammation disrupts the developmental program of white matter. *Annals of Neurology, 70*(4), 550-565. doi:10.1002/ana.22489

Filipovic, R., & Zecevic, N. (2008). The effect of CXCL1 on human fetal oligodendrocyte progenitor cells. *Glia, 56*(1), 1-15. doi:10.1002/glia.20582

Fleiss, B., Gressens, P., & Stolp, H. B. (2020). Cortical Gray Matter Injury in Encephalopathy of Prematurity: Link to Neurodevelopmental Disorders. *Front Neurol, 11*, 575-575. doi:10.3389/fneur.2020.00575

Fleiss, B., Van Steenwinckel, J., Bokobza, C., K Shearer, I., Ross-Munro, E., & Gressens, P. (2021). Microglia-Mediated Neurodegeneration in Perinatal Brain Injuries. *Biomolecules, 11*(1), 99. doi:10.3390/biom11010099

Genç, E., Fraenz, C., Schlüter, C., Friedrich, P., Hossiep, R., Voelkle, M. C., . . . Jung, R. E. (2018). Diffusion markers of dendritic density and arborization in gray matter predict differences in intelligence. *Nat Commun, 9*(1), 1905. doi:10.1038/s41467-018-04268-8

Gonchar, Y., Wang, Q., & 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

Gussenhoven, R., Klein, L., Ophelders, D. R. M. G., Habets, D. H. J., Giebel, B., Kramer, B. W., . . . Wolfs, T. G. A. M. (2019). Annexin A1 as Neuroprotective Determinant for Blood-Brain Barrier Integrity in Neonatal Hypoxic-Ischemic Encephalopathy. *Journal of Clinical Medicine, 8*(2), 137. doi:10.3390/jcm8020137

Gussenhoven, R., Westerlaken, R. J. J., Ophelders, D. R. M. G., Jobe, A. H., Kemp, M. W., Kallapur, S. G., . . . Wolfs, T. G. A. M. (2018). Chorioamnionitis, neuroinflammation, and injury: timing is key in the preterm ovine fetus. *Journal of Neuroinflammation, 15*(1), 113. doi:10.1186/s12974-018-1149-x

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., & Gressens, P. (2015). The role of inflammation in perinatal brain injury. *Nat Rev Neurol, 11*(4), 192-208. doi:10.1038/nrneurol.2015.13

Hagemeyer, N., Hanft, K. M., Akriditou, M. A., Unger, N., Park, E. S., Stanley, E. R., . . . Prinz, M. (2017). Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood. *Acta Neuropathol, 134*(3), 441-458. doi:10.1007/s00401-017-1747-1

Hansen-Pupp, I., Hellstrom, A., Hamdani, M., Tocoian, A., Kreher, N. C., Ley, D., & Hallberg, B. (2017). Continuous longitudinal infusion of rhIGF-1/rhIGFBP-3 in extremely preterm infants: Evaluation of feasibility in a phase II study. *Growth Horm IGF Res, 36*, 44-51. doi:10.1016/j.ghir.2017.08.004

Hansen-Pupp, I., Hellstrom-Westas, L., Cilio, C. M., Andersson, S., Fellman, V., & Ley, D. (2007). Inflammation at birth and the insulin-like growth factor system in very preterm infants. *Acta Paediatr, 96*(6), 830-836. doi:10.1111/j.1651-2227.2007.00276.x

Hansen-Pupp, I., Hovel, H., Hellstrom, A., Hellstrom-Westas, L., Lofqvist, C., Larsson, E. M., . . . Ley, D. (2011a). Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J Clin Endocrinol Metab, 96*(4), 1129-1135. doi:10.1210/jc.2010-2440

Hansen-Pupp, I., Hovel, H., Lofqvist, C., Hellstrom-Westas, L., Fellman, V., Huppi, P. S., . . . Ley, D. (2013). Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatr Res, 74*(5), 564-569. doi:10.1038/pr.2013.135

Hansen-Pupp, I., Lofqvist, C., Polberger, S., Niklasson, A., Fellman, V., Hellstrom, A., & Ley, D. (2011b). Influence of insulin-like growth factor I and nutrition during phases of postnatal growth in very preterm infants. *Pediatr Res, 69*(5 Pt 1), 448-453. doi:10.1203/PDR.0b013e3182115000

Harding, D. R., Humphries, S. E., Whitelaw, A., Marlow, N., & Montgomery, H. E. (2007). Cognitive outcome and cyclo-oxygenase-2 gene (-765 G/C) variation in the preterm infant. *Arch Dis Child Fetal Neonatal Ed, 92*(2), F108-112. doi:10.1136/adc.2006.099499

Hellström, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Ramenghi, L. A., Löfqvist, C., . . . Hård, A. L. (2016). Role of Insulinlike Growth Factor 1 in Fetal Development and in the Early Postnatal Life of Premature Infants. *Am J Perinatol, 33*(11), 1067-1071. doi:10.1055/s-0036-1586109

Herz, J., Bendix, I., & Felderhoff-Müser, U. (2021). Peripheral immune cells and perinatal brain injury: a double-edged sword? *Pediatric Research*. doi:10.1038/s41390-021-01818-7

Hoffmann, I., Bueter, W., Zscheppang, K., Brinkhaus, M. J., Liese, A., Riemke, S., . . . Dammann, C. E. L. (2010). Neuregulin-1, the fetal endothelium, and brain damage in preterm newborns. *Brain, behavior, and immunity, 24*(5), 784-791. doi:10.1016/j.bbi.2009.08.012

Holgersen, K., Gao, X., Narayanan, R., Gaur, T., Carey, G., Barton, N., . . . Sangild, P. T. (2020). Supplemental Insulin-Like Growth Factor-1 and Necrotizing Enterocolitis in Preterm Pigs. *Front Pediatr, 8*, 602047. doi:10.3389/fped.2020.602047

Horsch, S., Parodi, A., Hallberg, B., Malova, M., Björkman-Burtscher, I. M., Hansen-Pupp, I., . . . Ley, D. (2020). Randomized Control Trial of Postnatal rhIGF-1/rhIGFBP-3 Replacement in Preterm Infants: Post-hoc Analysis of Its Effect on Brain Injury. *Front Pediatr, 8*, 517207. doi:10.3389/fped.2020.517207

Hortensius, L. M., van Elburg, R. M., Nijboer, C. H., Benders, M. J. N. L., & de Theije, C. G. M. (2019). Postnatal Nutrition to Improve Brain Development in the Preterm Infant: A Systematic Review From Bench to Bedside. *Front Physiol, 10*(July), 1-18. doi:10.3389/fphys.2019.00961

Hurtado-Chong, A., Yusta-Boyo, M. J., Vergaño-Vera, E., Bulfone, A., de Pablo, F., & Vicario-Abejón, C. (2009). IGF-I promotes neuronal migration and positioning in the olfactory bulb and the exit of neuroblasts from the subventricular zone. *Eur J Neurosci, 30*(5), 742-755. doi:10.1111/j.1460-9568.2009.06870.x

Inder, T., Neil, J., Kroenke, C., Dieni, S., Yoder, B., & Rees, S. (2005). Investigation of Cerebral Development and Injury in the Prematurely Born Primate by Magnetic Resonance Imaging and Histopathology. *Developmental Neuroscience, 27*(2-4), 100-111. doi:10.1159/000085981

Jantzie, L. L., & Robinson, S. (2015). Preclinical Models of Encephalopathy of Prematurity. *Dev Neurosci, 37*(4-5), 277-288. doi:10.1159/000371721

Jellema, R. K., Lima Passos, V., Zwanenburg, A., Ophelders, D. R. M. G., De Munter, S., Vanderlocht, J., . . . Kramer, B. W. (2013a). Cerebral inflammation and mobilization of the peripheral immune system following global hypoxia-ischemia in preterm sheep. *Journal of Neuroinflammation, 10*(1), 807. doi:10.1186/1742-2094-10-13

Jellema, R. K., Wolfs, T. G., Lima Passos, V., Zwanenburg, A., Ophelders, D. R., Kuypers, E., . . . Kramer, B. W. (2013b). Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia. *PLoS One, 8*(8), e73031. doi:10.1371/journal.pone.0073031

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

Johnson, S., Fawke, J., Hennessy, E., Rowell, V., Thomas, S., Wolke, D., & Marlow, N. (2009). Neurodevelopmental disability through 11 years of age in children born before 26 weeks of gestation. *Pediatrics, 124*(2), e249-257. doi:10.1542/peds.2008-3743

Johnson, S., & Marlow, N. (2011). Preterm Birth and Childhood Psychiatric Disorders. *Pediatric Research, 69*(8), 11-18. doi:10.1203/PDR.0b013e318212faa0

Jumani, K., Advani, S., Reich, N. G., Gosey, L., & Milstone, A. M. (2013). Risk factors for peripherally inserted central venous catheter complications in children. *JAMA Pediatr, 167*(5), 429-435. doi:10.1001/jamapediatrics.2013.775

Kaindl, A. M., Favrais, G., & Gressens, P. (2009). Molecular Mechanisms Involved in Injury to the Preterm Brain. *Journal of Child Neurology, 24*(9), 1112-1118. doi:10.1177/0883073809337920

Kalaszczynska, I., & 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

Kannan, S., Dai, H., Navath, R. S., Balakrishnan, B., Jyoti, A., Janisse, J., . . . Kannan, R. M. (2012). Dendrimer-based postnatal therapy for neuroinflammation and cerebral palsy in a rabbit model. *Science translational medicine, 4*(130), 130ra146-130ra146. doi:10.1126/scitranslmed.3003162

Kapitanović Vidak, H., Catela Ivković, T., Jokić, M., Spaventi, R., & Kapitanović, S. (2012). The association between proinflammatory cytokine polymorphisms and cerebral palsy in very preterm infants. *Cytokine, 58*(1), 57-64. doi:10.1016/j.cyto.2011.12.018

Kaplan, H. M., Mishra, P., & Kohn, J. (2015). The overwhelming use of rat models in nerve regeneration research may compromise designs of nerve guidance conduits for humans. *Journal of Materials Science: Materials in Medicine, 26*(8), 226. doi:10.1007/s10856-015-5558-4

Kassis, I., Vaknin-Dembinsky, A., & Karussis, D. (2011). Bone marrow mesenchymal stem cells: agents of immunomodulation and neuroprotection. *Curr Stem Cell Res Ther, 6*(1), 63-68. doi:10.2174/157488811794480762

Kelsom, C., & Lu, W. (2013). Development and specification of GABAergic cortical interneurons. *Cell & Bioscience, 3*(1), 19. doi:10.1186/2045-3701-3-19

Kersbergen, K. J., Makropoulos, A., Aljabar, P., Groenendaal, F., de Vries, L. S., Counsell, S. J., & Benders, M. J. (2016). Longitudinal Regional Brain Development and Clinical Risk Factors in Extremely Preterm Infants. *J Pediatr, 178*, 93-100.e106. doi:10.1016/j.jpeds.2016.08.024

Keunen, K., Kersbergen, K. J., Groenendaal, F., Isgum, I., de Vries, L. S., & Benders, M. J. (2012). Brain tissue volumes in preterm infants: prematurity, perinatal risk factors and neurodevelopmental outcome: a systematic review. *J Matern Fetal Neonatal Med, 25 Suppl 1*, 89-100. doi:10.3109/14767058.2012.664343

Keunen, K., Van Elburg, R. M., Van Bel, F., & Benders, M. J. N. L. (2015). Impact of nutrition on brain development and its neuroprotective implications following preterm birth. *Pediatric Research, 77*(1), 148-155. doi:10.1038/pr.2014.171

Kim, E. S., Ahn, S. Y., Im, G. H., Sung, D. K., Park, Y. R., Choi, S. H., . . . Park, W. S. (2012). Human umbilical cord blood–derived mesenchymal stem cell transplantation attenuates severe brain injury by permanent middle cerebral artery occlusion in newborn rats. *Pediatric Research, 72*, 277. doi:10.1038/pr.2012.71

Kim, H., Xu, R., Padmashri, R., Dunaevsky, A., Liu, Y., Dreyfus, C. F., & Jiang, P. (2019). Pluripotent Stem Cell-Derived Cerebral Organoids Reveal Human Oligodendrogenesis with Dorsal and Ventral Origins. *Stem Cell Reports, 12*(5), 890-905. doi:10.1016/j.stemcr.2019.04.011

Kinney, H. C., & Volpe, J. J. (2012). Modeling the Encephalopathy of Prematurity in Animals: The Important Role of Translational Research. *Neurology Research International, 2012*, 295389. doi:10.1155/2012/295389

Krishnan, M. L., Van Steenwinckel, J., Schang, A.-L., Yan, J., Arnadottir, J., Le Charpentier, T., . . . Gressens, P. (2017). Integrative genomics of microglia implicates DLG4 (PSD95) in the white matter development of preterm infants. *Nat Commun, 8*(1), 428-428. doi:10.1038/s41467-017-00422-w

Lacaille, H., Vacher, C. M., Bakalar, D., O'Reilly, J. J., Salzbank, J., & Penn, A. A. (2019). Impaired Interneuron Development in a Novel Model of Neonatal Brain Injury. *eNeuro, 6*(1). doi:10.1523/eneuro.0300-18.2019

Lee, W. H., Clemens, J. A., & Bondy, C. A. (1992). Insulin-like growth factors in the response to cerebral ischemia. *Mol Cell Neurosci, 3*(1), 36-43. doi:10.1016/1044-7431(92)90006-n

Lee, W. H., Wang, G. M., Seaman, L. B., & Vannucci, S. J. (1996). Coordinate IGF-I and IGFBP5 Gene Expression in Perinatal Rat Brain after Hypoxia-Ischemia. *Journal of Cerebral Blood Flow & Metabolism, 16*(2), 227-236. doi:10.1097/00004647-199603000-00007

Lei, J., Rosenzweig, J. M., Mishra, M. K., Alshehri, W., Brancusi, F., McLane, M., . . . Burd, I. (2017). Maternal dendrimer-based therapy for inflammation-induced preterm birth and perinatal brain injury. *Sci Rep, 7*(1), 6106. doi:10.1038/s41598-017-06113-2

Ley, D., Hallberg, B., Hansen-Pupp, I., Dani, C., Ramenghi, L. A., Marlow, N., . . . Hellström, A. (2019). rhIGF-1/rhIGFBP-3 in Preterm Infants: A Phase 2 Randomized Controlled Trial. *J Pediatr, 206*, 56-65.e58. doi:10.1016/j.jpeds.2018.10.033

Ley, D., Hansen-Pupp, I., Niklasson, A., Domellof, M., Friberg, L. E., Borg, J., . . . Hellstrom, A. (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*(1), 68-74. doi:10.1038/pr.2012.146

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Allison, B. J., . . . Miller, S. L. (2017). Term vs. preterm cord blood cells for the prevention of preterm brain injury. *Pediatr Res, 82*(6), 1030-1038. doi:10.1038/pr.2017.170

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., . . . Miller, S. L. (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., . . . Miller, S. L. (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

Li, W. J., Mao, F. X., Chen, H. J., Qian, L. H., & Buzby, J. S. (2015). Treatment with UDP-glucose, GDNF, and memantine promotes SVZ and white matter self-repair by endogenous glial progenitor cells in neonatal rats with ischemic PVL. *Neuroscience, 284*, 444-458. doi:10.1016/j.neuroscience.2014.10.012

Li, W. Y., Chang, Y. C., Lee, L. J., & Lee, L. J. (2014). Prenatal infection affects the neuronal architecture and cognitive function in adult mice. *Dev Neurosci, 36*(5), 359-370. doi:10.1159/000362383

Liang, X., Ding, Y., Zhang, Y., Tse, H. F., & Lian, Q. (2014). Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant, 23*(9), 1045-1059. doi:10.3727/096368913×667709

Lim, L., Mi, D., Llorca, A., & Marín, O. (2018). Development and Functional Diversification of Cortical Interneurons. *Neuron, 100*(2), 294-313. doi:10.1016/j.neuron.2018.10.009

Lin, H. Y., Liou, C. W., Chen, S. D., Hsu, T. Y., Chuang, J. H., Wang, P. W., . . . Chuang, Y. C. (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

Lin, S., Fan, L. W., Rhodes, P. G., & Cai, Z. (2009). Intranasal administration of IGF-1 attenuates hypoxic-ischemic brain injury in neonatal rats. *Exp Neurol, 217*(2), 361-370. doi:10.1016/j.expneurol.2009.03.021

Linsell, L., Johnson, S., Wolke, D., O'Reilly, H., Morris, J. K., Kurinczuk, J. J., & Marlow, N. (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*(4), 363-370. doi:10.1136/archdischild-2017-313414

Madhavan, M., Nevin, Z. S., Shick, H. E., Garrison, E., Clarkson-Paredes, C., Karl, M., . . . Tesar, P. J. (2018). Induction of myelinating oligodendrocytes in human cortical spheroids. *Nature Methods, 15*(9), 700-706. doi:10.1038/s41592-018-0081-4

Mahrouf-Yorgov, M., Augeul, L., Da Silva, C. C., Jourdan, M., Rigolet, M., Manin, S., . . . Rodriguez, A. M. (2017). Mesenchymal stem cells sense mitochondria released from damaged cells as danger signals to activate their rescue properties. *Cell Death Differ, 24*(7), 1224-1238. doi:10.1038/cdd.2017.51

Maisonneuve, E., Lorthe, E., Torchin, H., Delorme, P., Devisme, L., L'Hélias, L. F., . . . Kayem, G. (2020). Association of Chorioamnionitis with Cerebral Palsy at Two Years after Spontaneous Very Preterm Birth: The EPIPAGE-2 Cohort Study. *J Pediatr, 222*, 71-78.e76. doi:10.1016/j.jpeds.2020.03.021

Malik, S., Vinukonda, G., Vose, L. R., Diamond, D., Bhimavarapu, B. B. R., Hu, F., . . . Ballabh, P. (2013). Neurogenesis continues in the third trimester of pregnancy and is suppressed by premature birth. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 33*(2), 411-423. doi:10.1523/JNEUROSCI.4445-12.2013

Marín, O. (2012). Interneuron dysfunction in psychiatric disorders. *Nat Rev Neurosci, 13*(2), 107-120. doi:10.1038/nrn3155

Martin, J. L., & Baxter, R. C. (2011). Signalling pathways of insulin-like growth factors (IGFs) and IGF binding protein-3. *Growth Factors, 29*(6), 235-244. doi:10.3109/08977194.2011.614237

Masters, B. A., Werner, H., Roberts, C. T., Jr., LeRoith, D., & Raizada, M. K. (1991). Insulin-like growth factor I (IGF-I) receptors and IGF-I action in oligodendrocytes from rat brains. *Regul Pept, 33*(2), 117-131. doi:10.1016/0167-0115(91)90207-W

Matthews, L. G., Inder, T. E., Pascoe, L., Kapur, K., Lee, K. J., Monson, B. B., . . . Anderson, P. J. (2018). Longitudinal Preterm Cerebellar Volume: Perinatal and Neurodevelopmental Outcome Associations. *Cerebellum (London, England), 17*(5), 610-627. doi:10.1007/s12311-018-0946-1

McClendon, E., Chen, K., Gong, X., Sharifnia, E., Hagen, M., Cai, V., . . . Back, S. A. (2014). Prenatal cerebral ischemia triggers dysmaturation of caudate projection neurons. *Ann Neurol, 75*(4), 508-524. doi:10.1002/ana.24100

McClendon, E., Wang, K., Degener, O., Brien, K., Hagen, M. W., Gong, X., . . . Back, S. A. (2019). Transient Hypoxemia Disrupts Anatomical and Functional Maturation of Preterm Fetal Ovine CA1 Pyramidal Neurons. *The Journal of Neuroscience, 39*(40), 7853. doi:10.1523/JNEUROSCI.1364-19.2019

McMorris, F. A., Smith, T. M., DeSalvo, S., & Furlanetto, R. W. (1986). Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development. *Proc Natl Acad Sci U S A, 83*(3), 822-826. doi:10.1073/pnas.83.3.822

Mei, L., & Nave, K.-A. (2014). Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases. *Neuron, 83*(1), 27-49. doi:10.1016/j.neuron.2014.06.007

Miron, V. E., Boyd, A., Zhao, J. W., Yuen, T. J., Ruckh, J. M., Shadrach, J. L., . . . Ffrench-Constant, C. (2013). M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat Neurosci, 16*(9), 1211-1218. doi:10.1038/nn.3469

Molina-Gonzalez, I., & Miron, V. E. (2019). Astrocytes in myelination and remyelination. *Neurosci Lett, 713*, 134532. doi:10.1016/j.neulet.2019.134532

Moretti, R., Pansiot, J., Bettati, D., Strazielle, N., Ghersi-Egea, J.-F., Damante, G., . . . Gressens, P. (2015). Blood-brain barrier dysfunction in disorders of the developing brain. *Frontiers in Neuroscience, 9*(40). doi:10.3389/fnins.2015.00040

Morioka, C., Komaki, M., Taki, A., Honda, I., Yokoyama, N., Iwasaki, K., . . . Morita, I. (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

Mueller, M., Oppliger, B., Joerger-Messerli, M., Reinhart, U., Barnea, E., Paidas, M., . . . Schoeberlein, A. (2017). Wharton's Jelly Mesenchymal Stem Cells Protect the Immature Brain in Rats and Modulate Cell Fate. *Stem Cells Dev, 26*(4), 239-248. doi:10.1089/scd.2016.0108

Mukai, T., Mori, Y., Shimazu, T., Takahashi, A., Tsunoda, H., Yamaguchi, S., . . . Nagamura-Inoue, T. (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

Mukhopadhyay, A., McGuire, T., Peng, C. Y., & Kessler, J. A. (2009). Differential effects of BMP signaling on parvalbumin and somatostatin interneuron differentiation. *Development, 136*(15), 2633-2642. doi:10.1242/dev.034439

Nag, O. K., & Delehanty, J. B. (2019). Active Cellular and Subcellular Targeting of Nanoparticles for Drug Delivery. *Pharmaceutics, 11*(10). doi:10.3390/pharmaceutics11100543

Nair, S., Rocha-Ferreira, E., Fleiss, B., Nijboer, C. H., Gressens, P., Mallard, C., & Hagberg, H. (2021). Neuroprotection offered by mesenchymal stem cells in perinatal brain injury: Role of mitochondria, inflammation, and reactive oxygen species. *J Neurochem, 158*(1), 59-73. doi:10.1111/jnc.15267

Ness, J. (2002). Insulin-like Growth Factor I, but Not Neurotrophin-3, Sustains Akt Activation and Provides Long-Term Protection of Immature Oligodendrocytes from Glutamate-Mediated Apoptosis. *Molecular and Cellular Neuroscience, 20*(3), 476-488. doi:10.1006/mcne.2002.1149

Nieto-Estévez, V., Defterali, Ç., & Vicario-Abejón, C. (2016). IGF-I: A Key Growth Factor that Regulates Neurogenesis and Synaptogenesis from Embryonic to Adult Stages of the Brain. *Frontiers in Neuroscience, 10*, 52. doi:10.3389/fnins.2016.00052

Nosarti, C., Al-Asady, M. H., Frangou, S., Stewart, A. L., Rifkin, L., & Murray, R. M. (2002). Adolescents who were born very preterm have decreased brain volumes. *Brain, 125*(Pt 7), 1616-1623. doi:10.1093/brain/awf157

Nosarti, C., Giouroukou, E., Healy, E., Rifkin, L., Walshe, M., Reichenberg, A., . . . Murray, R. M. (2008). Grey and white matter distribution in very preterm adolescents mediates neurodevelopmental outcome. *Brain, 131*(Pt 1), 205-217. doi:10.1093/brain/awm282

Oldenburg, K. S., O'Shea, T. M., & Fry, R. C. (2020). Genetic and epigenetic factors and early life inflammation as predictors of neurodevelopmental outcomes. *Semin Fetal Neonatal Med, 25*(3), 101115. doi:10.1016/j.siny.2020.101115

Ophelders, D. R., Wolfs, T. G., Jellema, R. K., Zwanenburg, A., Andriessen, P., Delhaas, T., . . . Kramer, B. W. (2016). Mesenchymal Stromal Cell-Derived Extracellular Vesicles Protect the Fetal Brain After Hypoxia-Ischemia. *Stem Cells Transl Med, 5*(6), 754-763. doi:10.5966/sctm.2015-0197

Oppliger, B., Joerger-Messerli, M., Mueller, M., Reinhart, U., Schneider, P., Surbek, D. V., & Schoeberlein, A. (2016). Intranasal Delivery of Umbilical Cord-Derived Mesenchymal Stem Cells Preserves Myelination in Perinatal Brain Damage. *Stem Cells and Development, 25*(16), 1234-1242. doi:10.1089/scd.2016.0027

Oppliger, B., Joerger-Messerli, M. S., Simillion, C., Mueller, M., Surbek, D. V., & 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*(7), 829-838. doi:10.1016/j.jcyt.2017.03.075

Ormel, P. R., Vieira de Sá, R., van Bodegraven, E. J., Karst, H., Harschnitz, O., Sneeboer, M. A. M., . . . Pasterkamp, R. J. (2018). Microglia innately develop within cerebral organoids. *Nat Commun, 9*(1). doi:10.1038/s41467-018-06684-2

Paliwal, S., Chaudhuri, R., Agrawal, A., & Mohanty, S. (2018). Regenerative abilities of mesenchymal stem cells through mitochondrial transfer. *J Biomed Sci, 25*(1), 31. doi:10.1186/s12929-018-0429-1

Panda, S., Dohare, P., Jain, S., Parikh, N., Singla, P., Mehdizadeh, R., . . . Ballabh, P. (2018). Estrogen Treatment Reverses Prematurity-Induced Disruption in Cortical Interneuron Population. *J Neurosci, 38*(34), 7378-7391. doi:10.1523/jneurosci.0478-18.2018

Pang, Y., Zheng, B., Fan, L. W., Rhodes, P. G., & Cai, Z. (2007). IGF-1 protects oligodendrocyte progenitors against TNFalpha-induced damage by activation of PI3K/Akt and interruption of the mitochondrial apoptotic pathway. *Glia, 55*(11), 1099-1107. doi:10.1002/glia.20530

Park, W. S., Ahn, S. Y., Sung, S. I., Ahn, J. Y., & Chang, Y. S. (2018). Strategies to enhance paracrine potency of transplanted mesenchymal stem cells in intractable neonatal disorders. *Pediatr Res, 83*(1-2), 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., . . . Chang, Y. S. (2016). Optimal Timing of Mesenchymal Stem Cell Therapy for Neonatal Intraventricular Hemorrhage. *Cell Transplant, 25*(6), 1131-1144. doi:10.3727/096368915×689640

Passera, S., Boccazzi, M., Bokobza, C., Faivre, V., Mosca, F., Van Steenwinckel, J., . . . Fleiss, B. (2021). Therapeutic potential of stem cells for preterm infant brain damage: Can we move from the heterogeneity of preclinical and clinical studies to established therapeutics? *Biochem Pharmacol, 186*, 114461. doi:10.1016/j.bcp.2021.114461

Paton, M. C. B., Allison, B. J., Fahey, M. C., Li, J., Sutherland, A. E., Pham, Y., . . . McDonald, C. A. (2019). Umbilical cord blood versus mesenchymal stem cells for inflammation-induced preterm brain injury in fetal sheep. *Pediatric Research*. doi:10.1038/s41390-019-0366-z

Paton, M. C. B., Allison, B. J., Li, J., Fahey, M. C., Sutherland, A. E., Nitsos, I., . . . Miller, S. L. (2018). Human Umbilical Cord Blood Therapy Protects Cerebral White Matter from Systemic LPS Exposure in Preterm Fetal Sheep. *Dev Neurosci, 40*(3), 258-270. doi:10.1159/000490943

Paton, M. C. B., McDonald, C. A., Allison, B. J., Fahey, M. C., Jenkin, G., & Miller, S. L. (2017). Perinatal Brain Injury As a Consequence of Preterm Birth and Intrauterine Inflammation: Designing Targeted Stem Cell Therapies. *Frontiers in Neuroscience, 11*, 200. doi:10.3389/fnins.2017.00200

Pozas, E., & Ibáñez, C. F. (2005). GDNF and GFRα1 Promote Differentiation and Tangential Migration of Cortical GABAergic Neurons. *Neuron, 45*(5), 701-713. doi:10.1016/j.neuron.2005.01.043

Raabe, T. D., Suy, S., Welcher, A., & DeVries, G. H. (1997). Effect of neu differentiation factor isoforms of neonatal oligodendrocyte function. *Journal of neuroscience research, 50*(5), 755-768. doi:10.1002/(SICI)1097-4547(19971201)50:5<755::AID-JNR12>3.0.CO;2-0

Reid, M. V., Murray, K. A., Marsh, E. D., Golden, J. A., Simmons, R. A., & Grinspan, J. B. (2012). Delayed myelination in an intrauterine growth retardation model is mediated by oxidative stress upregulating bone morphogenetic protein 4. *J Neuropathol Exp Neurol, 71*(7), 640-653. doi:10.1097/NEN.0b013e31825cfa81

Rezaie, P., & Dean, A. (2002). Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. *Neuropathology, 22*(3), 106-132. doi:10.1046/j.1440-1789.2002.00438.x

Rittchen, S., Boyd, A., Burns, A., Park, J., Fahmy, T. M., Metcalfe, S., & Williams, A. (2015). Myelin repair in vivo is increased by targeting oligodendrocyte precursor cells with nanoparticles encapsulating leukaemia inhibitory factor (LIF). *Biomaterials, 56*, 78-85. doi:10.1016/j.biomaterials.2015.03.044

Robinson, S., Li, Q., Dechant, A., & 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

Scafidi, J., Hammond, T. R., Scafidi, S., Ritter, J., Jablonska, B., Roncal, M., . . . Gallo, V. (2014). Intranasal epidermal growth factor treatment rescues neonatal brain injury. *Nature, 506*(7487), 230-234. doi:10.1038/nature12880

Schmitz, T., Ritter, J., Mueller, S., Felderhoff-Mueser, U., Chew, L.-J., & Gallo, V. (2011). Cellular Changes Underlying Hyperoxia-Induced Delay of White Matter Development. *The Journal of Neuroscience, 31*(11), 4327. doi:10.1523/JNEUROSCI.3942-10.2011

Scruggs, B. A., Semon, J. A., Zhang, X., Zhang, S., Bowles, A. C., Pandey, A. C., . . . Bunnell, B. A. (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*(10), 797-807. doi:10.5966/sctm.2013-0026

Segovia, K. N., McClure, M., Moravec, M., Luo, N. L., Wan, Y., Gong, X., . . . Back, S. A. (2008). Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann Neurol, 63*(4), 520-530. doi:10.1002/ana.21359

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., & Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology, 106-107*, 1-16. doi:10.1016/j.pneurobio.2013.04.001

Shah, J., Jefferies, A. L., Yoon, E. W., Lee, S. K., & Shah, P. S. (2015). Risk Factors and Outcomes of Late-Onset Bacterial Sepsis in Preterm Neonates Born at < 32 Weeks' Gestation. *Am J Perinatol, 32*(7), 675-682. doi:10.1055/s-0034-1393936

Shiow, L. R., Favrais, G., Schirmer, L., Schang, A. L., Cipriani, S., Andres, C., . . . Rowitch, D. H. (2017). Reactive astrocyte COX2-PGE2 production inhibits oligodendrocyte maturation in neonatal white matter injury. *Glia, 65*(12), 2024-2037. doi:10.1002/glia.23212

Sivakumar, V., Ling, E.-A., Lu, J., & Kaur, C. (2010). Role of glutamate and its receptors and insulin-like growth factors in hypoxia induced periventricular white matter injury. *Glia, 58*(5), 507-523. doi:10.1002/glia.20940

Spoto, G., Amore, G., Vetri, L., Quatrosi, G., Cafeo, A., Gitto, E., . . . Di Rosa, G. (2021). Cerebellum and Prematurity: A Complex Interplay Between Disruptive and Dysmaturational Events. *Frontiers in Systems Neuroscience, 15*, 54. doi:10.3389/fnsys.2021.655164

Steinberg, G. K., Kondziolka, D., Wechsler, L. R., Lunsford, L. D., Coburn, M. L., Billigen, J. B., . . . Schwartz, N. E. (2016). Clinical Outcomes of Transplanted Modified Bone Marrow-Derived Mesenchymal Stem Cells in Stroke: A Phase 1/2a Study. *Stroke, 47*(7), 1817-1824. doi:10.1161/strokeaha.116.012995

Stewart, A., Tekes, A., Huisman, T. A., Jennings, J. M., Allen, M. C., Northington, F. J., . . . Graham, E. M. (2013). Glial fibrillary acidic protein as a biomarker for periventricular white matter injury. *Am J Obstet Gynecol, 209*(1), 27.e21-27. doi:10.1016/j.ajog.2013.02.049

Stolp, H. B., Fleiss, B., Arai, Y., Supramaniam, V., Vontell, R., Birtles, S., . . . 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

Thion, M. S., Mosser, C. A., Férézou, I., Grisel, P., Baptista, S., Low, D., . . . Audinat, E. (2019). Biphasic Impact of Prenatal Inflammation and Macrophage Depletion on the Wiring of Neocortical Inhibitory Circuits. *Cell Rep, 28*(5), 1119-1126.e1114. doi:10.1016/j.celrep.2019.06.086

Thorne, R. G., Pronk, G. J., Padmanabhan, V., & Frey, W. H., 2nd. (2004). Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. *Neuroscience, 127*(2), 481-496. doi:10.1016/j.neuroscience.2004.05.029

Tibrewal, M., Cheng, B., Dohare, P., Hu, F., Mehdizadeh, R., Wang, P., . . . Ballabh, P. (2018). Disruption of Interneuron Neurogenesis in Premature Newborns and Reversal with Estrogen Treatment. *J Neurosci, 38*(5), 1100-1113. doi:10.1523/jneurosci.1875-17.2017

Traiffort, E., Kassoussi, A., Zahaf, A., & Laouarem, Y. (2020). Astrocytes and Microglia as Major Players of Myelin Production in Normal and Pathological Conditions. *Frontiers in Cellular Neuroscience, 14*, 79. doi:10.3389/fncel.2020.00079

Ullah, M., Liu, D. D., & Thakor, A. S. (2019). Mesenchymal Stromal Cell Homing: Mechanisms and Strategies for Improvement. *iScience, 15*, 421-438. doi:10.1016/j.isci.2019.05.004

Vaes, J. E. G., Vink, M. A., de Theije, C. G. M., Hoebeek, F. E., Benders, M., & Nijboer, C. H. A. (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

van Haastert, I. C., Groenendaal, F., Uiterwaal, C. S., Termote, J. U., van der Heide-Jalving, M., Eijsermans, M. J., . . . de Vries, L. S. (2011). Decreasing incidence and severity of cerebral palsy in prematurely born children. *J.Pediatr., 159*, 86-91.

Van Steenwinckel, J., Schang, A. L., Krishnan, M. L., Degos, V., Delahaye-Duriez, A., Bokobza, C., . . . Gressens, P. (2019). Decreased microglial Wnt/β-catenin signalling drives microglial pro-inflammatory activation in the developing brain. *Brain, 142*(12), 3806-3833. doi:10.1093/brain/awz319

van Tilborg, E., Achterberg, E. J. M., van Kammen, C. M., van der Toorn, A., Groenendaal, F., Dijkhuizen, R. M., . . . Nijboer, C. H. A. (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*(1), 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., . . . Nijboer, C. H. (2018b). Origin and dynamics of oligodendrocytes in the developing brain: Implications for perinatal white matter injury. *Glia, 66*(2), 221-238. doi:10.1002/glia.23256

van Tilborg, E., Heijnen, C. J., Benders, M. J., van Bel, F., Fleiss, B., Gressens, P., & Nijboer, C. H. (2016). Impaired oligodendrocyte maturation in preterm infants: Potential therapeutic targets. *Progress in Neurobiology, 136*(1873-5118 (Electronic)), 28-49. doi:10.1016/j.pneurobio.2015.11.002

van Velthoven, C. T., Kavelaars, A., & Heijnen, C. J. (2012). 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., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain, Behavior and Immunity, 24*(3), 387-393. doi:10.1016/j.bbi.2009.10.017

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2011). Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. *Brain, Behavoir and Immunity, 25*(7), 1342-1348. doi:10.1016/j.bbi.2011.03.021

Varma Shrivastav, S., Bhardwaj, A., Pathak, K. A., & Shrivastav, A. (2020). Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3): Unraveling the Role in Mediating IGF-Independent Effects Within the Cell. *Frontiers in Cell and Developmental Biology, 8*(286). doi:10.3389/fcell.2020.00286

Varner, M. W., Costantine, M. M., Jablonski, K. A., Rouse, D. J., Mercer, B. M., Leveno, K. J., . . . Caritis, S. N. (2020). Sex-Specific Genetic Susceptibility to Adverse Neurodevelopmental Outcome in Offspring of Pregnancies at Risk of Early Preterm Delivery. *Am J Perinatol, 37*(3), 281-290. doi:10.1055/s-0039-1678535

Vasistha, N. A., Pardo-Navarro, M., Gasthaus, J., Weijers, D., Müller, M. K., García-González, D., . . . Khodosevich, K. (2020). Maternal inflammation has a profound effect on cortical interneuron development in a stage and subtype-specific manner. *Molecular Psychiatry, 25*(10), 2313-2329. doi:10.1038/s41380-019-0539-5

Veraart, J., Nunes, D., Rudrapatna, U., Fieremans, E., Jones, D. K., Novikov, D. S., & Shemesh, N. (2020). Noninvasive quantification of axon radii using diffusion MRI. *eLife, 9*, e49855. doi:10.7554/eLife.49855

Verney, C., Pogledic, I., Biran, V., Adle-Biassette, H., Fallet-Bianco, C., & 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*(3), 251-264. doi:10.1097/NEN.0b013e3182496429

Volpe, J. J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurology, 8*(1), 110-124. doi:10.1016/s1474-4422(08)70294-1

Volpe, J. J. (2019). Dysmaturation of Premature Brain: Importance, Cellular Mechanisms, and Potential Interventions. *Pediatr Neurol, 95*, 42-66. doi:10.1016/j.pediatrneurol.2019.02.016

Volpe, J. J. (2021). Commentary - Cerebellar underdevelopment in the very preterm infant: Important and underestimated source of cognitive deficits. *J Neonatal Perinatal Med, 14*(4), 451-456. doi:10.3233/npm-210774

Volpe, J. J., Kinney, H. C., Jensen, F. E., & Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *International Journal of Developmental Neuroscience, 29*(4), 423-440. doi:10.1016/j.ijdevneu.2011.02.012

Voronova, A., Yuzwa, S. A., Wang, B. S., Zahr, S., Syal, C., Wang, J., . . . Miller, F. D. (2017). Migrating Interneurons Secrete Fractalkine to Promote Oligodendrocyte Formation in the Developing Mammalian Brain. *Neuron, 94*(3), 500-516.e509. doi:10.1016/j.neuron.2017.04.018

Wagenaar, N. (2019). *Advances in perinatal stroke: from early prediction to future therapies*. Utrecht University, Utrecht.

Wilson, H. C., Onischke, C., & Raine, C. S. (2003). Human oligodendrocyte precursor cells in vitro: phenotypic analysis and differential response to growth factors. *Glia, 44*(2), 153-165. doi:10.1002/glia.10280

Winerdal, M., Winerdal, M. E., Kinn, J., Urmaliya, V., Winqvist, O., & Adén, U. (2012). Long lasting local and systemic inflammation after cerebral hypoxic ischemia in newborn mice. *PLoS One, 7*(5), e36422. doi:10.1371/journal.pone.0036422

Wlodarczyk, A., Holtman, I. R., Krueger, M., Yogev, N., Bruttger, J., Khorooshi, R., . . . Owens, T. (2017). A novel microglial subset plays a key role in myelinogenesis in developing brain. *Embo j, 36*(22), 3292-3308. doi:10.15252/embj.201696056

Xiao, Y., Geng, F., Wang, G., Li, X., Zhu, J., & 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

Xu, J., Hu, C., Chen, S., Shen, H., Jiang, Q., Huang, P., & Zhao, W. (2017). Neuregulin-1 protects mouse cerebellum against oxidative stress and neuroinflammation. *Brain Research, 1670*, 32-43. doi:10.1016/j.brainres.2017.06.012

Yap, V., & Perlman, J. M. (2020). Mechanisms of brain injury in newborn infants associated with the fetal inflammatory response syndrome. *Seminars in Fetal and Neonatal Medicine*, 101110. doi:10.1016/j.siny.2020.101110

Yue, S. L., Eke, A. C., Vaidya, D., Northington, F. J., Everett, A. D., & Graham, E. M. (2021). Perinatal blood biomarkers for the identification of brain injury in very low birth weight growth-restricted infants. *Journal Of Perinatology, 41*(9), 2252-2260. doi:10.1038/s41372-021-01112-8

Yumani, D. F., Lafeber, H. N., & van Weissenbruch, M. M. (2015). Dietary proteins and IGF I levels in preterm infants: determinants of growth, body composition, and neurodevelopment. *Pediatr Res, 77*(1-2), 156-163. doi:10.1038/pr.2014.172

Yumani, D. F. J., Calor, A. K., & van Weissenbruch, M. M. (2020). The Course Of IGF-1 Levels and Nutrient Intake in Extremely and Very Preterm Infants During Hospitalisation. *Nutrients, 12*(3). doi:10.3390/nu12030675

Zeddou, M., Briquet, A., Relic, B., Josse, C., Malaise, M. G., Gothot, A., . . . Beguin, Y. (2010). The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. *Cell Biol Int, 34*(7), 693-701. doi:10.1042/cbi20090414

Zhang, Y., Inder, T. E., Neil, J. J., Dierker, D. L., Alexopoulos, D., Anderson, P. J., & Van Essen, D. C. (2015). Cortical structural abnormalities in very preterm children at 7 years of age. *NeuroImage, 109*, 469-479. doi:10.1016/j.neuroimage.2015.01.005

Zhang, Z., Li, Z., Deng, W., He, Q., Wang, Q., Shi, W., . . . Xu, X. (2016). Ectoderm mesenchymal stem cells promote differentiation and maturation of oligodendrocyte precursor cells. *Biochem Biophys Res Commun, 480*(4), 727-733. doi:10.1016/j.bbrc.2016.10.115

Zhao, J., Chen, Y., Xu, Y., & Pi, G. (2013). Effect of intrauterine infection on brain development and injury. *International Journal of Developmental Neuroscience, 31*(7), 543-549. doi:10.1016/j.ijdevneu.2013.06.008

Zonouzi, M., Scafidi, J., Li, P., McEllin, B., Edwards, J., Dupree, J. L., . . . Gallo, V. (2015). GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury. *Nature Neuroscience, 18*(5), 674-682. doi:10.1038/nn.3990

11

Appendices

NEDERLANDSE SAMENVATTING (SUMMARY IN DUTCH)

In Nederland wordt ongeveer 7-8% van alle kinderen te vroeg geboren, dat wil zeggen na een zwangerschapsduur van minder dan 37 weken, in plaats van de normale duur van circa 40 weken. Een deel van deze kinderen wordt *extreem* te vroeg geboren, na een zwangerschapsduur onder de 28 weken. Deze groep kinderen wordt ook wel 'extreem prematuur' genoemd. Na extreme vroeggeboorte zijn belangrijke organen nog onrijp en kunnen hun functie nog niet naar behoren uitvoeren. Zo kunnen onderontwikkelde longen leiden tot schommelingen in de zuurstofvoorziening van het lichaam, terwijl een onderontwikkeld immuunsysteem baby's kwetsbaarder maakt voor infecties. Deze schadelijke processen kunnen de verdere ontwikkeling van onrijpe organen verstoren en zo levenslange gezondheidsproblemen veroorzaken.

De hersenen zijn normaal gesproken volop in ontwikkeling gedurende het laatste trimester van de zwangerschap, en daardoor erg kwetsbaar voor de schadelijke processen geassocieerd met extreme vroeggeboorte. De ontwikkeling van de witte stof in de hersenen, verantwoordelijk voor effectieve communicatie tussen verschillende hersengebieden, vindt precies plaats rond de periode na extreme vroeggeboorte. Daardoor is de witte stof van de hersenen het meest aangedaan in extreem premature kinderen. De ernst van witte stof schade varieert. In het verleden ging het vaak om grote cystes in de witte stof (dit werd cysteuze periventriculaire leukomalacie (cPVL) genoemd), het resultaat van hersenweefselsterfte. Kinderen met cPVL hebben vaak een ernstige motorische handicap (cerebrale parese). Door verbeteringen in de ondersteunende zorg voor de allerkleinsten (bijv. in beademingstechniek) op de neonatale intensive care unit (NICU) komt diffuse witte stof schade, een mildere variant, tegenwoordig vaker voor. Diffuse witte stof schade lijkt een gevolg van verstoorde ontwikkeling van de witte stof, zonder overmatige sterfte van cellen. Grote studies hebben laten zien dat diffuse witte stof schade onder andere kan leiden tot verminderde cognitieve prestaties en psychiatrische problemen, zoals autismespectrumstoornis (ASS). Recent onderzoek laat zien dat extreme vroeggeboorte naast de ontwikkeling van witte stof, ook de ontwikkeling van andere hersencellen/structuren belemmert. De term 'Encephalopathy of Prematurity' (EoP) wordt de laatste jaren gebruikt om het gehele spectrum van hersenschade in de extreem te vroeg geboren baby te beschrijven.

Op dit moment zijn er geen geneesmiddelen beschikbaar om hersenontwikkeling na extreme vroeggeboorte te stimuleren. Meer onderzoek naar nieuwe behandelopties om hersenschade na extreme vroeggeboorte te voorkomen of te herstellen is dan ook noodzakelijk. Dit proefschrift, een combinatie van preklinisch en klinisch onderzoek (van het laboratorium naar de te vroeg geboren baby in de couveuse; 'from bench to incubator'), richt zich op het ontdekken en klinisch vertalen van veelbelovende behandelopties om hersenontwikkeling in extreem prematuur geboren baby's te verbeteren.

In **hoofdstuk 2, 3** en **4** wordt de meest recente kennis over de onderliggende mechanismen van hersenschade na extreme vroeggeboorte samengevat. Naast zenuwcellen (neuronen) bevat het brein ook ondersteunende celtypes (glia), met ieder een eigen taak. Zo zijn oligodendrocyten verantwoordelijk voor het produceren van myeline, het isolerende laagje dat zenuwuitlopers in de witte stof omvat. Myeline beschermt zenuwuitlopers en is essentieel voor efficiënte signaaloverdracht tussen neuronen. Microglia zijn een belangrijk onderdeel van het afweersysteem van de hersenen. Blootstelling aan schadelijke processen (zoals infecties of zuurstofschommelingen) leidt tot activatie van microglia, met als gevolg een ontstekingsreactie in het brein (pro-inflammatoire activatie). Tijdens deze ontstekingsreactie scheiden microglia schadelijke stoffen (pro-inflammatoire cytokines) uit. Astrocyten maken deel uit van de bloed-hersenbarrière en spelen een belangrijke rol in het reguleren van het hersenmilieu, bijvoorbeeld door het uitscheiden van groeifactoren en wegvangen van schadelijke stoffen en neurotransmitters. Astrocyten kunnen na activatie ook bijdragen aan ontstekingsreacties, door middel van het uitscheiden van pro-inflammatoire cytokines. Na extreme vroeggeboorte kunnen zuurstofschommelingen, infecties en ontstekingsreacties (gemedieerd door microglia en astrocyten) de ontwikkeling van oligodendrocyten verstoren, met minder myeline productie en witte stof schade als gevolg. Behandelmethodes die aangrijpen op de processen die ten grondslag liggen aan witte stof schade, bijvoorbeeld het direct stimuleren van de uitrijping van oligodendrocyten of het remmen van pro-inflammatoire activatie van microglia en astrocyten, kunnen mogelijk hersenschade na extreme vroeggeboorte voorkomen of verminderen.

In **hoofdstuk 3** bespreken we een mogelijk veelbelovende nieuwe behandelmethode voor EoP: mesenchymale stamcel (MSC) therapie. Aan de hand van de resultaten uit eerdere onderzoeken naar MSCs voor de behandeling van andere soorten hersenschade (bij volwassenen en kinderen) wordt het mogelijke werkingsmechanisme en

meest optimale behandelprotocol (celtype, methode van toediening etc.) bediscussieerd. **Hoofdstuk 4** richt zich op het gebruik van groeifactoren en anti-inflammatoire cytokines voor het voorkomen en herstellen van EoP. In dit hoofdstuk wordt de meest recente kennis over de rol van een groot aantal factoren in normale hersenontwikkeling en hun mogelijke rol in het herstel van schade aan oligodendrocyten, microglia, astrocyten en (inter)neuronen samengevat.

Translationeel onderzoek vertaalt resultaten uit fundamenteel onderzoek naar toepassing in de kliniek. Door ziektes na te bootsen in diermodellen en celkweken kan er onder gecontroleerde omstandigheden meer inzicht worden verkregen in de onderliggende mechanismen die een rol spelen in het ontstaan van ziekte en de werking van potentiele nieuwe behandelmethoden. In **hoofdstuk 5** beschrijven we een nieuw EoP muis model. Door de dieren bloot te stellen aan vroege postnatale ontsteking en zuurstoftekort ontstaat er een patroon van hersenschade wat veel gelijkenissen heeft met de klinische situatie op de NICU, met verstoorde ontwikkeling van oligodendrocyten en activatie van microglia en astrocyten. Daarnaast hebben de EoP muizen functionele beperkingen in denkvermogen en motorisch gedrag.

In **hoofdstuk 5** gebruiken we ons muismodel om te onderzoeken of behandeling met MSCs, toegediend via neusdruppels (intranasaal), gunstige effecten heeft op hersenontwikkeling na EoP. Intranasale toediening van MSCs, enkele dagen na induceren van schade, leidt tot herstel van myelinisatie (de aanleg van myeline laagjes om de zenuwuitlopers door oligodendrocyten), afname van het ontstekingsproces en functioneel herstel. Daarnaast hebben we de onderliggende werkingsmechanismen van MSCs in EoP onderzocht door middel van primaire glia celkweken. Onze bevindingen laten zien dat MSCs belangrijke groeifactoren en anti-inflammatoire factoren uitscheiden, en zo direct oligodendrocyte uitrijping stimuleren plus microglia- en astrocyte activatie dempen. Hoewel vroege MSC therapie veelbelovend lijkt, laten we in **hoofdstuk 5** zien dat effectiviteit van behandeling beperkt is bij late toediening van MSCs (6-10 dagen na inductie van schade). In een vervolgstudie, beschreven in **hoofdstuk 6**, onderzoeken we de beperkte werking van MSCs op latere tijdstippen en bekijken we of het tijdsinterval voor effectieve behandeling van EoP met MSCs verlengd kan worden door tijdelijk verhoogde uitscheiding (hypersecretie) van groeifactoren te induceren in de stamcellen. In dit onderzoek laten we zien dat toediening van deze aangepaste MSCs met hypersecretie van insulin-like growth factor I (IGF1),

leukemia inhibitory factor (LIF) of interleukin-11 (IL11) leidt tot verlenging van effectieve behandeling van EoP als de MSC behandeling op een later tijdstip wordt gestart.

IGF1, een groeifactor geproduceerd in bijna elk orgaan van het lichaam, speelt een essentiële rol in foetale en postnatale hersenontwikkeling. Eerder onderzoek heeft aangetoond dat IGF1 levels in het bloed lager zijn na extreme vroeggeboorte dan foetale levels rond een vergelijkbare zwangerschapsduur (m.a.w. als de baby nog in de baarmoeder zou zijn). In **hoofdstuk 7** van dit proefschrift bestuderen we of behandeling met intranasale IGF1 druppels gunstige effecten heeft op het herstellen van myelinisatie en dempen van ontstekingsprocessen in ons muismodel van EoP. Intranasale behandeling met IGF1 na inductie van EoP leidt tot verbetering van myelinisatie van de hersenen, motoriek en denkvermogen. Aanvullend onderzoek gericht op de onderliggende werkingsmechanismen van IGF1 liet zien dat de groeifactor met name direct oligodendrocyte ontwikkeling stimuleert, maar geen (remmend) effect lijkt te hebben op de ontstekingsreactie in de hersenen.

Hoofdstuk 8 richt zich op een celtype wat eerder over het hoofd werd gezien, het interneuron. Uit zeer recent onderzoek is gebleken dat de ontwikkeling van interneuronen ook plaatsvindt in het laatste trimester van de zwangerschap, en daardoor zeer waarschijnlijk verstoord raakt na extreme vroeggeboorte. Verstoringen in de hoeveelheid en verdeling van interneuronen in de hersenen zijn geassocieerd met psychiatrische aandoeningen, zoals ASS, welke vaker gezien worden na extreme vroeggeboorte. In **hoofdstuk 8** gebruiken we twee gevalideerde (knaag)diermodellen van EoP om verschillende types interneuron afwijkingen op te sporen. In beide diermodellen worden specifieke interneuron afwijkingen gevonden, daarnaast vertonen muizen met EoP afwijkend sociaal gedrag in vergelijking met gezonde soortgenoten. Tevens laten we in het muismodel zien dat intranasale behandeling met MSCs of met IGF1 interneuron afwijkingen grotendeels herstelt en sociaal gedrag verbetert.

Op dit moment is MRI-beeldvorming van de hersenen rond de aterme leeftijd (~40 weken) de gouden standaard voor het identificeren van de afwijkingen geassocieerd met EoP. Echter, ons onderzoek laat zien dat vroege herkenning van EoP noodzakelijk lijkt voor optimaal herstel van hersenontwikkeling, immers MSC en IGF1 behandeling werkt beter als er vroeg gestart kan worden. Gevalideerde biomarkers om in een vroeg stadium te herkennen welke te vroeg geboren baby's het meeste risico hebben op EoP zijn er momenteel niet. In **hoofdstuk 9** onderzoeken we de relatie

tussen bloed levels van IGF1 en het bindingseiwit IGFBP3 in de eerste levensweken en neurologische uitkomst in een populatie van extreem te vroeg geboren baby's. In ons cohort kinderen waren hogere levels van IGFBP3 in de eerste levensweek geassocieerd met een betere neurologische uitkomst. Bloed levels van IGF1 waren, in tegenstelling tot eerder onderzoek in andere landen, niet geassocieerd met hersenontwikkeling in onze populatie. Meer onderzoek is nodig om een potentiele rol van IGFBP3 als biomarker voor EoP te bevestigen.

Vooralsnog blijft EoP een groot probleem na extreme vroeggeboorte. Nieuwe, veelbelovende therapieën, zoals neusdruppels met MSCs of IGF1, bieden perspectief in de behandeling van EoP. Aanvullend onderzoek is echter noodzakelijk, met name gericht op de veiligheid en het vaststellen van het meest optimale klinische behandelprotocol. Door te blijven samenwerken kunnen we hopelijk stappen maken in de vertaling van deze behandelingen naar de kliniek, zodat we in de nabije toekomst hersenontwikkeling na extreme vroeggeboorte beter kunnen waarborgen.

LIST OF COAUTHORS

Wilhelmina Children's Hospital, UMC Utrecht, Utrecht University, the Netherlands

Department for Developmental Origins of Disease (DDOD)

Cora H. Nijboer
Caroline G.M. de Theije
Freek E. Hoebeek
Myrna J.V. Brandt
Caren M. van Kammen
Rik van Vliet
Mirjam Maas
Nikki Wanders
Marit Vink
Suzanne M. Onstwedder
Chantal M. Kosmeijer
Marthe Kaal

Department of Neonatology

Manon J.N.L. Benders
Floris Groenendaal
Frank van Bel
Niek E. van der Aa
Lisanne M. Baak
Femke Lammertink
Maria Luisa Tataranno
Jeroen Dudink

Center for Image Sciences, UMC Utrecht, Utrecht University, The Netherlands

Biomedical MR Imaging and Spectroscopy Group

Annette van der Toorn
Rick M. Dijkhuizen

MERLN Institute for Technology-Inspired Regenerative Medicine, Maastricht University, The Netherlands

Department of Instructive Biomaterials Engineering

Sabine H. van Rijt
Chloe Trayford
Eva Gubbins

Max Planck Institute of Experimental Medicine, Göttingen, Germany

Electron Microscopy Core Unit, Department of Neurogenetics

Wiebke Möbius
Torben Ruhwedel

INSERM, Université de Paris, France

NeuroDiderot

Pierre Gressens

LIST OF PUBLICATIONS

Published

Frank van Bel, Josine Vaes, Floris Groenendaal (2019) Prevention, reduction and repair of brain injury of the preterm infant. Frontiers in Physiology.

Josine E.G. Vaes, Marit Vink, Caroline G.M. de Theije, Freek E. Hoebeek, Manon J.N.L. Benders, Cora H.A. Nijboer (2019) The potential of stem cell therapy to repair white matter injury in preterm infants: Lessons learned from experimental models. Frontiers in Physiology.

Josine E.G. Vaes, Chantal M. Kosmeijer, Marthe Kaal, Rik Van Vliet, Myrna J.V. Brandt, Manon J.N.L Benders, Cora H. Nijboer (2020) Regenerative therapies to restore interneuron disturbances in experimental models of encephalopathy of prematurity. International Journal of Molecular Sciences

J.E.G. Vaes, C.M. van Kammen, C.Trayford, A. van der Toorn, T. Ruhwedel, M.J.N.L. Benders, R.M. Dijkhuizen, W. Möbius, S.H. van Rijt, C.H. Nijboer (2021) Intranasal mesenchymal stem cell therapy to boost myelination after encephalopathy of prematurity. GLIA.

Josine E.G. Vaes, Myrna J.V. Brandt, Nikki Wanders, Manon J.N.L. Benders, Caroline G.M. de Theije, Pierre Gressens, Cora H. Nijboer (2021). The impact of trophic and immunomodulatory factors on oligodendrocyte maturation: Potential treatments for encephalopathy of prematurity. GLIA.

Submitted

J.E.G. Vaes, L.M. Baak, C.M. van Kammen, F. Groenendaal, M.J.N.L Benders, C.H. Nijboer. Insulin-like growth factor 1 therapy to restore hypomyelination in encephalopathy of prematurity.

Josine E.G. Vaes, Suzanne M. Onstwedder, Chloe Trayford, Eva Gubbins, Mirjam Maas, Sabine H. van Rijt, C.H. Nijboer. Modifying the secretome of MSCs prolongs the regenerative treatment window for encephalopathy of prematurity.

In preparation

J.E.G. Vaes, N.E. van der Aa, F. Groenendaal, L.M. Baak, F. Lammertink, M.L. Tataranno, J. Dudink, C.H. Nijboer, M.J.N.L. Benders. The association between insulin-like growth factor 1 and insulin-like binding protein 3 serum levels and brain development after extreme preterm birth.

CURRICULUM VITAE

Josine Vaes was born in Nederweert, the Netherlands, on September 30th, 1989. After graduating high school (HAVO), she studied Applied Psychology at the Fontys Hogeschool Eindhoven. After the first year, she moved to Utrecht and continued her studies at the University of Amsterdam. In 2013 she obtained her bachelor's degree in Psychobiology, and got accepted for the Selective Utrecht Medical Master (SUMMA) at Utrecht University. During SUMMA she got interested in pediatrics, with a particular interest in neonatal and pediatric neurology. After a successful research internship and graduation, she was accepted as a PhD-candidate at the Department for Developmental Origins of Disease (DDOD) and Department of Neonatology in the Wilhelmina Children's Hospital in Utrecht in 2017. Her translational research, under the supervision of prof. Benders and associate profs. Nijboer and Groenendaal, focused on the preclinical search for novel regenerative strategies to reduce preterm birth related brain injury, as well as clinical assessment of a potential therapeutic target in preterm neonates admitted to the NICU. In the course of her PhD Josine has set up multiple new projects and (international) collaborations.

During her PhD, Josine participated in the PhD curriculum 'Training Upcoming Leaders In Pediatric Science' (TULIPS). In addition, she supervised multiple students during their research internship, and was active as a mentor for medical students.

At the end of 2020 she started as a resident not in training (ANIOS) at the department of pediatrics in the St. Antonius hospital. In 2022 she joined the pediatric residency program at Leiden University Medical Center. Aside from work, Josine enjoys travelling, photography and spending time with family and friends. She lives together with Dion in Almere.

DANKWOORD (ACKNOWLEDGEMENTS)

Dit proefschrift is tot stand gekomen dankzij alle mensen met wie ik heb mogen samenwerken, die mij geholpen hebben of me vanaf de zijlijn hebben gesteund. Dank jullie wel.

Waarom moeilijk doen, als het samen kan
Loesje

Lieve Cora, waar moet ik beginnen? Ik heb ongelooflijk veel van je geleerd. Dit proefschrift is de kroon op onze samenwerking. Al heel vroeg in het traject zag je precies wat ik nodig had om te groeien, misschien omdat we in veel opzichten (inclusief ons gevoel voor humor) op elkaar bleken te lijken. Door me vrijheid en verantwoordelijkheid te geven zorgde je ervoor dat ik het allerbeste uit mezelf kon halen. Je scherpe blik en indrukwekkende kennis hielpen me altijd weer vooruit. Bedankt voor je rotsvaste vertrouwen, de wijze lessen en je onmisbare steun de afgelopen jaren bij alle (persoonlijke) hoogte- en dieptepunten. Je bent een buitengewoon goede onderzoeker, maar bovenal een buitengewoon fijn persoon. Ik heb heel veel bewondering voor alles wat je doet en ben super trots dat ik deel mocht uitmaken van je groep. Ik hoop dat we nog lang blijven samenwerken!

Lieve Manon, je aanstekelijke enthousiasme en passie voor het onderzoek leidde tot mijn stageplek bij het DDOD, waar het voor mij allemaal begon. Je eerlijke, soms kritische feedback zorgde ervoor dat ik scherp bleef en we samen tot het beste resultaat kwamen. Door deuren te openen gaf je me de ruimte om me te blijven ontwikkelen en nieuwe projecten op te zetten. Bedankt voor je enthousiasme, vertrouwen en steun bij alle obstakels op mijn pad, in het werk en daarbuiten. Ik hoop dat we elkaar tegen blijven komen!

Beste Floris, wat fijn dat jij aangehaakt bent als copromotor. Je staat altijd klaar om te helpen, ongeacht hoe vol je agenda is. Bedankt voor je enthousiasme, waardevolle adviezen en feedback voor vele hoofdstukken in dit proefschrift en je onmisbare hulp met statistiek.

Geachte beoordelingscommissie, prof. dr. Hol, prof. dr. Braun, prof. dr. van der Ent, prof. dr. Kemner en prof. dr. van Weissenbruch, veel dank voor het lezen en beoordelen van dit proefschrift.

Lieve Caro(line), wat ben ik blij dat ik jou mocht leren kennen! Klein van stuk maar niet van persoonlijkheid, ik heb genoten van je gezelligheid en aanstekelijke lach. Ik bewonder je bereidheid om altijd een ander te helpen en bovenal je talent om altijd en overal lichtpuntjes in te blijven zien. Hoogte- en dieptepunten konden we samen delen. Bedankt voor je steun, wijze woorden en hulp de afgelopen jaren.

Beste Freek, bedankt voor je betrokkenheid en oprechte interesse. Ik bewonder hoe je naast oog voor de inhoud ook altijd oog houdt voor het persoonlijke. Ik heb je luisterend oor en adviezen (en bbq skills) erg gewaardeerd.

Mijn 'brein groep' collega's, bedankt voor de onvergetelijke tijd en al jullie hulp bij heel veel experimenten! Erik, voor je hulp bij het opstarten en goede voorbeeld. Caren, voor alle technieken die ik van je heb mogen leren en alles wat we samen hebben bereikt, met als hoogtepunt het preklinische MSC stuk. Mirjam, Rebecca, Dominique en Karima, voor al jullie hulp en het meedenken met experimenten! Roeland, voor je kritische blik, het was leuk om samen een nieuw project op te zetten (met wisselend succes). Rik en Diederick, voor alle koffie (tegenwoordig maak ik 'm maar zelf), goede grappen en oneindige hulp met organiseren, kleuringen en analyses. Rik, terecht mijn paranimf en coauteur na de EM analyses. Marijke en Marian, voor jullie ondersteuning en gezelligheid. Mijn collega PhDs, Myrna, Eva, Sara, Judit, Chantal en Fieke, bedankt voor de leuke momenten op de AIO-kamer en het lab. Myrna, bedankt voor je hulp met de gedragsexperimenten en de samenwerking voor het review (mooi stuk, maar wat een werk) en het labuitje! Chantal, van student naar collega, wat was het leuk om je te begeleiden en samen te mogen werken aan het interneuron project.

De collega's van de 'pijngroep', in het bijzonder Niels, Hanneke, Judith, Ramin, Mohammed, Sabine, Christian, Fanny en Kim, bedankt voor de inhoudelijke discussies en bovenal de goede sfeer op de werkvloer.

De studenten die ik met veel plezier heb mogen begeleiden, Angela, Michiel, Lisanne, Nikki, Marit, Marthe, Chantal en Suzanne. Bedankt voor jullie vertrouwen, harde werk en het goede gezelschap tijdens ontelbaar veel glia kweken en andere experimenten.

Alle GDL-medewerkers, in het bijzonder Anja, bedankt voor alle hulp en jullie inzet voor ons onderzoek.

Lieve (oud)collega-onderzoekers van de neonatologie, samen leren en groeien als onderzoekers, samen op congres of een klein koffiemoment op de dag, wat was het fijn om onderdeel van jullie groep te mogen zijn! Bedankt voor het delen van de pieken en dalen van het onderzoek en alles daarbuiten. Nienke, bedankt voor jouw warme welkom, verbindende kracht en altijd wijze raad. Ik bewonder je harde werk en doorzettingsvermogen. Nathalie, voor de gezelligheid op congres en je oprechte interesse. Kim, voor jouw steun, warmte en de herkenning. Ik waardeer onze vriendschap enorm. Raymond, voor je nuchtere blik, rust en droge humor. Elise, voor je goede koffie (nespresso uit je kluisje), verfrissende ideeën en goede adviezen. Femke, voor je hulp met imaging en analyses (hoofdstuk 9!), relativeringsvermogen en de fijne gesprekken over werk en alles daarbuiten. Maaike, voor je aanstekelijke vrolijkheid en de leuke samenwerking. Lisa, onderzoeks- en TULIPS-genootje, voor het samen optrekken in de pieken- en dalen van het onderzoek. Lisanne, voor de samenwerking en harde werken tijdens je stage bij het DDOD, hulp bij het IGF1-project en bovenal de gezelligheid!

Beste Niek, ondanks je overvolle agenda nam je de tijd om mij te helpen met een DTI-project. Ontzettend bedankt voor je heldere uitleg en begeleiding. We hebben samen een mooi paper neergezet. Ook veel dank voor je opbeurende woorden en goede adviezen als het even tegenzat in mijn traject, dat heb ik erg gewaardeerd!

Beste Linda, bedankt voor het delen van je indrukwekkende kennis op het gebied van de neonatale neurologie tijdens alle onderwijsmomenten en bij het scoren van MRI-scans. Ik heb je betrokkenheid bij de arts-onderzoekers en lieve sinterklaasgedichten enorm gewaardeerd. Wat leuk dat we elkaar weer tegenkomen in Leiden!

Beste Frank, door jouw enthousiaste presentatie tijdens mijn studie kwam ik bij DDOD/afdeling neonatologie terecht. Altijd een sfeermaker op congres of tijdens een etentje. Bedankt voor je betrokkenheid en goede adviezen.

Neonatologen, PA-ers en verpleegkundigen van de afdeling neonatologie, bedankt voor jullie ondersteuning bij het klinische project. In het bijzonder Jeroen en Maria Luisa, veel dank voor jullie hulp met het scoren van MRI's en onderhouden van de

prematuren database. Barbra, voor je behulpzaamheid en het coördineren van afspraken in onmogelijk drukke agenda's.

Onze samenwerkingspartners, in binnen- en buitenland. Leden van de gebruikerscommissie van het IGF1 project (hersenstichting), bedankt voor jullie waardevolle suggesties en input om ons onderzoek naar een hoger niveau te tillen. Prof. dr. Rick Dijkhuizen en Annette van der Toorn, bedankt voor jullie bijdrage aan het ontwikkelen van ons EoP model. Dr. Sabine van Rijt, Chloe Trayford en Eva Gubbins, bedankt voor de samenwerking in het tracen van MSCs. Met behulp van jullie innovatieve technieken hebben we samen twee mooie stukken neergezet! Dr. Wiebke Möbius and Torben Ruhwedel, I enjoyed setting up our collaboration after meeting at the myelin conference. Your expertise in electron microscopy has been a valuable addition to our preclinical work. Prof. dr. Pierre Gressens, thank you for your insightful comments and contribution to our review article, you are a true pioneer in the field of perinatal brain injury.

Mijn lieve TULIPS clubje, wat was het fijn om alle hoogte- en dieptepunten met elkaar te kunnen delen en samen verder te kunnen groeien. Dankjewel voor jullie steun, kwetsbaarheid en de gezellige dagen en weekenden. Ik hoop dat we elkaar blijven ontmoeten. Myrthe, wat leuk en bijzonder dat wij samen aan de opleiding zijn begonnen!

Lieve collega's uit het St. Antonius ziekenhuis, kinderartsen, arts-assistenten, verpleegkundigen en poli-medewerkers, ik kan geen betere plek bedenken om te mogen starten als jonge dokter. Bedankt voor alle wijze lessen, de ruimte om mezelf te zijn en jullie aanmoediging richting de opleiding. Hopelijk tot ziens!

Mijn nieuwe collega's in het LUMC, bedankt voor het warme welkom. Wat fijn om onderdeel van jullie team te mogen zijn. Ik kijk er naar uit om de komende jaren van jullie te mogen leren.

Graag wil ik ook mijn lieve vrienden en familie bedanken. Jullie belangstelling en steun de afgelopen jaren was minstens zo belangrijk in de totstandkoming van dit proefschrift. Mijn lieve opa en oma Lucas, altijd zo trots en positief. Mijn schoonfamilie, Terry en Sjon, bedankt voor jullie interesse en behulpzaamheid. Mijn 'oudste' vriendinnen, Loes en Miriam, van tieners op de middelbare school naar nu ieder een

eigen volwassen leven, een gedeeld pad met waardevolle herinneringen (van letterlijk vallen en opstaan). Ook al zien we elkaar een tijdje niet, het voelt altijd vertrouwd. Lieve Anouk, je staat altijd klaar voor een ander en bent het beste luisterend oor. Dank voor je opbeurende woorden en wie je bent. Lieve Melissa en Django (en Boaz), een bruiloft in de Ardennen heeft geleid tot een onmisbare vriendschap, bedankt voor jullie oprechte interesse, steun en alle gezellige momenten. Nieuwe vriendschappen, Sharleen, Aafke en Shaden, bedankt voor de onvergetelijke ANIOS-tijd gevuld met fijne koffie momenten en goede adviezen.

Lieve Tamara, sinds het eerste jaar van SUMMA zijn we onafscheidelijk! Wat is het een feestje om met jou samen dit pad te mogen afleggen. Ik ben zo trots op wie je bent en hoe je je eigen weg durft te kiezen buiten de gebaande paden. Bedankt voor je luisterend oor, wijze woorden en bovenal dat je me altijd weer aan het lachen maakt. Laten we altijd samen blijven dromen (van koffiebar tot verlos- kinderarts duo). Mijn paranimf en mijn dierbare vriendin, ik hoop dat we nog veel avonturen met elkaar mogen beleven.

Lieve Wouter, we zijn soms water en vuur (vul jij maar in wie wat is) maar weten elkaar altijd te vinden als het ertoe doet. Bedankt dat je achter me staat en me aanmoedigt. Lieve Joyce, wat fijn dat jij deel uitmaakt van ons gezin! Ik heb oneindig veel bewondering voor hoe jullie het leven opnieuw vormgeven na een ongelooflijk moeilijke tijd. Lieve Olivia, jij bent altijd een zonnestraal, hoe donker ook de dag. Lieve Juliette, zo lief, zo klein en zo kwetsbaar. Voor jou mogen zorgen heeft een onuitwisbare indruk op me gemaakt en me meer geleerd dan ik in woorden kan uitdrukken. Ik denk heel vaak aan je!

Peter, gelukkig al vele jaren onderdeel van ons gezin. Bedankt voor al je goede zorgen!

Mijn ouders, jullie zijn de basis van elke stap. Lieve papa, je hebt me zoveel meegegeven in korte tijd. Je stimuleerde me nieuwsgierig en doortastend te zijn, kwaliteiten die mij een betere onderzoeker maken. Samen leerden we hoe kwetsbaar het leven kan zijn, dat samen genieten van de weg zoveel belangrijker is dan de bestemming. Ik neem je altijd met me mee. Lieve mama, bedankt voor je onvoorwaardelijke steun en vertrouwen. Blijven zitten op de HAVO of 3x uitgeloot worden voor geneeskunde, met jou naast me lukte het om door te zetten. Ik bewonder jouw levenslust en zorg-

zaamheid. Het allerbelangrijkste wat je me hebt meegegeven is veerkracht. Soms struikelen en vallen, maar altijd weer opstaan.

Lieve Dion, ik vind het moeilijk om in woorden te beschrijven wat jij voor mij betekent. Je geeft me de ruimte om mijn weg te vinden, mijn ambities waar te maken en steunt me onvoorwaardelijk als het tegenzit. Je scherpe blik, relativeringsvermogen en humor zijn je kracht om elk obstakel te overwinnen. Ik ben zo ongelooflijk trots op jou. Bij jou ben ik thuis.

UMC Utrecht

Universiteit Utrecht

ISBN 978-94-6458-431-8