Towards improved treatment options for white matter injury in preterm infants

Erik van Tilborg

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Richting verbeterde behandelmethoden voor witte stof schade in prematuur geboren kinderen

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TOWARDS IMPROVED TREATMENT OPTIONS FOR WHITE MATTER INJURY IN PRETERM INFANTS

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The most beautiful thing we can experience is the mysterious. It is the source of all true art and science.

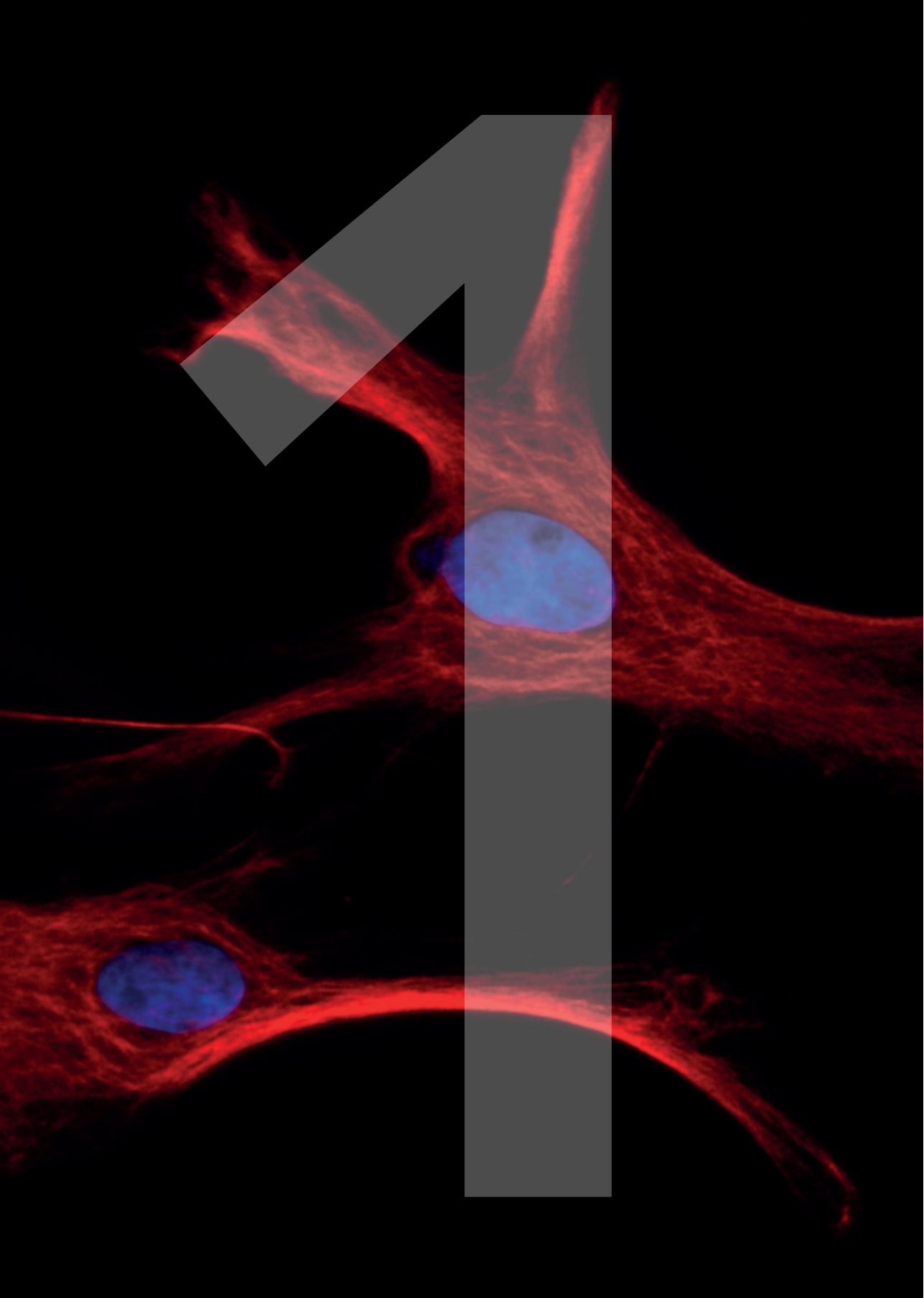
- *Albert Einstein*

Voor mijn ouders

The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory.

Santiago Ramón y Cajal

Table of contents



CHAPTER 1

General introduction

Diffuse white matter injury (WMI) in the brain is a major problem in infants born prematurely. This thesis aims at bringing potential therapeutic treatments closer to clinical application by investigating underlying mechanisms and novel treatment strategies for diffuse WMI. In this general introduction, the clinical aspects of diffuse WMI are briefly described, together with the underlying cellular pathology. Furthermore, current advances on WMI research are stated from a methodological perspective, and various potential therapeutic options are described. Chapters 2 and 3 review the clinical and biological background of diffuse WMI in preterm infants. This general introduction will conclude with the outline of this thesis.

Diffuse white matter injury in preterm infants

10% of all babies are born prematurely (i.e. before 37 weeks gestational age)[1] and preterm infants are at high risk to develop neurological morbidity[2]. Cystic periventricular leukomalacia (cPVL) has been a common diagnosis in preterm infants for several decades. cPVL is associated with large cystic lesions in the white matter causing severe motor disabilities such as cerebral palsy[3-5]. Fortunately, the incidence of cPVL has declined over the past decades, but at the same time more diffuse types of white matter injury (WMI) have become more prevalent[6, 7]. Diffuse WMI is clinically characterized by decreased white matter volumes, altered white matter fractional anisotropy values, and the presence of small punctate white matter lesions[8]. Compared to cPVL, the neurological consequences of diffuse WMI are less severe, but are still devastating to patients and their families, as they include poor cognitive performance, impaired motor development and increased risk of neurodevelopmental disorders, including autism-spectrum disorders and schizophrenia[2, 3, 9, 10].

The exact mechanisms underlying diffuse WMI in preterm infants are difficult to define due to the heterogeneity of each individual case. However, clinical studies revealed that exposure to inflammatory insults (e.g. maternal infections, postnatal sepsis) and unstable cerebral oxygenation (due to e.g. lung disease, underdeveloped cerebral vascularization, ventilation therapy) are important risk factors for white matter abnormalities[11-19]. Furthermore, it has been proposed that exposure to recurrent and/or multiple different perinatal insults is an important risk factor to develop neonatal WMI with early (e.g *in utero*) insults sensitizing the brain to later (e.g. postnatal) insults[20]. Indeed, clinical data show that exposure to multiple hits dramatically increases the risk of WMI[21, 22].

Impeded oligodendrocyte maturation

Glial cells play an important role in the etiology of diffuse WMI in preterm infants. Activation of microglia and astrogliosis are important pathological hallmarks in the white matter of deceased WMI patients[23-26]. Furthermore, the developmental program of oligodendrocytes, the cells responsible for providing axons with myelin sheaths, seems to be disrupted[23, 24, 26]. To elaborate, during healthy brain development oligodendrocytes are derived from oligodendrocyte precursor cells (OPCs) that differentiate into premyelinating oligodendrocytes, and eventually mature into end-stage myelinating oligodendrocytes[27]. However, pathology studies on human post-mortem brain tissue revealed that the maturation of oligodendrocytes is impaired in diffuse WMI[23, 24, 26, 28]. Whereas the exact pathways that contribute to this developmental arrest of oligodendrocyte maturation remain unknown, inflammatory mediators and the consequences of oxygen fluctuations likely play important roles. Such perinatal insults can directly affect developing oligodendrocytes, or can indirectly create an unfavorable environment for OPCs by activating microglia and astrocytes.

Research methods to study diffuse WMI

Considering the high prevalence of diffuse WMI, the long-term consequences and the limited treatment options that are available, it is essential to perform more research into underlying mechanisms and novel therapeutic options. Clinical imaging and epidemiological studies may aid our understanding of the neuroanatomical and functional consequences of preterm birth-related insults, may identify new risk factors, and may elucidate the effects of specific white matter abnormalities on neurological outcome. However, in order to fully understand the cellular and molecular basis of diffuse WMI, to identify specific therapeutic targets and to develop novel treatments, it is essential to use pre-clinical models in a translational setting. In recent years, various rodent models have been developed to study non-cystic diffuse WMI, however most consider only a single inflammatory or a single hypoxic insult to induce WMI[5]. In this thesis, we propose that in particular a combination of both types of insults is highly clinically relevant.

An important aim of this thesis is to expand the tools available to study mechanisms underlying diffuse WMI. A novel multiple-hit rat model of diffuse WMI comprising both an inflammatory and a hypoxic component was developed and characterized in great detail. Furthermore, a new histology-based technique to analyze the microstructural organization of myelinated axons in the rodent cortex is proposed.

Treatment options

Shockingly, no treatment options are currently clinically available for preterm infants with diffuse WMI. Therefore, the development of new therapeutic strategies is urgently needed. Taking into account the complex pathophysiology underlying diffuse WMI, various strategies may have beneficial effects. For example, anti-inflammatory treatments may reduce the pro-inflammatory state of the brain, thereby creating a more favorable environment for oligodendrocytes to develop normally[29-31]. Alternatively, the use of mesenchymal stem cells is a promising therapeutic option that has previously been shown to promote recovery of the white matter in animal models of neonatal hypoxic-ischemic brain damage[32, 33]. Another strategy is to pharmacologically target glial cells in the brain. One potential pathway to target is the MAP kinase JNK signaling pathway, which has been previously associated with neonatal WMI and has been shown to negatively regulate the expression of myelin genes[34-36]. Furthermore, earlier research in our lab showed that inhibition of JNK signaling has neuroprotective effects on both white and gray matter in a rodent model of neonatal hypoxia-ischemia[37, 38]. Inhibition of JNK signaling may therefore be a promising therapeutic strategy to reduce inflammation and promote oligodendrocyte differentiation in the preterm brain. In this thesis, we demonstrate that inhibition of JNK signaling has beneficial effects in a rat model of diffuse neonatal WMI.

Aim and outline of this thesis

In summary, the main aims of this thesis were to develop new methodology to aid research into diffuse WMI, to identify the cellular and molecular mechanisms underlying impaired oligodendrocyte maturation in WMI, and to develop a novel treatment option to combat diffuse WMI.

Chapters 2 and 3 focus on the clinical and biological background of WMI in preterm infants. **Chapter 2** specifically reviews the cellular pathology in neonatal WMI, implicating microglia and astrocytes in the negative regulation of oligodendrocyte maturation in preterm infants. Potential therapeutic targets to protect against WMI are highlighted. **Chapter 3** describes the cellular mechanisms underlying healthy white matter development, together with an update on current advances in our understanding of oligodendrocyte biology. Furthermore, a comparison is made between rodent and human white matter development and implications of novel concepts in the field of oligodendrocyte biology for neonatal WMI are discussed.

Chapters 4, 5 and 6 highlight the development of new methodology to study diffuse WMI in a translational setting. In **chapter 4**, a novel multiple-hit model of neonatal diffuse WMI in newborn rats is described in great detail, comprising histological findings, MRI scans and short-term behavioral outcome. **Chapter 5** describes previously used methods of studying rodent myelination using histology, and demonstrates several newly developed analysis methods to investigate the microstructural organization of myelination in the rodent cortex. In **chapter 6**, long-term cognitive performance in the novel two-hit rat model of diffuse WMI is investigated.

Chapters 7 and 8 focus on unraveling the mechanisms underlying impaired oligodendrocyte development and on testing a novel treatment option to combat diffuse WMI in preterm infants. More specifically, in **chapter 7**, the therapeutic potential of the JNK inhibiting peptide D-JNKi is investigated using *in vitro* primary OPC cultures and the newly developed rat model of diffuse WMI (for the model see **chapter 5**). In **chapter 8** RNA sequencing was used to characterize how inflammation alters the transcriptional profile of differentiating OPCs, thereby identifying novel genes that potentially contribute to WMI. These insights may provide an important basis for future research. Finally, **chapter 9** contains a summary of this thesis and a general critical discussion of the findings described in this thesis.

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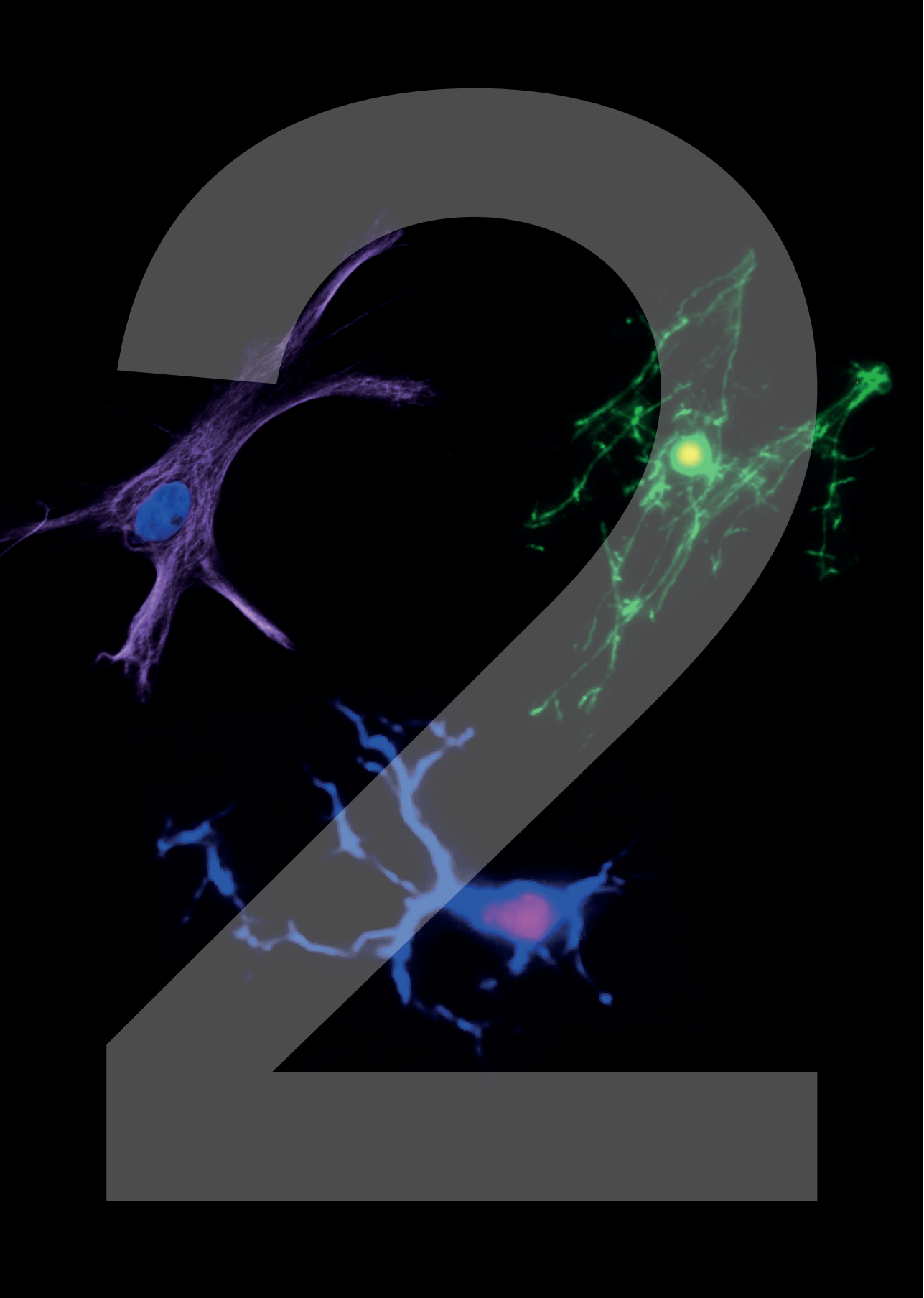

CHAPTER 2

Impaired oligodendrocyte maturation in preterm infants: potential therapeutic targets

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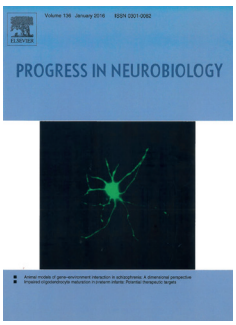

HIGHLIGHTS

- Diffuse white matter injury in the brain is a highly prevalent morbidity in babies born prematurely.
- Perinatal inflammation and imbalanced oxygen supply are important risk factors for diffuse white matter injury.
- In perinatal diffuse white matter injury, arrested maturation of oligodendrocytes causes impaired myelination.
- The interplay between active microglia/astrocytes and oligodendrocyte precursors contributes to diffuse white matter injury.
- Promising therapeutic strategies include growth factor treatment, mesenchymal stem cell therapy and anti-inflammatory drugs.

ABSTRACT

Preterm birth is an evolving challenge in neonatal health care. Despite declining mortality rates among extremely premature neonates, morbidity rates remain very high. Currently, perinatal diffuse white matter injury (WMI) is the most commonly observed type of brain injury in preterm infants and has become an important research area. Diffuse WMI is associated with impaired cognitive, sensory and psychological functioning and is increasingly being recognized as a risk factor for autism-spectrum disorders, ADHD, and other psychological disturbances. No treatment options are currently available for diffuse WMI and the underlying pathophysiological mechanisms are far from being completely understood. Preterm birth is associated with maternal inflammation, perinatal infections and disrupted oxygen supply which can affect the cerebral microenvironment by causing activation of microglia, astrogliosis, excitotoxicity, and oxidative stress. This intricate interplay of events negatively influences oligodendrocyte development, causing arrested oligodendrocyte maturation or oligodendrocyte cell death, which ultimately results in myelination failure in the developing white matter. This review discusses the current state in perinatal WMI research, ranging from a clinical perspective to basic molecular pathophysiology. The complex regulation of oligodendrocyte development in healthy and pathological conditions is described, with a specific focus on signaling cascades that may play a role in WMI. Furthermore, emerging concepts in the field of WMI and issues regarding currently available animal models are put forward. Novel insights into the molecular mechanisms underlying impeded oligodendrocyte maturation in diffuse WMI may aid the development of novel treatment options which are desperately needed to improve the quality-of-life of preterm neonates.

Keywords: Preterm birth, White matter injury, Therapeutic strategies, Oligodendrocyte, Microglia, Astrogliosis.

Review outline

1. INTRODUCTION

Worldwide, over 10% of all babies are born prematurely and their mortality accounts for 35% of all neonatal deaths (Liu et al., 2012a). Over the past years advances in neonatal care have led to decreased neonatal mortality in Western society (EUROCAT, 2013). However, many survivors of preterm birth show considerable morbidity including necrotizing enterocolitis, bronchopulmonary dysplasia, retinopathy of prematurity and/or neurological damage (Costeloe et al., 2012; Stoll et al., 2015). Currently, the most common type of brain injury in preterm neonates is diffuse perinatal white matter injury (WMI), in which impaired oligodendrocyte (OL) maturation and myelination result in decreased cognitive, behavioral, and sensory abilities as well as psychological problems later in life (reviewed in Back and Miller, 2014; Volpe et al., 2011). No treatment for diffuse WMI is currently available. This review explores clinical observations regarding diffuse WMI, describes cellular pathophysiological processes underlying arrested OL development in diffuse WMI and highlights several intracellular pathways that may contribute to impeded OL maturation. Furthermore, several suggestions for future research and potential therapeutic strategies are provided.

2. PERINATAL WHITE MATTER INJURY: CLINICAL OBSERVATIONS AND ETIOLOGY

2.1. Types of WMI

Perinatal WMI occurs in various forms, ranging from severe cystic white matter lesions to subtle changes in the white matter microenvironment (see Table 1). In both clinical and experimental literature, the term WMI is often used regardless of the pattern of injury. The ambiguous term "WMI" can be confusing, considering that this term can pertain to multiple patterns of injury that may have different underlying pathophysiology and different long-term consequences. Therefore, we argue that different aspects of perinatal WMI should be more clearly defined so they can be studied separately. Using different neuroimaging techniques, various types of WMI can be distinguished (for reviews see Benders et al., 2014; de Vries et al., 2013; Ment et al., 2009; Rutherford et al., 2010).

In the most severe type of WMI in preterm neonates, hemorrhagic infarction (mostly of intraventricular origin) results in the formation of large *porencephalic cysts* in the white matter (Fig. 1A). This type of injury generally occurs unilaterally and can lead to severe disabilities such as hemiplegia. *Cystic periventricular leukomalacia* (cPVL) is another

Table 1

Type of WMI	Visible on cranial ultrasound?	Anatomical pathophysiology	Major clinical outcome
Porencephalic cysts	Yes	Severe, often unilateral brain injury in the shape of large porencephalic cysts in the white matter, often resulting from intraventricular hemorrhage.	Depending on location of lesion, severe motor disabilities including hemiplegia
Cystic periventricular leukomalacia	Yes	Bilateral cysts in the white matter, often associated with inflammatory and ischemic insults due to hypotension or hypocarbia.	Severe motor deficits including cerebral palsy
Punctate white matter lesions	As inhomogeneous echogenicity	Microscopic cysts in the white matter, often localized around the lateral ventricles, that may disappear with age. May originate from hemorrhagic or ischemic origin.	Limited data available, but seems to be related to mild cognitive impairment and behavioral problems
Diffuse WMI	Certain aspects: ventriculomegaly, increased size of interhemispheric fissure	White matter atrophy resulting in e.g. ventriculomegaly, thinning of the corpus callosum and accumulated cerebrospinal fluid surrounding the brain. Associated with altered white matter microstructure as indicated by low FA values.	Associated with mild cognitive impairment, behavioral problems and psychological problems

Different types of white matter injury and clinical features (for reviews see Benders et al., 2014; de Vries et al., 2013; Rutherford et al., 2010).

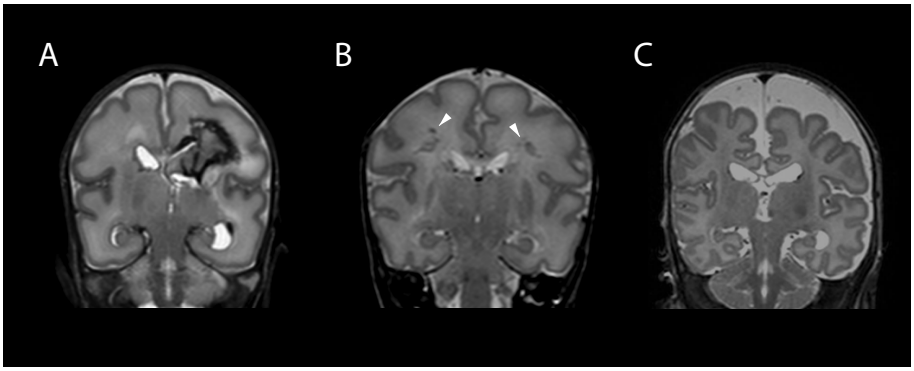

Figure 1 Various types of white matter injury in the preterm neonate as demonstrated by T1-weighted MRI scans. A. Periventricular hemorraghic infaction has led to severe injury (porencephalic cyst) in the periventricular white matter. B. Punctate white matter lesions (arrowheads) in the preterm brain. C. Extreme case of diffuse white matter injury at term equivalent age of a neonate born at 23 weeks gestational age. White matter atrophy has resulted in ventriculomegaly, thinning of the corpus callosum and large amounts of CSF around the brain.

type of severe WMI with a different etiology, as it is mostly associated with cerebral inflammation and ischemia that lead to the bilateral formation of focal necrotic cysts in the white matter. cPVL typically results in motor disabilities such as cerebral palsy (reviewed in Volpe, 2009). Both large porencephalic cysts and cPVL can be visualized relatively easy by early cranial ultrasound, or by magnetic resonance imaging (MRI) (reviewed in de Vries et al., 2013). Over the past twenty-five years, the incidence and the severity of cPVL has decreased considerably (Hamrick et al., 2004; van Haastert et al., 2011), with large cystic white matter abnormalities being present in only 10% of all WMI patients (e.g. Inder et al., 2003; Maalouf et al., 1999; Miller et al., 2003). However, with technologic advances in imaging methods it has become clear that milder forms of WMI without the presence of macroscopic necrotic cysts, are commonly observed in preterm infants and affect the majority of the WMI cases (reviewed in Back and Miller, 2014).

Using MRI, we and others observed that around 35% of children born before 28 weeks of gestation showed *punctate white matter lesions* (PWML) (Fig. 1B) (M. Benders, *unpublished data*; Buser et al., 2012). These are more subtle micro-lesions in the white matter, which are mostly hemorrhagic or ischemic in nature, are often localized around the lateral ventricles, and are often too small to be detected by cranial ultrasound. Using different MRI-based analyses, a distinction can be made between the multiple

types of PWML: whereas PWML resulting from hemorrhagic insults are clearly visible on susceptibility-weighted images, PWML from ischemic origin are more clearly visible on T1-weighted and diffusion-weighted images (Kersbergen et al., 2014; Niwa et al., 2011). PWML can disappear with age, leaving behind a glial scar that may, depending on its location, have negative consequences on neuronal connectivity and long-term outcome (Kersbergen et al., 2014; Niwa et al., 2011). Although data on long-term outcome are still limited, the effects of PWML on neurodevelopemental outcome seem to depend on lesion origin (hemorrhagic or ischemic) and the total number of lesions (reviewed in Benders et al., 2014).

Another MRI-based observation in preterm neonates is the presence of *diffuse excessive high signal intensities* (DEHSI) in the white matter. It has previously been suggested that these signal intensities may reflect a pattern of dysmaturation of the preterm brain (Counsell et al., 2003). However, several studies have pointed out that no relationship exists between the presence of DEHSI and long-term outcome (de Bruine et al., 2011; Jeon et al., 2012). Rather than being a pathophysiological feature of neurological injury, DEHSI likely reflect a normal developmental process.

Diffuse WMI, which is characterized by *loss of white matter* (Fig. 1C), is another pattern of injury that is very commonly observed in preterm infants at term equivalent age. Although the prevalence of severe diffuse non-cystic WMI is declining (Gano et al., 2015), this type of injury, especially the milder form of diffuse WMI, remains present in the majority of WMI patients, sometimes in combination with other types of WMI including PWML (reviewed in Back and Miller, 2014; Khwaja and Volpe, 2008; Volpe et al., 2011). Diffuse WMI can be visualized using MRI, and can result in decreased brain volumes, increased volumes of the ventricles and atrophy of important white matter tracts reflected by e.g. thinning of the corpus callosum. Notably, signs of diffuse WMI are often observed in both the cerebrum and the cerebellum (e.g. Hart et al., 2010; Wang et al., 2014). MRI-based diffusion weighted imaging revealed that diffuse WMI is often accompanied by low fractional anisotropy (FA) values, which are indicative of decreased microstructural integrity of the white matter (reviewed in Ment et al., 2009). Therefore, decreased FA values are considered a pathophysiological feature of diffuse WMI. Low FA values remain visible throughout adulthood and have been associated with adverse long-term outcome (Allin et al., 2011; van Kooij et al., 2011, 2012).Each of the above mentioned types of WMI may have different underlying causes, risk factors and consequences, and they may be interrelated. In this review, the main focus will be diffuse WMI.

2.2. Long-term consequences of perinatal diffuse WMI

Preterm birth is associated with a wide range of neuropsychological implications later in life including attention problems like ADHD, executive function disorders, perceptual difficulties, social-emotional problems like autism, motor disabilities, impaired cognition, and psychiatric conditions (Dudova et al., 2014; Johnson et al., 2010; Pyhala et al., 2014; Yang et al., 2015). Whereas cystic WMI can lead to severe consequences for motor coordination like cerebral palsy, non-cystic diffuse WMI results in more subtle consequences affecting mostly neurodevelopmental scores, perceptual abilities and cognitive functioning (Counsell et al., 2008; Kontis et al., 2009; Northam et al., 2011; Nosarti et al., 2008; Skranes et al., 2007; Soria-Pastor et al., 2008; Spittle et al., 2009; van Kooij et al., 2012; Woodward et al., 2005, 2006). Behavioral abnormalities persist throughout adolescence and adulthood. For instance, in prematurely born male adolescents thinning of the corpus callosum remains associated with impaired verbal abilities and in adults altered white matter microstructure is indicative of cognitive impairment (Allin et al., 2011; Nosarti et al., 2004). Preterm birth increases the risk of developing psychiatric disorders later in life (Johnson et al., 2010; Lund et al., 2011; Nosarti et al., 2012). For example, a link between preterm birth and autism spectrum disorders is increasingly being recognized (Guy et al., 2015; Pyhala et al., 2014) and altered white matter integrity and neuroinflammation are becoming major topics in the field of schizophrenia research (Lener et al., 2014; Najjar and Pearlman, 2014). Even though studies linking diffuse WMI to psychiatric diseases like schizophrenia remain scarce, it is likely that a causal relationship exists (reviewed in Chew et al., 2013). Taken together, the above mentioned observations suggest that diffuse WMI can lead to permanent disabilities, including cognitive deficits and psychiatric problems, which greatly impair quality-of-life of patients and their families. Monitoring extremely prematurely born children throughout their childhood and providing adequate individual support may benefit neurodevelopment of these patients and could provide important insights into the developmental consequences of diffuse white matter abnormalities.

2.3. Etiology of perinatal diffuse WMI

Importantly, preterm birth is typically accompanied by maternal inflammation and/or perinatal infections which are considered important risk factors for WMI, as perinatal inflammation is strongly associated with impaired neurological outcome and increased risk of WMI (Chau et al., 2009, 2012; Glass et al., 2008; Goepfert et al., 2004; Mwaniki et al., 2012; Procianoy and Silveira, 2012; van Vliet et al., 2013; Viscardi et al., 2004). The negative effects of inflammation on the white matter are likely mediated by

increased levels of pro-inflammatory cytokines and the activation of microglial cells in the brain (see Section 6.1). Both bacterial and viral infections are observed in preterm infants and both types of infections have been associated with adverse outcome and increased risk of WMI, although underlying pathophysiology may be different (Nijman et al., 2013; O'Shea et al., 2009; Shah et al., 2008; Verboon-Maciolek et al., 2008). Viral infections observed in preterm infants that have been associated with WMI include rotavirus, cytomegalovirus and human parechovirus (Nijman et al., 2013; Verboon-Maciolek et al., 2008, 2012).

During the perinatal period, cerebral oxygen supply can be disrupted by a variety of factors. First of all, the cardiovascular and respiratory systems of the preterm neonate are underdeveloped and the premature lungs are vulnerable to damage resulting from perinatal inflammation (Viscardi et al., 2004). Compromised respiratory and cardiovascular systems negatively affect cerebrovascular autoregulation, which can lead to low blood pressure, hypocarbia and low oxygen supply towards the brains of preterm neonates (Soul et al., 2007; Tsuji et al., 2000; reviewed in Fyfe et al., 2014). Hypocarbia (low blood carbon dioxide levels) is an important risk factor for WMI that contributes to impaired cerebral perfusion by inducing vasoconstriction (Resch et al., 2012; Shankaran et al., 2006). Second, premature infants are exposed to external oxygen levels much earlier compared to term neonates, causing an earlier rise in oxygen saturation levels in the preterm brain (Nuntnarumit et al., 2010; Schwaberger et al., 2015). Moreover, in neonatal clinics high oxygen levels are often administered to preterm neonates as ventilation therapy, in order to alleviate respiratory distress (Stoll et al., 2015). However, the optimal oxygen saturation level for preterm neonates in neonatal intensive care units remains an important topic of debate, and excessive respiratory support may cause hyperoxia (Castillo et al., 2008; reviewed in Lakshminrusimha et al., 2015).Imbalanced oxygenation poses an important risk factor for diffuse WMI, as it has been clinically associated with white matter injury, low FA values and adverse neurological outcome (Alexandrou et al., 2014; Leviton et al., 2010; Shankaran et al., 2006). Experimental data support the fact that perinatal insults like inflammation, hypoxia or hyperoxia negatively affect the developing white matter, causing WMI (e.g. Cai et al., 2000; Favrais et al., 2011; Felderhoff-Mueser et al., 2004). For instance, Curristin et al. (2002) have shown that exposure to hypoxia downregulates the expression of genes regulating myelination and oligodendrocyte development in mice. In conclusion, inflammation and disrupted oxygen supply are important risk factors for WMI and exposure of preterm neonates to such perinatal insults likely contributes to WMI by negatively affecting developing OLs.

3. EMERGING CONCEPTS IN THE FIELD OF WMI

3.1. The contribution of perinatal complications to WMI

Besides brain injury, preterm infants often suffer from a wide range of other complications that may contribute to increased vulnerability of the white matter. For instance, injury to the lungs or cardiovascular system may contribute to WMI by disturbing the oxygen supply towards the brain. Premature infants often suffer from lung diseases like bronchopulmonary dysplasia, which has been associated with increased risk of developing white matter abnormalities (Anjari et al., 2009). Additionally, in term neonates congenital heart disease has been linked to delayed neurodevelopment and white matter abnormalities (Dimitropoulos et al., 2013; Licht et al., 2009; Miller et al., 2007a,b).

Furthermore, complications in the gastrointestinal system might contribute to diffuse WMI. The gut and the brain interact through a variety of mechanisms, collectively named the gut-brain axis. For instance, mediators produced in the gut influence the brain via activation of the vagal nerve or via systemic circulation. Correct functioning of the gut-brain axis is crucial for normal brain development and depends on the composition of the host microbiota (Diaz Heijtz et al., 2011; Sudo et al., 2004). Bacterial colonization affects the immune response by release of immuno-regulatory short chain fatty acids and subsequent release of pro- or anti-inflammatory cytokines (Mirmonsef et al., 2012; Tan et al., 2014). The gut microbiota of preterm infants differs from term infants, as premature neonates lack the presence of e.g. bacterial species *Bifidobacterium* and *Lactobacillus,* two species that possess anti-inflammatory properties (Sagar et al., 2014). Furthermore, preterm infants show high inter-individual variability in gut-flora biodiversity (Barrett et al., 2013). Bacterial colonization of the gut coincides with critical neurodevelopmental processes and alterations in gut microbiota may contribute to neurological problems in WMI via gut-brain axis dysfunctioning (Borre et al., 2014). A diet containing probiotics may prevent gut-mediated negative effects on neurodevelopment (reviewed in Keunen et al., 2014). Furthermore, necrotizing enterocolitis, a disease of the gut commonly observed in preterm neonates, and inflammatory bowel disease have been associated with increased white matter abnormalities, further supporting the notion that gut-brain interactions may contribute to WMI (Ferro et al., 2014; Shah et al., 2008).

Exposure of premature neonates to surgery has also been shown to have adverse effects on white matter integrity and cognitive outcome (Filan et al., 2012; Vinall et

al., 2014). This effect has been suggested to be mediated by increased respiratory morbidity, inflammatory activation of the immune system and oxidative stress, which negatively affect developing OLs (Filan et al., 2012; Vinall et al., 2014). Other neonatal complications that have been associated with increased risk of WMI include placental inflammation, bacteremia and sepsis (O'Shea et al., 2009; Shah et al., 2008). In conclusion, adverse events in various peripheral organs of premature neonates may contribute to white matter damage in the brain. Importantly, most of these peripheral complications are accompanied by inflammation, which highlights a pro-inflammatory environment as a crucial risk factor for developing diffuse WMI in preterm neonates.

3.2. Perinatal WMI: Genetic predisposition

Even though pathophysiological mechanisms underlying WMI are steadily being elucidated, it remains unknown why some premature neonates are more prone to develop WMI than others. Genetic aspects might play a significant role in this regard. However, genetic studies into neurological vulnerability of premature infants remain scarce. In preterm infants, single nucleotide polymorphisms (SNPs) in genes encoding for pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6 can lead to increased bioavailability of these cytokines. These SNPs have been associated with adverse outcome and increased risk for developing cystic WMI and cerebral palsy (Harding et al., 2004; Kapitanovic Vidak et al., 2012; Resch et al., 2009). A SNP in the gene encoding for factor XIII (a gene related to blood clot formation) has been associated with decreased risk of developing severe forms of WMI after preterm birth, which might be related to reduced risk of hemorrhagic infarction (Gopel et al., 2002). Additionally, a SNP in the gene encoding cyclo-oxygenase 2 has been associated with adverse neurodevelopmental outcome at two and five years after preterm birth which might be related to impaired anti-inflammatory functioning (Harding et al., 2007). Furthermore, Boardman et al. (2014) have investigated the relationship between 13 candidate SNPs and white matter injury in 83 preterm infants. They found two SNPs in the 'armadillo repeat gene deleted in velocardiofacial syndrome' and 'fatty acid desaturase 2' genes, which were associated with white matter abnormalities as observed using diffusion tensor imaging. This study indicates that SNPs may enhance vulnerability to WMI in preterm infants, but considering the small sample size that was used, the precise link between these genes and risk of WMI should be further investigated. Taken together, this small body of research shows that genetic factors may predispose neonates to WMI and adverse cognitive outcome. It is of great importance that more research is done into the discovery of genes that facilitate vulnerability of the brain to injury associated with preterm birth. Collaboration

between medical centers may greatly benefit genetic research by enabling access to larger patient groups. Studies investigating genetic aspects of other white matter diseases may also be relevant for WMI. Genetic research may point to new genes and proteins that play a role in the etiology of WMI, which can be translated into new therapeutic strategies. Moreover, research into genetic predisposing factors may aid the development of new screening methods to characterize high-risk patients for whom early preventive treatment could be beneficial.

3.3. Lessons learned from other white matter diseases

Research on different white matter diseases like multiple sclerosis (MS), vanishing white matter disease (VWMD) or other hypomyelinating disorders can provide valuable insights into the mechanisms underlying white matter development in normal and pathological conditions (reviewed in Franklin et al., 2012; Pouwels et al., 2014). VWMD is a disease in which genetic mutation of the eukaryotic initiation factor 2 gene – a gene important in the regulation of protein synthesis – causes demyelination, resulting in both cystic and diffuse white matter pathologies without typical glial scar formation. Like WMI, VWMD is associated with impaired OL maturation (see Section 6). VWMD highlights the importance of proper regulation of protein synthesis during myelination for proper white matter development (Bugiani et al., 2013). Similarities between VWMD and WMI may prove useful for understanding the mechanisms underlying (re) myelination failure.

Like perinatal diffuse WMI, MS is an inflammatory disease of the white matter in which inflammation and oxidative stress are important mediators that cause demyelination. As in WMI, in MS patients it has been shown that newly generated OL precursor cells (OPCs) migrate towards demyelinating lesions, however they do not mature into fully developed OLs (Kuhlmann et al., 2008). Successful therapies for MS may also benefit diffuse WMI patients. For instance, the quinolizidine alkaloid *matrine* has been shown to suppress excitotoxicity, inflammation and demyelination in an animal model of MS, and may therefore also be of therapeutic potential for perinatal diffuse WMI (Kan et al., 2014). Although stimulation of myelination may be the ultimate goal in both MS and WMI, an important difference is that in MS research the focus lies on *remyelination*, i.e. remyelinating axons that were previously myelinated in an adult brain, likely in the absence of temporal cues to stimulate OL differentiation and myelination. In diffuse WMI, the aim is rather boosting proper *myelination* in white matter tracts during normal brain development (i.e. generation, rather than regeneration).

Table 2

Model	Species	Pathophysiological features	References
Maternal LPS exposure	Rats	Increased pro-inflammatory cytokines, decreased myelination (not in Rousset et al., 2013), astrogliosis, increased cell death in white and gray matter, delayed development of motor skills	Cai et al., 2000; Kumral et al., 2007; Rousset et al., 2006;Rousset et al., 2013; Tuzun et al., 2012
Systemic IL-1β exposure	Mice	Transient increase in microglial density, increased serum TNFα levels, altered white matter microstructure, decreased myelination, impaired OL maturation, impaired memory performance, no increased cell death	Favrais et al., 2011
Hyperoxia exposure	Mice, rats	Neuronal and immature OL cell death with subsequent repopulation of oligodendrotypic cells causing delayed myelination, altered GFAP expression, long-term microstructural abnormalities	Felderhoff-Mueser et al., 2004; Gerstner et al., 2008; Ritter et al., 2013; Schmitz et al., 2011; Vottier et al., 2011
Hypoxic rearing	Mice	Enlarged ventricles, neuronal damage, delayed myelination, apoptosis of immature OLs, long-term myelin abnormalities, impaired memory performance and motor skills	Back et al., 2006; Chahboune et al., 2009; Scafidi et al., 2014; Weiss et al., 2004
Maternal LPS and hyperoxic rearing	Mice	Decreased number of OLs in cortex and hippocampus, microglial activation, increased levels of apoptosis marker cleaved caspase-3	Graf et al., 2014
Postnatal LPS exposure + hyperoxia	Rats	Altered white matter microstructure, decreased myelination, increased OL death, impaired OL maturation	Brehmer et al., 2012
LPS exposure + hypoxia-ischemia at postnatal day 6	Mice	Focal necrosis, activation of microglia, astrogliosis, myelin loss	Shen et al., 2010; Shen et al., 2012
LPS exposure + hypoxia-ischemia at postnatal day 2	Rats	Decreased myelination, apoptotic premyelinating OLs, activation of microglia, blood-brain-barrier leakage	Wang et al., 2010

Neonatal rodent models aimed at inducing a subtle pattern of white matter injury, in order to study diffuse perinatal white matter injury.

4. ANIMAL MODELS OF DIFFUSE WMI

Studies on human brain tissue of diffuse WMI patients remain scarce, due to low mortality rates. Non-invasive imaging methods, identification of important risk factors and knowledge of genetic predispositions can provide clues regarding the mechanisms underlying WMI. However, in order to investigate how risk factors such as neuro-inflammation affect the cellular and molecular environment we need to rely on cell culture experiments and animal models. Therefore, most knowledge regarding the cellular pathophysiology underlying diffuse WMI that is discussed in this review has been gathered from *in vitro* and *in vivo* experimental models. To which extent such processes can be translated towards the human situation is an important issue.

Fundamental pathophysiological mechanisms underlying WMI, such as the effects of pro-inflammatory cytokines and disrupted oxygenation on glial reactivity and OL health, are often assessed *in vitro* using primary glial cell (co-)cultures. However, in order to accurately investigate the complex interplay of different organs, cell types and molecular pathways underlying diffuse WMI, it is essential to use animal models. Various rodent, ovine and primate models have been used to mimic neonatal brain injury, but most focus on severe types of injury like hypoxia-ischemia or cPVL (e.g. Drobyshevsky et al., 2007; Kannan et al., 2012; reviewed in Hagberg et al., 2002; Silbereis et al., 2010). The small number of rodent models that specifically focuses on diffuse WMI is summarized in Table 2. In the listed animal models, the types of insults used to induce diffuse WMI vary greatly: most investigators use a single inflammatory insult or a disruption of the oxygen supply to induce a diffuse pattern of WMI. The increasing use of inflammatory or infectious agents to model diffuse WMI is strongly supported by epidemiological studies that focus on the role on inflammation in WMI etiology (Goldenberg et al., 2008; Leviton et al., 2011). Furthermore, it is of particular interest that both hypoxic and hyperoxic insults are used in diffuse WMI animal models (e.g. Curristin et al., 2002; Felderhoff-Mueser et al., 2004; Gerstner et al., 2006). As discussed in Section 2.3, it might be argued that preterm infants are generally exposed to both conditions: low oxygen levels and impaired perfusion occur as a consequence of an underdeveloped and affected respiratory system and hypocarbia, however the periods of (severe) hypoxic distress are often treated with ventilation therapy causing hyperoxia. Therefore, both hypoxia and hyperoxia seem clinically relevant insults to study WMI in animal models, although it should be taken into account that underlying pathophysiology may be different.

With regard to the rodent models listed in Table 2, it is interesting to note that in most long-term studies immunohistochemical analysis of brain sections revealed that the insults lead to an initial transient decrease in myelination which returns to normal as rodents mature (Rousset et al., 2013; Schmitz et al., 2011). In contrast, using diffusion tensor imaging it has been shown that in such rodent models the white matter microstructure generally remains altered for at least several months, which translates to adulthood in humans (Chahboune et al., 2009; Favrais et al., 2011; Scafidi et al., 2014; Schmitz et al., 2011). Behavioral consequences in rodent models of diffuse WMI are mostly observed in the shape of impaired cognitive abilities, but in several models motor skills are also affected (Chahboune et al., 2009; Favrais et al., 2011; Rousset et al., 2013; Scafidi et al., 2014; Weiss et al., 2004). As the link between WMI and later psychological functioning is increasingly being recognized (Guy et al., 2015; Pyhala et al., 2014), it would be useful to expand the behavioral parameters that are widely used in animal models (i.e. motor skills and cognitive abilities) with behavioral paradigms developed to assess depression-, autism-, or schizophrenia-like behavior later in life.

Animal models that are more clinically relevant could aid our understanding of which fundamental mechanisms underlie diffuse WMI and, moreover, such *in vivo* models can be used to investigate potential treatment strategies. Considering the various aspects that play a role in WMI etiology, it will be useful to develop animal models in which multiple hits induce a subtle, yet durable pattern of injury that translates well to the human situation in terms of the type of insults, underlying pathophysiology and behavioral consequences (reviewed in Kaindl et al., 2009). By using a multiple hit model, Brehmer et al. (2012) were able to investigate differential effects of inflammation and hyperoxia on WMI, as well as the interaction between these factors. They concluded that in OLs, inflammation attenuates OL maturation, whereas hyperoxia induces OL cell death. The authors did not investigate long-term effects or behavioral consequences in their model. Besides developing more clinically relevant animal models, the use of conditional knockdown or knockout models could provide a means to investigate the importance of genes and proteins that play an important role in proper white matter development, as well as pathways that mediate the negative effects of preterm birth-related insults on OLs. When making use of neonatal rodent models, it should always be taken into account that rodents are born prematurely in terms of brain development when compared to the human situation. More specifically, rodents are born at a time that corresponds to the beginning of the third trimester in humans when looking at their neurodevelopmental stage (reviewed in Semple et al., 2013). The fact that rodents are born at such a relatively early neurodevelopmental stage, provides a convenient

time window to investigate the effects of perinatal insults on brain development at an age that translates to a gestational age of 24-32 weeks in humans – the age at which preterm infants are at high risk to develop neurological injury (Back et al., 2001). To which extent findings in rodent models can be translated towards the human situation remains an important issue. However, rodent models have provided valuable insights in how preterm birth-related insults negatively affect the ability of OLs to survive and fully mature, and are useful tools to explore novel treatment strategies.

5. THE STRICT REGULATION OF OLIGODENDROCYTE DEVELOPMENT

OLs provide neuronal axons with sheaths of myelin allowing efficient saltatory conduction of electrophysiological signals and providing axons with trophic support (reviewed in Simons and Lyons, 2013). Research in the past decades has shed light on the complex mechanisms underlying OL maturation. In the first stage of OL development, neural stem cells differentiate into OPCs (Fig. 2A and D). Under strictly timed and regulated conditions, OPCs proliferate and differentiate into pre-myelinating OLs (pre-OLs) with multiple branches (Fig. 2B and E). As these pre-OLs mature into OLs, their processes contact axons and start to produce myelin (Fig. 2C and F). Neonates born between 24 and 30 weeks of gestation are at high risk for developing WMI, since vulnerable OPCs and pre-OLs are the predominating cells of the OL lineage in the brain at this gestational age. OL maturation and myelination only start after approximately 32 weeks of gestation (Back et al., 2001). Insults that disrupt OL maturation (e.g. inflammation and disrupted oxygenation) during this critical and vulnerable period at a gestational age of 24-32 weeks will therefore have major consequences on white matter development.

Extensive analysis of post-mortem tissue of human preterm neonates revealed the absence of neuronal and cortical injury in non-cystic WMI (Back et al., 2005a; Pierson et al., 2007). Similarly, in ovine models of preterm birth no association was found between non-cystic WMI and neuronal damage or axonal degeneration (Dean et al., 2013; Riddle et al., 2012). These observations point out that impaired myelination, rather than neuronal damage, is the primary problem in diffuse non-cystic WMI. Exactly how myelination failure affects neuronal connectivity and functional outcome remains an important topic for future research.

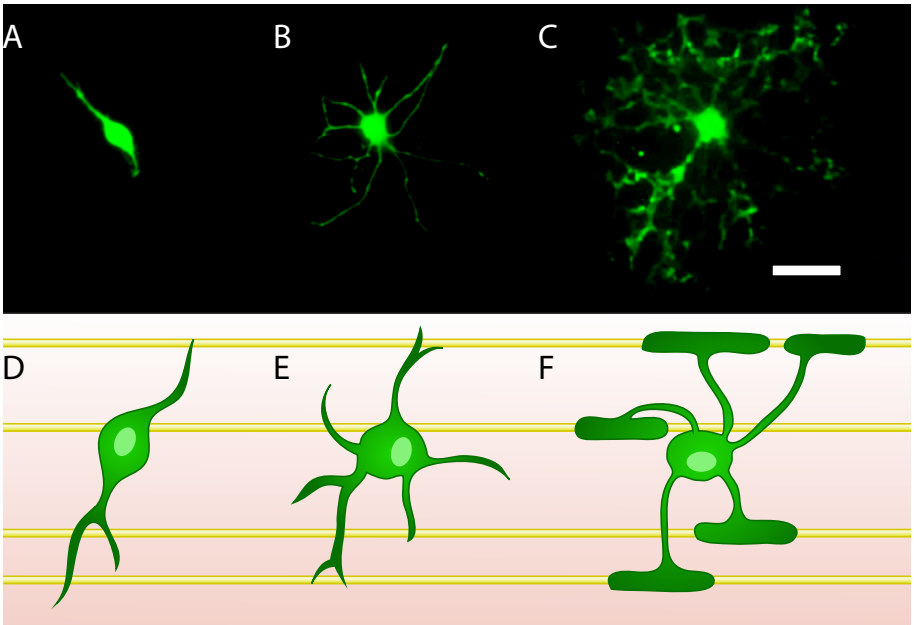

Figure 2 Different developmental stages of cultured primary oligodendrocytes (A-C) and corresponding schematic representations (D-F). A,D. Oligodendrocyte precursor cell showing a typical bi- or tripolar morphology. B,E. Pre-myelinating oligodendrocytes display a round cell body with multiple processes. C,F. Fully matured oligodendrocytes possess networks of myelinating fibers that enwrap axons (yellow) in an *in vivo* situation. Scale bar = 20 µm.

Each transition of the developing OL requires precise timing and regulation of proliferation and differentiation, which is orchestrated by a variety of growth factors and signaling pathways. Much knowledge regarding the regulation of OL development has been gathered from experimental *in vitro* studies. Extracellular signals play an important role in the regulation of OL maturation. For example, platelet-derived growth factor (PDGF) acts via the OPC-specific PDGF receptor α (PDGFRα) to promote proliferation, but at the same time PDGF inhibits differentiation of OPCs. During periods of proliferation, differentiation-stimulating transcription factors like Olig1/2 and Sox10 are inhibited by factors like Inhibitor of differentiation (ID)2, ID4 and Hes5 (for an extensive overview of OL differentiation inhibitors see Kremer et al., 2011). Hence, when differentiation is initiated, the inhibitory effects on the differentiation-promoting factors are relieved, a mechanism designated as the "derepression" model of OL differentiation (Fig. 3). Disinhibition of differentiation-inhibiting factors allows

activation of a transcriptional network, which includes transcription factors Nkx2.2, Olig1/2, Sox10 and MYRF (myelin regulatory factor), that promotes expression of genes involved in OL differentiation and myelin production (reviewed in Li et al., 2009; Emery, 2010). Disinhibition can be achieved via (epi)genetic regulators like microRNAs (miRNAs) and histone deacetylases (HDACs). miRNAs influence gene expression by post-transcriptional interference with messenger RNA. For instance, miRNA-219 and miRNA-338 promote differentiation of OPCs by suppressing the expression of differentiation-inhibiting factors like PDGFRα and Hes5 (Dugas et al., 2010; Zhao et al., 2010). Furthermore, miRNA-199a is thought to interact with transcription factor MYRF, an important transcription factor regulating the expression of myelin-associated genes (Bujalka et al., 2013; Emery et al., 2009; Letzen et al., 2010).

Chromatin remodeling is another important epigenetic process in which chromatin remodeling enzymes regulate gene expression by modifying DNA accessibility for transcription factors. For instance, HDACs negatively regulate accessibility and expression of genes by removing acetyl groups from histones and they are essential for proper OL fate determination and OPC differentiation (Conway et al., 2012). Different types of HDACs exist, organized in different classes. Most importantly, class I HDAC1 and HDAC2 regulate OL differentiation by decreasing expression of differentiation-inhibiting factors such as PDGFRα, ID2, ID4 and TCF4. Furthermore, class IV HDAC11 is involved in regulating terminal differentiation and myelination by activating myelin-associated genes (reviewed in Jacob et al., 2011). Brg1 is an additional chromatin remodeling enzyme that cooperates with Olig2 to enhance accessibility and expression of genes involved in OL differentiation, including MYRF (Yu et al., 2013). This mechanism promotes, but is not crucial for, proper OL differentiation since mice in which Brg1 was conditionally knocked out in OPCs still showed presence of mature myelinating OLs (albeit ~40% less), which illustrates the complexity of OL development (Bischof et al., 2015). In conclusion, OL maturation is tightly regulated by maintaining an accurate balance between factors that either stimulate or inhibit differentiation.

After reaching their final developmental stage, OLs contact neuronal axons and start the production of a large amount of myelin and myelin-associated proteins. OLs have been described as being among the most prolific producers of cell membrane and it is approximated that OLs can produce up to 5000 μm^2 of myelin per day (reviewed in Pfeiffer et al., 1993). The extent of myelination of individual OLs is determined during a very short critical time-window of 5-12 hours (Czopka et al., 2013; Watkins et al., 2008). As to be expected, the large amount of protein synthesis occurring during the

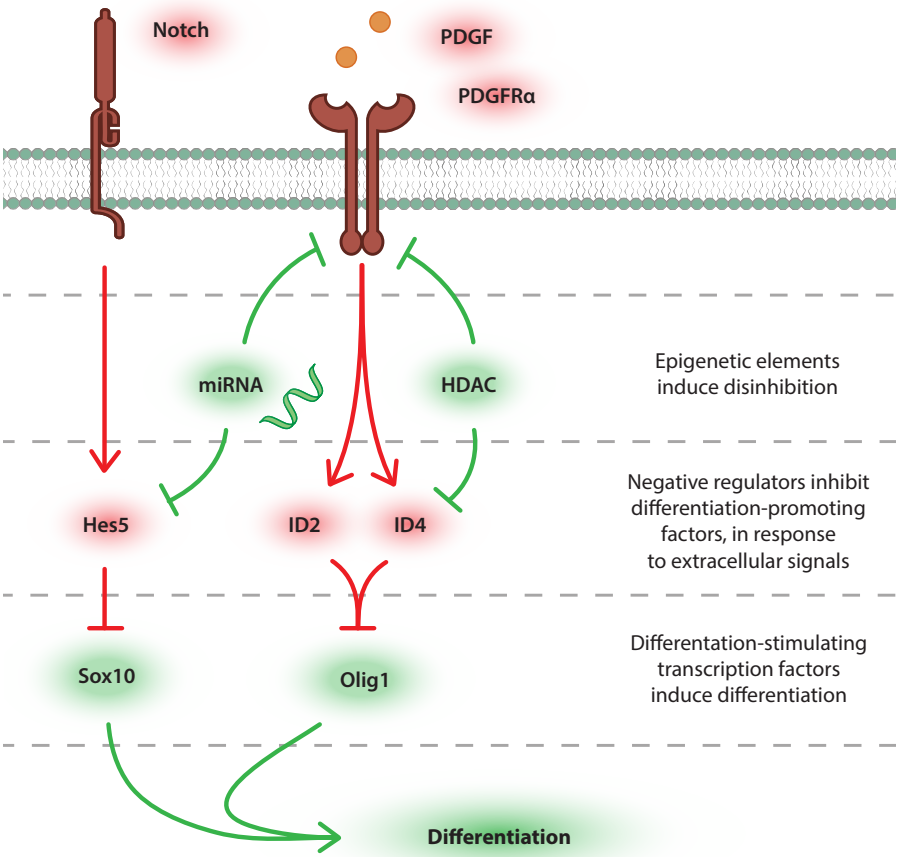

Figure 3 Simplified schematic overview of the "derepression model of OPC differentiation" with examples of differentiation-promoting signals (green) and differentiation-inhibiting signals (red) that interact to regulate OL lineage progression. In a proliferating state, mitogens inhibit differentiation-promoting signals. In a differentiating state, this inhibition is relieved by epigenetic regulators like histone deacetylases (HDACs) and microRNAs (miRNAs).

final stage of OL development is accompanied by an enormous increase in cellular metabolic activity (reviewed in Chrast et al., 2011). Mammalian target of rapamycin (mTOR) is an important regulator of protein synthesis and is therefore proposed to play a key role in determining the amount of myelin that OLs produce (Wahl et al., 2014). Furthermore, myelin associated transcription factors like MYRF are important in mature OLs to maintain proper myelination (Koenning et al., 2012). More knowledge on exactly how healthy OLs initiate, maintain and abrogate myelin production is required

to fully understand the process of myelination. It is likely that insults like inflammation, oxidative stress and/or excitotoxicity during this crucial period negatively influence the extent of myelin production, thereby drastically affecting overall myelination and white matter development.

6. ARRESTING OL MATURATION: CRUCIAL UNDERLYING PHENOMENA

Studies investigating post-mortem brain tissue of preterm neonates with WMI, demonstrate a *loss* of OLs (Haynes et al., 2003; Haynes and van Leyen, 2013; Robinson et al., 2006), while others point out that the complex regulation of OL *maturation* may be disrupted (Billiards et al., 2008; Buser et al., 2012; Verney et al., 2012). In animal models, OL loss is followed by a proliferative response generating a new pool of OPCs leading to normalization in OL numbers, but a developmental arrest causes OLs to not fully mature, resulting in impaired myelination (Rousset et al., 2006; Segovia et al., 2008). Whether a lack of fully-developed myelinating OLs results from delayed maturation of original OLs, or whether a maturational arrest occurs in a newly generated pool of OPCs is currently unknown. Multiple intra- and extracellular regulators of OL differentiation may be affected by perinatal insults like inflammation and disrupted oxygen supply in preterm neonates, thereby causing an arrest in OL maturation.

6.1. Neuroinflammation and activation of microglia

Microglia orchestrate the immune response in the central nervous system (CNS). After exposure to environmental cues, microglia proliferate and shift towards a pro- or anti-inflammatory state, adjusting their secretion profile of factors that are released into the cerebral environment. Microglia-activating signals include cytokines, pathogen-associated molecular patterns (PAMPs) from viral or bacterial origin, damage-associated molecular patterns (DAMPs) from injured cells (reviewed in Hagberg et al., 2012), but also imbalanced oxygen supply (Liu et al., 2015; Lu et al., 2006). Histological analysis of human brain tissue of preterm neonates with diffuse WMI revealed that microglia are active and maintained in a pro-inflammatory state (Buser et al., 2012; Haynes et al., 2003; Verney et al., 2012). Accordingly, high levels of pro-inflammatory cytokines like TNF-α, IL-1β, IL-2 and IL-17 and have been found in brains of preterm infants with WMI (Kadhim et al., 2002; Kadhim et al., 2001). Importantly, these cytokines have been demonstrated to exert negative effects on proliferation, differentiation and survival of immature OLs in experimental *in vivo* and *in vitro* models (Favrais et al.,

2011; Paintlia et al., 2011; Pang et al., 2010a; Steelman and Li, 2011; Taylor et al., 2010). Experimental studies further indicate that multiple complex mechanisms underlie the negative effects of pro-inflammatory cytokines on immature OLs. For instance, TNF-α can target developing OLs directly by impairing mitochondrial function and increasing vulnerability to excitotoxic injury (Aden et al., 2010; Bonora et al., 2014), or indirectly via the activation of astrocytes (see Section 6.2) (Li et al., 2008). The increased release of pro-inflammatory cytokines by activated microglia may have evolved as a protective mechanism, since microglia activation promotes the survival of *mature* OLs *in vitro* (Miller et al., 2007a,b; Taylor et al., 2010). However, in the preterm neonate mature OLs have yet to emerge from their progenitor counterparts which are, in contrast, extremely vulnerable to pro-inflammatory cytokines (Back et al., 2001; Craig et al., 2003).

In addition, microglia that display a pro-inflammatory (M1) phenotype can also negatively affect OL maturation via cytokine-independent mechanisms. For instance, *in vitro* studies indicate that activated microglia reduce the release of several trophic factors like insulin-like growth factor 1 (IGF-1) and ciliary neurotrophic factor (CNTF) which play important roles in OL development and protection, respectively (Albrecht et al., 2007; Pang et al., 2010a; Talbott et al., 2007). Furthermore, pro-inflammatory M1 microglia produce harmful free radicals, like nitric oxide (as demonstrated in human neonatal WMI brains (Haynes et al., 2003)), and increase vulnerability of the neonatal rat brain to excitotoxic injury (Rousset et al., 2008). In conclusion, the microglial response to perinatal inflammation plays a key role in impeded OL development and WMI through e.g. increased release of pro-inflammatory cytokines and free radicals, and decreased release of trophic factors.

Besides taking on a pro-inflammatory role, microglia can also acquire an anti-inflammatory (M2) phenotype which results in the production and release of anti-inflammatory cytokines. Microglia in the neonatal brain are capable of acquiring such a phenotype (P. Gressens, *unpublished data*). Miron et al. (2013) showed that M2 microglia promote OL differentiation *in vitro* and stimulate remyelination in a mouse model of MS. Furthermore, treatment with IL-10, a potent stimulator of anti-inflammatory polarization of microglia, reduces white matter damage in an animal model of cystic preterm brain injury (Chhor et al., 2013; Mesples et al., 2003). Even though the role of M2 microglia in diffuse WMI remains to be investigated, these data indicate that anti-inflammatory polarization of microglia may aid the development of OLs in the white matter.

Increasing evidence is being found indicating that microglia can be primed before being activated (reviewed in Norden et al., 2014). Microglia may be primed by (epi)genetic predisposition, inflammatory insults or stress, to exert an increased inflammatory response after a secondary insult, or "second hit". For instance, stress-induced exposure to glucocorticoids increases the microglial inflammatory response to subsequent exposure to inflammation-inducing lipopolysaccharide (LPS) in rats (Frank et al., 2010; Frank et al., 2012). Furthermore, LPS induces a behavioral pain response in rats only when preceded by a surgical intervention that primes microglia (Hains et al., 2010). Similar processes have been suggested to be involved in neurodegenerative disorders (reviewed in Perry and Holmes, 2014). Even though evidence from human brain tissue is lacking, pre-clinical studies indicate that microglia priming may contribute to diffuse WMI. For instance, neonatal mice exposed to hypoxia-ischemia on postnatal day 6 develop a diffuse pattern of WMI which is exacerbated when the insult is preceded by LPS-induced activation of the immune system (Shen et al., 2012). Also, in more severe mouse models of neonatal brain injury, pre-treatment with LPS dramatically increases injury after hypoxia-ischemia and is required for increased expression of pro-inflammatory cytokines IL-1β and IL-6 (Bonestroo et al., 2015; Wang et al., 2007; Wang et al., 2009). In preterm neonates, priming or sensitization of microglia might be the consequence of prenatal stress or maternal infections such as chorioamnionitis. Secondary hits like disturbed oxygenation, surgery or postnatal infections may eventually trigger microglia to exert an amplified inflammatory response. This idea is supported by the observation that exposure to multiple inflammatory conditions, rather than exposure to a single insult, dramatically increases the risk of WMI (Glass et al., 2008; Korzeniewski et al., 2014).

6.2. Astrogliosis

Another prominent hallmark of diffuse WMI is astrocyte reactivity or astrogliosis, as demonstrated in post-mortem brain tissue of preterm infants with WMI (Billiards et al., 2008; Buser et al., 2012; Haynes et al., 2003; Verney et al., 2012). Astrocytes are glial cells that serve many physiological functions in the CNS including provision of nutritional support, regulation of the ion balance, regulation of cerebral blood flow, and glutamate uptake. In response to microglial activation and/or hypoxia, astrocytes proliferate and demarcate lesion sites forming a so-called *glial scar*. Astrocyte reactivity may have evolved as a protective mechanism, as it modulates blood flow, potentiates reconstruction of the blood-brain-barrier (BBB), provides a physical barrier between damaged and healthy tissue, and promotes tissue repair. However, astrogliosis can also exert negative effects on developmental and regenerative properties of the CNS

by inhibiting axonal growth and remyelination. Importantly, astrocytes play an essential role in regulating OPC differentiation by releasing both differentiation-promoting, as well as differentiation-inhibiting factors (reviewed in Lundgaard et al., 2014). Several studies indicate that reactive astrocytes may benefit OL maturation. For instance, increased STAT3 activation has been observed in reactive astrocytes in human post-mortem WMI tissue and ablation of STAT3 exacerbates WMI in mice (Nobuta et al., 2012). Interestingly, microglial TGF-β release was shown to be crucial for the observed STAT3 upregulation in astrocytes (Nobuta et al., 2012). Additionally, reactive astrocytes may increase release of CXCL12, thereby protecting OL proliferation and differentiation as observed in a mouse model of MS (Patel et al., 2012). In contrast, reactive astrocytes negatively affect OL maturation and survival through release of e.g. bone morphogenetic proteins (BMPs), endothelin-1 or pro-inflammatory cytokines TNF-α and IL-1β, as indicated by experimental studies (Deng et al., 2014; Hammond et al., 2014; Su et al., 2011; Wang et al., 2011). Furthermore, astrogliosis may contribute to diffuse WMI by increasing the production of hyaluronan and adenosine, and reducing glutamate uptake as discussed below.

The glycosaminoglycan hyaluronan is a component of the extracellular matrix and has been shown to potently inhibit OL maturation in experimental settings (Back et al., 2005b; Dean et al., 2011; Sloane et al., 2010). Interestingly, hyaluronan accumulation is observed in brains of MS patients, which indicates that hyaluronan accumulation might contribute to the remyelination failure in MS (Back et al., 2005b; Sloane et al., 2010). Hyaluronan acts via its receptor CD44, but it has been shown that the negative effects of hyaluronan on OPC differentiation are also at least partly dependent on toll-like receptor 2 activation (Sloane et al., 2010). Back et al. (2005b) demonstrated that degradation of hyaluronan significantly improves OL differentiation in astrocyte-OPC co-cultures, indicating that astrocytes indeed have the capacity to negatively regulate OL maturation by production of hyaluronan. In post-mortem tissue of patients with perinatal WMI, Buser et al. (2012) demonstrated an increased expression of hyaluronan receptor CD44 on reactive astrocytes, as well as increased hyaluronan accumulation surrounding reactive astrocytes in the white matter. Collectively, these data indicate that accumulation of astrocyte-derived hyaluronan may contribute to perinatal WMI by inhibiting OL maturation.

It has been demonstrated that cultured astrocytes exposed to TNF-α increase the expression of CD73, which catalyzes the conversion of adenosine monophosphate to adenosine, thereby increasing adenosine production (Brisevac et al., 2012). Several

studies report that hypoxia may also increase astrocytic adenosine production and release *in vitro*, although this observation is still a subject of debate (Frenguelli et al., 2003; Fujita et al., 2012; Kulik et al., 2010; Lin et al., 2008; Vangeison and Rempe, 2009). On the one hand, increased adenosine levels have been shown to stimulate OL differentiation and myelination *in vitro*, possibly through activation of adenosine 2B receptors (A2BR) (Lin et al., 2008; Stevens et al., 2002). On the other hand, adenosine can have detrimental effects in the developing white matter via its other receptors A1R, A2AR and A3R, which have all found to be expressed by OPCs (Stevens et al., 2002). Importantly, immature OLs express more A1Rs compared to mature OLs *in vitro*, and in neonatal rodent models A1R stimulation induces white matter loss and mediates hypoxia-induced ventriculomegaly (Ma et al., 2011; Turner et al., 2003; Turner et al., 2002). Moreover, adenosine signaling via A2AR inhibits OPC differentiation *in vitro* and A3R signaling mediates ischemia-induced apoptosis of OLs in the rat optic nerve (Coppi et al., 2013; Gonzalez-Fernandez et al., 2014). Other *in vitro* studies indicate that A2AR activation results in increased nitric oxide release by microglia, increased neuronal glutamate release and decreased glutamate uptake by astrocytes, thereby aggravating excitotoxicity (see Section 6.3) (Matos et al., 2013; Saura et al., 2005; Sperlagh et al., 2007). Nonspecific blocking of adenosine signaling protects the neonatal brain in a murine model of hypoxia-induced WMI (Back et al., 2006). However, this treatment was not protective against excitotoxicity-induced WMI in neonatal mice (Bahi et al., 2001). Furthermore, specific blockade of A2A receptors has been shown to reduce demyelination and TNF-α levels in an animal model of spinal cord injury (Paterniti et al., 2011). In human preterm neonates, treatment with the nonspecific adenosine blocking agent *caffeine* has beneficial effects on white matter microstructure, but side-effects (including increased risk of necrotizing enterocolitis) limit its applicability (Doyle et al., 2010; Taha et al., 2014). These findings indicate that adenosine signaling plays a complex role in regulating OL development and likely contributes to diffuse WMI. Therefore, attenuating adenosine signaling by antagonizing specific purinergic receptors is an interesting therapeutic strategy to further explore, as this strategy may cause less peripheral side effects compared to nonspecific adenosine inhibitors.

6.3. Excitotoxicity

Glutamate signaling contributes to the regulation of proper OL development and myelination of axons (Fannon et al., 2015). However, excessive glutamate levels can have detrimental effects on developing OLs. As previously mentioned, an important function of astrocytes is to remove excess glutamate from the extracellular space in order to protect the brain from excitotoxicity. The developing white matter is

particularly vulnerable to excitotoxic injury, especially under pro-inflammatory conditions as demonstrated in rodents (Dommergues et al., 2000; Rousset et al., 2008). This region-specific vulnerability is caused by a variety of factors, for instance OPCs in the preterm brain express glutamatergic AMPA and NMDA receptors which are more Ca^{2+}-permeable compared to those in the adult brain, due to the composition of their receptor subunits (Deng et al., 2003; Jantzie et al., 2013b). In order to protect the vulnerable white matter from excitotoxicity, astrocytes in this region show higher numbers of glutamate transporters and the activity of glutamate transporters per synapse is higher than that of cortical areas (Goursaud et al., 2009; Hassel et al., 2003). This high rate of glutamate uptake results in more effective glutamate clearance in white matter as opposed to gray matter and consequently, the amount of glutamate present in healthy white matter is only half of that found in gray matter (Hassel et al., 2003).

Notably, several studies indicate that in the pathological situation of perinatal WMI glutamate uptake is decreased, causing increased availability of glutamate and, consequently, excitotoxic injury to the vulnerable white matter. Namely, in astrocytes of human WMI patients altered expression of glutamate transporter GLT-1 has been observed (Desilva et al., 2008). Consistently, in a neonatal mouse model of chronic hypoxia, reactive astrocytes were shown to reduce expression of glutamate transporters GLAST (glutamate aspartate transporter) and GLT-1, and in an ovine model of WMI, low blood oxygen levels were associated with relatively high extracellular glutamate levels in the cerebral white matter (Loeliger et al., 2003; Raymond et al., 2011). Furthermore, it has recently been demonstrated in a murine model of hypoxia-induced diffuse WMI that GABA-ergic signaling (which can relieve cells from excitotoxic injury) is impaired in cerebellar oligodendrocytes, causing impaired maturation and myelination (Zonouzi et al., 2015). To conclude, glutamate-induced excitoxicity is considered an important factor in the etiology of diffuse WMI and preventing excitotoxicity might prove an effective strategy to combat perinatal WMI.

6.4. Oxidative stress

Evidence of oxidative stress in immature OLs has been found in human neonatal white matter lesions (Back et al., 2005a; Haynes et al., 2003). Oxidative stress in OPCs can be caused by e.g. inflammation-induced release of nitric oxide by microglia in close vicinity of OPCs, by variations in oxygen levels, or by glutamate-dependent Ca^{2+}, which result in the formation and accumulation of detrimental reactive oxygen species (ROS) within OPCs. ROS accumulation is considered to play an important role in the

etiology of perinatal WMI (for reviews see Perrone et al., 2013; Volpe et al., 2011), as OPCs in the preterm brain are known to be specifically vulnerable to oxidative stress, due to a developmental lack of antioxidant enzymes (e.g. superoxide dismutase-1 and -2), their ability to accumulate iron for differentiation and increased expression of ROS-producing 12/15 lipoxygenase as demonstrated in human brain tissue (Folkerth et al., 2004; Haynes and van Leyen, 2013). High levels of oxidative stress are associated with apoptotic cell death, for instance in animal models of neonatal hypoxic-ischemic injury (e.g. Nijboer et al., 2013; Nijboer et al., 2011). In primary OPC cultures oxidative stress decreases the expression of important differentiation-promoting genes (Sox10, Olig1, Olig2), while it increases the expression of certain differentiation-inhibiting genes (ID2, ID4), ultimately resulting in an arrest of OL maturation, but without increased apoptotic cell death (French et al., 2009). In line with these observations, in a neonatal rat model of intrauterine growth retardation, oxidative stress was shown to inhibit OL maturation and to delay myelination by increasing expression of bone morphogenetic protein (BMP)4 (Reid et al., 2012). These findings indicate that oxidative stress most likely plays an important role in the etiology of diffuse WMI, impeding OL development and even inducing OL cell death as observed in tissue of human WMI patients (Billiards et al., 2008; Buser et al., 2012; Haynes et al., 2003; Haynes and van Leyen, 2013; Robinson et al., 2006; Verney et al., 2012).

6.5. Other micro-environmental factors

Besides factors produced by microglia and astrocytes, other micro-environmental aspects that may occur during WMI can also negatively regulate OL differentiation. For example, an acidic environment, which might be present around microscopic white matter lesions, has been shown to promote OPC adhesion and migration, but to inhibit OPC differentiation *in vitro* (Jagielska et al., 2013). This could explain findings of increased numbers of OPCs and pre-OLs in human WMI brain tissue (Billiards et al., 2008; Buser et al., 2012).

A reduction in BBB integrity might be an additional factor that contributes to diffuse WMI. Animal experiments point out that the BBB of neonatal rodents becomes leaky after e.g. hypoxic-ischemic injury (e.g. Wang et al., 2010; Wang et al., 2012). Decreased BBB integrity results in the infiltration of peripheral immune cells into the brain which can contribute to WMI by aggravating neuro-inflammation, as demonstrated in rodent models (Winerdal et al., 2012; Yang et al., 2014). Moreover, recent experimental studies indicate that OPCs actively interact with the vasculature of the BBB, and that disruption of this crosstalk may have detrimental effects on BBB integrity and white

matter development (Miyamoto et al., 2014). Human and rodent OPCs are in direct contact with pericytes within the BBB and these two cell types seem to reciprocally stimulate each other's proliferation (Maki et al., 2015a). Other experimental studies indicate that OPCs promote BBB integrity via TGF-β signaling, that normal HIF1/2 signaling in OPCs is required for proper angiogenesis within the white matter, and that OPCs promote proliferation of endothelial cells in the BBB (Seo et al., 2014; Yuen et al., 2014). These findings highlight the recent increased interest in the interactions between OLs and the BBB and future research should reveal how these interactions contribute to normal brain development and precisely how their disruption contributes to diffuse WMI.

7. SIGNALING PATHWAYS POTENTIALLY CONTRIBUTING TO ARRESTED OL DEVELOPMENT

As explained in Section 5, OL development is a complex process that requires many different cues within a narrow time window. In diffuse WMI the balance in signals that regulate proliferation, differentiation and myelination is disturbed as a consequence of preterm birth-related insults. Despite the difficulties of investigating cellular pathophysiology in human tissue, the combination of clinical knowledge on risk factors and pathophysiology of WMI, and fundamental knowledge about which signals regulate OL development *in vitro* and in animal models allows us to speculate about signaling pathways that contribute to arrested OL maturation (Fig. 4).

7.1. MAPK signaling

From *in vitro* and *in vivo* studies, we know that the mitogen activated protein kinases (MAPKs) p38, ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) play important roles in the regulation of OL development. Whereas p38 and ERK stimulate OPC proliferation and are required for differentiation (Baron et al., 2000; Chew et al., 2010; Chung et al., 2015; Dai et al., 2014; Guardiola-Diaz et al., 2012; Ishii et al., 2013), evidence from *in vitro* studies indicates that JNK activation promotes proliferation but inhibits differentiation (Chew et al., 2010; Zhang et al., 2014a).

JNK signaling can be activated in cultured developing OLs by pro-inflammatory cytokines, toll-like receptor 4 activation or excitotoxicity (Liu et al., 2002; Taylor et al., 2010). Furthermore, in both adult and neonatal animal models it has been demonstrated that ischemic insults increase JNK phosphorylation in OLs (Melani et al., 2009; Wang et al., 2012). As neuro-inflammation and oxygen imbalance pose important risk factors for

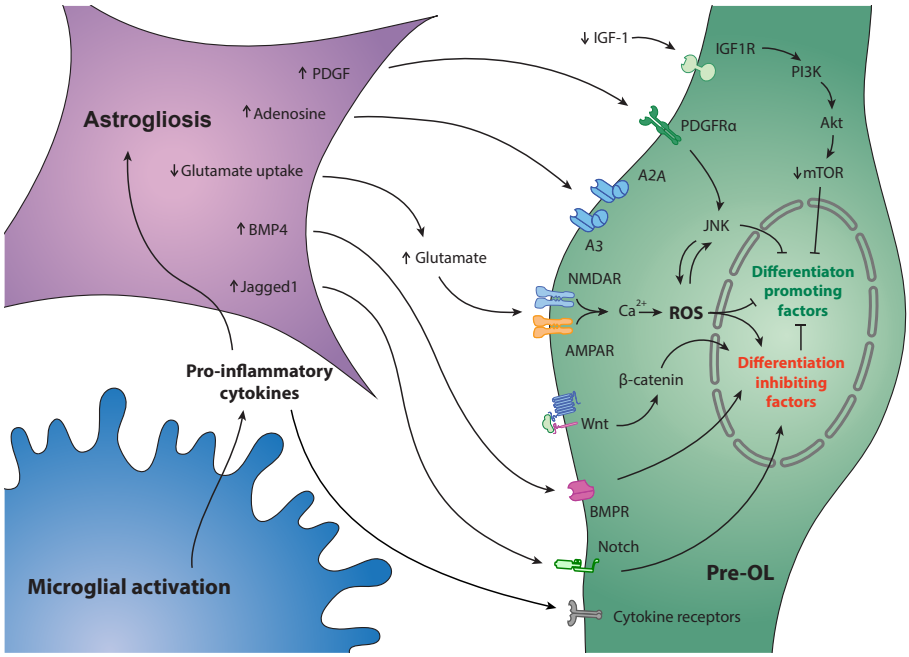

Figure 4 Proposed pathways that may underlie arrested oligodendrocyte (OL) maturation in preterm neonates, as indicated by experimental models. Preterm birth-related insults induce microglial activation and astrogliosis, setting in motion a number of changes in the environment of OL precursor cells and pre-myelinating OLs (pre-OLs) that may negatively influence their capacity to differentiate into mature OLs. Ligands bind to their corresponding receptors on pre-OLs, stimulating the downstream formation of reactive oxygen species (ROS) and activating downstream pathways that (1) stimulate the activity of transcription factors that inhibit OPC differentiation (including inhibitor of differentiation (ID)2, ID4, Tcf4 and Hes5) and (2) decrease the activity of transcription factors that promote OPC differentiation (including Olig1, Olig2 and Sox10).

diffuse WMI and are two important activators of the JNK pathway, these data suggest an important role for JNK signaling in arrested OL maturation. Besides playing a role in OLs directly, in experimental models JNK signaling is also known to mediate the pro-inflammatory response of microglia and astrocytes, thereby contributing to the production of pro-inflammatory cytokines (Gao et al., 2013; Waetzig et al., 2005; Zhang et al., 2014b). Taken together, these data indicate that inhibition of the JNK pathway may be an interesting therapeutic strategy to attenuate neuro-inflammation and to stimulate OL differentiation in the neonatal white matter. One difficulty might be that the JNK signaling pathway regulates cellular processes in many cell types. Therefore,

side-effects should be warranted and strategies to inhibit JNK in a cell-specific, timed or local manner should be further explored. Alternatively, stimulation of the ERK pathway may also prove beneficial for stimulating OL growth and development and could be achieved by e.g. IGF-1, which promotes ERK activation as demonstrated in OLs *in vitro* (Bibollet-Bahena and Almazan, 2009; Pang et al., 2007; De Paula et al., 2014).

7.2. PDGF signaling

PDGF plays an important role in regulating the developmental program of OLs (see Section 5) and acts on OPCs via its receptor PDGFRα, of which expression seizes after differentiation. *In vitro*, PDGF stimulates proliferation, whereas it potently inhibits differentiation of OPCs (Baron et al., 2000; Gard and Pfeiffer, 1993). In early stages of OL development, PDGF stimulates activation of MAPKs p38, ERK and JNK in order to promote proliferation (Baron et al., 2000; Chew et al., 2010). Considering the differentiation-stimulating properties of p38 and ERK, it is likely that the differentiation-inhibiting effects of PDGF are mediated through activation of JNK or other, yet unknown pathways (Baron et al., 2000; Chew et al., 2010; Ishii et al., 2013). Astrocytes are an important source of PDGF for OPCs, and it has been shown that astrocytes increase PDGF production and release as a response to pro-inflammatory cytokines *in vitro*, thereby inhibiting OL differentiation (Gard et al., 1995; Silberstein et al., 1996). This may be an additional mechanism through which astrogliosis contributes to impaired OL maturation, although the exact relevance of astrocyte PDGF production in diffuse WMI should be verified using animal models and human tissue.

7.3. Notch signaling

Notch signaling plays an important role in regulating the timing of OPC differentiation. Notch1 receptors are expressed by OPCs and their activation by membrane-bound ligands like Jagged1 during cell-cell contact potently inhibits OPC differentiation *in vitro* via activation of transcription factor Hes5 (John et al., 2002; Wang et al., 1998; Yuan and Yu, 2010). During development of the rat optic nerve, downregulation of the Notch ligand Jagged1 occurs in parallel with myelination, suggesting a role for decreased Notch activation as a signal for OPCs to start differentiation and myelin production (Wang et al., 1998). Accordingly, knockdown of Notch1 in mice results in premature OPC differentiation and hypermyelination (Genoud et al., 2002; Givogri et al., 2002).

It has been demonstrated *in vitro* that Jagged1 is upregulated in both astrocytes and microglia in response to pro-inflammatory stimuli (Morga et al., 2009; John et al., 2002; Cao et al., 2008). Additionally, in various cell types, including astrocytes, it has been shown that Notch signaling is increased after hypoxia (Mutoh et al., 2012; Gustafsson et al., 2005). Furthermore, in a rodent model of MS, it has been demonstrated that reactive astrocytes within demyelinated lesions show an increase in Jagged1 expression, enabling interaction with Notch1 receptors on OPCs, thereby inhibiting OL maturation and remyelination (Hammond et al., 2014). Although Notch signaling remains an understudied topic in the field of neonatal WMI, a similar process might underlie arrested OL maturation in perinatal diffuse WMI. To conclude, Notch activation in OPCs caused by hypoxia or upregulation of Jagged1 in neighboring cells may play a significant role in WMI and if this is the case, suppressing Notch signaling may be a valid treatment strategy to restore proper maturation of OLs.

7.4. Wnt/β-catenin signaling

Wnt signaling has emerged as an important regulator of OL maturation and myelination, although the precise underlying mechanisms remain poorly understood (reviewed in Guo et al., 2015). Wnt acts via proteins Axin1 and β-catenin to activate transcription factor TCF4 which promotes early differentiation of OPCs into pre-OLs. In contrast, TCF4 activation seems to inhibit subsequent maturation of preOLs, indicating a potential dual role for TCF4 in the regulation of OL development (reviewed in Guo et al., 2015). Another target of Wnt is Axin2, which provides negative feedback to the Wnt/β-catenin pathway by promoting degradation of β-catenin. Fancy et al. (2011) showed that Axin2 is expressed by OPCs in human neonatal white matter lesions, but not in age-matched controls. Pharmacological stabilization of Axin2 promotes OPC differentiation and myelination after hypoxic and demyelinating injury in mice (Fancy et al., 2011). Furthermore, a recent study by Yuen et al. (2014) indicates that the negative effects of hypoxia on the murine white matter are mediated by increased levels of Wnt subtypes Wnt7a and Wnt7b. Together, these data demonstrate that an imbalance in the Wnt/β-catenin/TCF4 pathway may be involved in the developmental arrest of OLs that is observed in premature infants with diffuse WMI. Future research should further pinpoint how Wnt signaling contributes to disrupted OL development and diffuse WMI.

7.5. BMP4 signaling

BMP4 has emerged as a negative regulator of OL maturation. At early stages of OL development, BMP4 can shift the cellular fate of OPCs towards the astroglial lineage

and at later stages BMP4 negatively regulates the expression of OL-specific myelin-associated proteins (Feigenson et al., 2011; See et al., 2004). *In vitro* experiments using primary cultures of OPCs point out that BMP4 inhibits OL maturation by (1) downregulating differentiation-promoting transcription factors Olig1 and Olig2, (2) upregulating differentiation-inhibiting factors ID2 and ID4 and (3) enhancing the availability of genes downstream of the Wnt and Notch signaling pathways by attenuating HDAC activity (Cheng et al., 2007; Samanta and Kessler, 2004; Wu et al., 2012). Increased BMP4 levels have been associated with demyelination in a murine model of MS and reactive astrocytes from rats with traumatic spinal cord injury inhibit OPC differentiation by increasing expression of BMP2 and BMP4 (Wang et al., 2011). Moreover, in a rat model of intrauterine growth retardation, oxidative stress was shown to increase extracellular BMP4 which resulted in inhibition of OPC differentiation (Reid et al., 2012). Also, overexpression of the BMP antagonist Noggin was shown to protect the white matter from perinatal hypoxia-ischemia in mice (Dizon et al., 2011). These findings indicate that in a pathological situation BMP4 levels may be increased and that BMP4 signaling negatively regulates OL development. Therefore, BMP4 may contribute to arrested OL maturation in diffuse WMI.

7.6. mTOR signaling

The mammalian target of rapamycin (mTOR) signaling pathway is generally important in the regulation of protein synthesis, cell proliferation, differentiation and survival. Several *in vitro* and *in vivo* studies have shown that mTOR signaling is involved in the late stages of OL development, regulating the transition from immature OLs to mature myelinating OLs and determining the extent of myelination (Dai et al., 2014; Guardiola-Diaz et al., 2012; Tyler et al., 2011; Wahl et al., 2014). The differentiation-stimulating role of mTOR is mediated by an upregulation of several pro-differentiation factors and a downregulation of differentiation inhibitors (Tyler et al., 2009, 2011). Generally, mTOR signaling is centered around two major complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is strongly associated with regulating protein synthesis and conditional knockdown studies in mice point out that specifically mTORC1 is a crucial regulator of myelination (Bercury et al., 2014; Lebrun-Julien et al., 2014). Upstream regulators of mTOR signaling include IGF-1, epidermal growth factor (EGF) and estrogen, which act via the PI3K/Akt/mTOR pathway.

IGF-1 promotes mTOR signaling in cultured OLs (Ye and D'Ercole, 1999; De Paula et al., 2014; Pang et al., 2007; Bibollet-Bahena and Almazan, 2009). Interestingly, IGF-1 levels decrease rapidly after birth resulting in a much earlier decrease of IGF-1 levels

in the brains of preterm infants when compared to infants born at term. Decreased levels of circulating IGF-1 in preterm infants have been associated with decreased myelinated white matter volumes and impaired mental development (Hansen-Pupp et al., 2011; Hansen-Pupp et al., 2013). Therefore, reduced IGF-1 signaling may play a role in arrested OL maturation in diffuse WMI and treatment with IGF-1 may prove beneficial for increasing myelination in WMI. Accordingly, IGF-1 stimulation can protect cultured OLs from inflammation-induced injury and promotes myelination in a mTOR-dependent manner (Ye and D'Ercole, 1999; De Paula et al., 2014; Pang et al., 2007; Bibollet-Bahena and Almazan, 2009). Furthermore, IGF-1 attenuates inflammation-induced hypomyelination in both adult mice and neonatal rats (Cai et al., 2011; Ye et al., 2007; Pang et al., 2010b). Similarly, a ligand of estrogen receptor β enhanced remyelination by stimulating PI3K/Akt/mTOR signaling in a murine model of MS, further demonstrating the beneficial effects of mTOR signaling on restoring myelination (Kumar et al., 2013). Additionally, intranasal EGF treatment effectively enhances myelination in a murine model of hypoxia-induced WMI (Scafidi et al., 2014). This effect may be mediated by mTOR signaling as in many cell types EGF stimulates the PI3K/Akt/mTOR pathway (e.g. Diaz et al., 2012), although this mechanism has not yet been demonstrated in OLs. To summarize, the mTOR signaling pathway plays a crucial role in the regulation of OL development and stimulation of this pathway (e.g. by growth factors) seems to promote myelination *in vitro* and *in vivo*, and may therefore be an effective treatment option for diffuse WMI.

7.7. Thyroid hormone signaling

Thyroid hormones are critical for normal brain development, including OL maturation as evidenced by post-mortem human and experimental studies (reviewed in Bernal, 2007). Thyroid hormones act on nuclear thyroid hormone receptors that function as ligand-dependent transcription factors. Both *in vitro* and *in vivo* evidence indicates that thyroid hormones regulate (1) axonal development, which is critical for myelination, (2) the proliferation of OPCs and their maturation into OLs, and (3) the transcription of genes associated with myelination (Baas et al., 1997; Berbel et al., 1994; Billon et al., 2002).

The maternal thyroid gland supplies thyroid hormones during maturation of the fetal thyroid gland, and the maternal/fetal supply is inversely proportional to gestational age. As such, up to 85% of preterm infants suffer from hypothyroidism that can persist for several weeks after birth and this has been related to an increased risk of WMI (Reuss et al., 1996; Simpson et al., 2005). Contradictory data are available regarding

the protective effects of thyroid supplementation on white matter integrity in preterm infants (van Wassenaer et al., 2005; Vanhole et al., 1997) and experimental models of neonatal WMI (Schang et al., 2014b; Vose et al., 2013), for commentary see Schang et al. (2014a). Overall, clinical and experimental studies highlight the importance of thyroid hormones in WMI, but a better understanding of the complex interactions between inflammation and very specific epochs of OL maturation in preterm infants is required to make therapy with thyroid hormones viable.

7.8. Epigenetic regulation

As discussed in Section 5, epigenetic factors play an important role in regulating OL lineage specification and differentiation. Several lines of experimental evidence indicate that disrupted epigenetic regulation might contribute to white matter pathologies, including perinatal WMI. miRNAs are involved in the regulation of OL differentiation as they inhibit differentiation-suppressing factors. Using transgenic mouse models, it has been demonstrated that accurate regulation of miRNAs is crucial for correct OL maturation and white matter development (Dugas et al., 2010; Shin et al., 2009). Birch et al. (2014) recently demonstrated that hypoxia-ischemia induced a large increase in the expression of miRNA-21, miRNA-138 and miRNA-338 in the white matter of neonatal mice, but only after a delay of several days post-insult. The authors suggested that delayed expression of miRNAs negatively affects OPC proliferation and differentiation and may therefore contribute to arrested OL maturation in hypoxic-ischemic WMI. This notion was supported by the observation that ablation of miRNAs in OPCs by conditional knockdown of miRNA-processing enzyme Dicer, protected the white matter after hypoxia-ischemia in mice (Birch et al., 2014). Additionally, altered expression of a wide range of miRNAs has been observed in other white matter diseases such as MS, further demonstrating the importance of proper miRNA functioning in the white matter (Lopez-Ramirez et al., 2014; reviewed in Ma et al., 2014). Taken together, these findings highlight the importance of proper miRNA signaling in normal white matter development and indicate that preterm birth-related insults may negatively affect miRNA dynamics, thereby reducing myelination and contributing to diffuse WMI in preterm infants. Future research on experimental models and human brain tissue should reveal whether this is indeed the case and which specific miRNAs are crucial to the pathophysiology underlying perinatal WMI.

HDACs are epigenetic factors that control OL development by regulating DNA accessibility through histone modification (see Section 5). Interestingly, inhibition of class I and II HDACs has been shown to have beneficial effects on the white matter in rodent

models of ischemic stroke and neonatal inflammation-sensitized hypoxia-ischemia (Fleiss et al., 2012; Kim and Chuang, 2014; Liu et al., 2012b). The neuroprotective effect of HDAC inhibition was, however, not accompanied by a reduction in gene expression of OL differentiation-inhibiting factors ID2, ID4 and Hes5 (Fleiss et al., 2012). The protective effects of HDAC inhibition may alternatively be mediated by suppression of the pro-inflammatory response of microglia, although depending on the properties of specific HDAC inhibitors microglial cytokine expression and release may also be enhanced (Faraco et al., 2009; Singh et al., 2014; Zhang et al., 2008). HDAC inhibitors seem plausible therapeutic candidates for WMI. Future research should reveal precisely which HDAC classes/types play a role in the regulation of OL development and which mechanisms underlie neuroprotective effects of HDAC inhibition. Local administration of HDAC inhibitors might be favorable in order to exclude potential adverse effects.

8. POTENTIAL TREATMENT STRATEGIES

Currently, no treatment is available to prevent or improve the outcome of preterm neonates with diffuse WMI. However, several promising therapeutic candidates have been put forward (Fig. 5). One such promising treatment is erythropoietin (EPO), which has been shown to enhance oligodendrogenesis, survival of OLs, OL maturation and myelin production *in vitro* (Jantzie et al., 2013a). Furthermore, EPO has been shown to promote oligodendrogenesis after stroke in neonatal rodents and to protect the neonatal ovine brain from inflammation-induced white matter injury (Gonzalez et al., 2013; Rees et al., 2010). Recent clinical trials indicate that EPO treatment in preterm infants (1) improves white matter development as measured by FA values, (2) decreases risk of brain injury in the gray and white matter, and (3) does not have any major adverse consequences (Fauchere et al., 2015; Leuchter et al., 2014; O'Gorman et al., 2015). Other potential treatment candidates include the growth factors EGF and IGF-1, which stimulate OL differentiation and myelination *in vitro* likely via activation of the ERK and Akt/mTOR pathways (Pang et al., 2007; Bibollet-Bahena and Almazan, 2009). Accordingly, EGF and IGF-1 have been shown to exert protective effects in animal models of WMI (Cai et al., 2011; Scafidi et al., 2014), although in mice too high dosage of IGF-1 has adverse effects when combined with an inflammatory stimulus, increasing risk of intracerebral hemorrhage and leukocyte infiltration (Pang et al., 2010b). Clinical trials with intravenous IGF-1 administration in preterm neonates with a focus on retinopathy of prematurity have shown that this treatment is safe (Ley et al., 2013). Therefore, translation towards the clinic seems feasible. Other promising

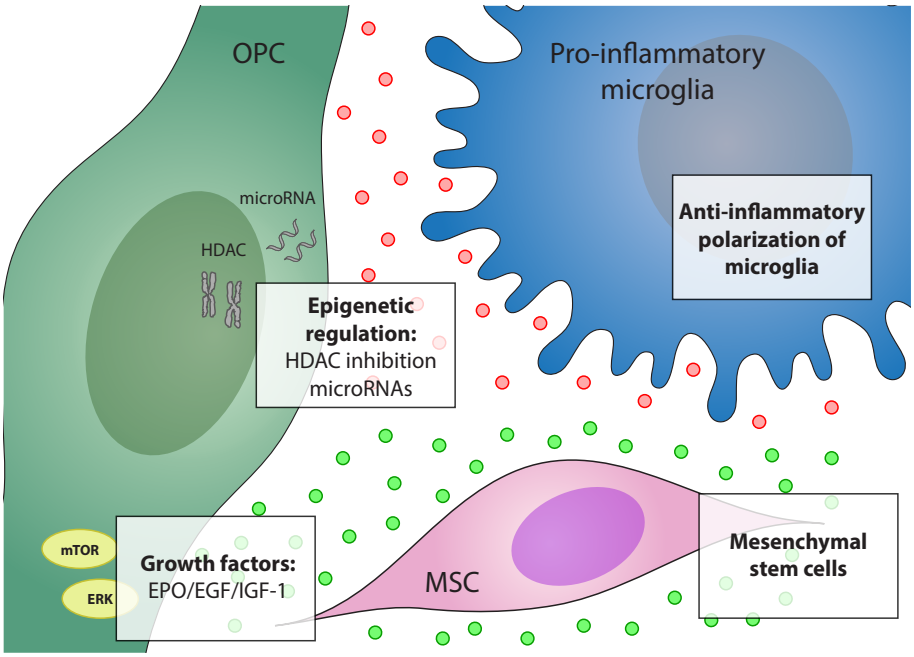

Figure 5 Summary of promising therapeutic strategies to stimulate oligodendrocyte maturation and myelination in preterm infants to combat perinatal diffuse white matter injury (WMI). Pre-clinical studies indicate that manipulation of histone deacetylases (HDACs) or microRNAs might protect the neonatal white matter from injury. Neuro-inflammation is an important risk factor for diffuse WMI and is accompanied by microglial release of pro-inflammatory cytokines (red dots) that are harmful for developing oligodendrocytes. Anti-inflammatory drugs might promote anti-inflammatory polarization of microglia, thereby decreasing release of pro-inflammatory cytokines and increasing the release of anti-inflammatory factors. Erythropoietin (EPO) has been shown to promote white matter development in preterm infants in clinical trials. Experimental evidence indicates that treatment with growth factors EGF or IGF-1 might stimulate oligodendrocyte differentiation and myelination, by activating the ERK and/or mTOR pathways. Other experimental studies demonstrated that mesenchymal stem cells produce factors (green dots) that can protect oligodendrocytes in the white matter from damage.

therapeutic candidates that have been found to protect the white matter in animal models include the adenosine antagonist caffeine and antioxidants melatonin and quercetin, which aim at counteracting oxidative stress (Back et al., 2006; Olivier et al., 2009; Qu et al., 2014).

We propose that novel treatment strategies might aim at suppressing the pro-inflammatory response in the brain, for instance by driving microglia towards an anti-

inflammatory state. Anti-inflammatory polarization of microglia might be achieved by IL-4 or IL-10 administration (Chhor et al., 2013; Mesples et al., 2003). In addition, BMP7 treatment has been shown to induce the transition of monocytes into anti-inflammatory macrophages and may have similar effects on microglia (Rocher and Singla, 2013). A different option would be to directly antagonize the function of involved pro-inflammatory cytokines like TNF-α, which has been shown to protect against excitotoxicity in the inflamed neonatal brain (Aden et al., 2010). It should be taken into consideration that anti-inflammatory treatments in preterm infants may increase their vulnerability to develop infections.

The use of mesenchymal stem cells (MSCs) provides an additional promising therapeutic strategy, as these cells have been shown to secrete factors that promote OL lineage specification, OL maturation and myelination *in vitro* (Jadasz et al., 2013). Research of our group has pointed out that intranasal administration of MSCs can protect both the gray and white matter from hypoxia-ischemia-induced injury in neonatal mice (Donega et al., 2013). Intranasally administered MSCs migrate towards the lesion site, but rather than homing and becoming a source of newly generated cells, the MSCs act as vehicles that secrete important growth factors, anti-inflammatory cytokines and chemokines to stimulate tissue repair and (re)myelination (Donega et al., 2014). Whether MSCs have beneficial effects in diffuse WMI, where apparent lesions are absent, remains to be investigated. In the future, stem cells may even be specifically designed to increase expression of inflammation-suppressing proteins and/or factors that promote OL maturation and myelination.

Alternatively, pharmacological intervention could target relevant signaling pathways (e.g. the Wnt pathway; Fancy et al., 2011) or epigenetic regulation (e.g. HDAC inhibition; Fleiss et al., 2012) in order to promote OL maturation and/or to stimulate myelination. Interference with the (epi)genetic machinery of OLs might prove an effective method to stimulate OL development and myelination. Dendrimers are nanoparticles designed to allow safe and controlled release of e.g. drugs or non-viral vectors into organs, including the brain. Kannan et al. (2012) demonstrated that treatment with dendrimers conjugated with the drug N-acetyl cysteine attenuated neuroinflammation and improved myelination in a neonatal rabbit model of cystic WMI. Furthermore, Nance et al. (2015) showed that treatment with N-acetyl cysteine conjugated dendrimers reduced WMI in a neonatal mouse model by attenuating the pro-inflammatory response, while retaining proper anti-inflammatory functioning of microglia. In the future, dendrimers may be used for gene delivery into the brain or to transfect human MSCs for stem

cell treatment with genes of e.g. growth factors (Kim et al., 2015; Zarebkohan et al., 2015). However, considering the current status of gene therapy in the central nervous system such treatments remain far from being applicable in neonatal neuropathies at present. The signals regulating OL proliferation and differentiation are mostly not OL-specific, which should be taken into consideration when targeting such general pathways as a potential treatment strategy because side effects might occur when cell-specific targeting is not possible. Ideally, novel treatments for diffuse WMI are administered intranasally or systemically as these routes of administration are most relevant in the preterm neonate, with intranasal administration having the advantage of applying treatments more locally which might circumvent adverse effects of drugs on peripheral organs.

9. CONCLUDING REMARKS

To summarize, diffuse WMI in preterm neonates is a highly prevalent, complex and multi-factorial type of brain injury, in which different inter- and intracellular signaling cascades are involved. Disruption of the OL developmental program and/or OL cell death appear to be key mechanisms in the etiology of diffuse WMI. Phenomena like inflammation and disrupted oxygen supply affect the cerebral environment in a way that is unfavorable for immature OLs to survive and fully mature, ultimately resulting in decreased myelination. Important cellular mediators in this process are pro-inflammatory microglia and reactive astrocytes, which lead to harmful release of cytokines, excitotoxicity and oxidative stress. We argue that different components of WMI should be more clearly defined, so different underlying mechanisms can be studied separately. Most of our current knowledge regarding mechanisms underlying perinatal WMI is derived from experimental research, which complicates rapid development of novel treatment strategies. Future research should elucidate exactly which of the reviewed pathways are most relevant in human preterm infants. Conditional knock-out animal models provide a means of revealing cell-specific targets that are directly or indirectly involved in arresting OL maturation in the context of diffuse WMI. Such knowledge can be used to establish feasible treatment options that should be investigated using clinically relevant "multiple-hit" animal models before being translated towards the patient. Clinical trials indicate that EPO- and IGF-1 treatment are both safe and may have beneficial effects on white matter development in preterm infants (Fauchere et al., 2015; Ley et al., 2013; O'Gorman et al., 2015). Pre-clinical studies highlight several other feasible therapeutic strategies, the most promising

being stimulation of the Akt/mTOR or ERK signaling pathways by EGF treatment, suppression of neuro-inflammation, and stem cell therapy (Fig. 5) (Aden et al., 2010; Donega et al., 2013; Scafidi et al., 2014). The development of new treatment options is urgently needed to combat diffuse WMI to improve both neurological prospects and quality-of-life of preterm neonates.

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3

CHAPTER 3

Origin and dynamics of oligodendrocytes in the developing brain: implications for perinatal white matter injury

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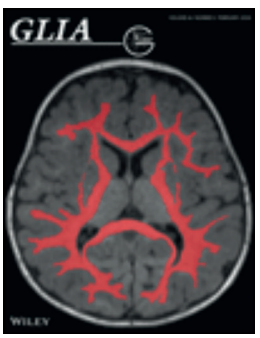

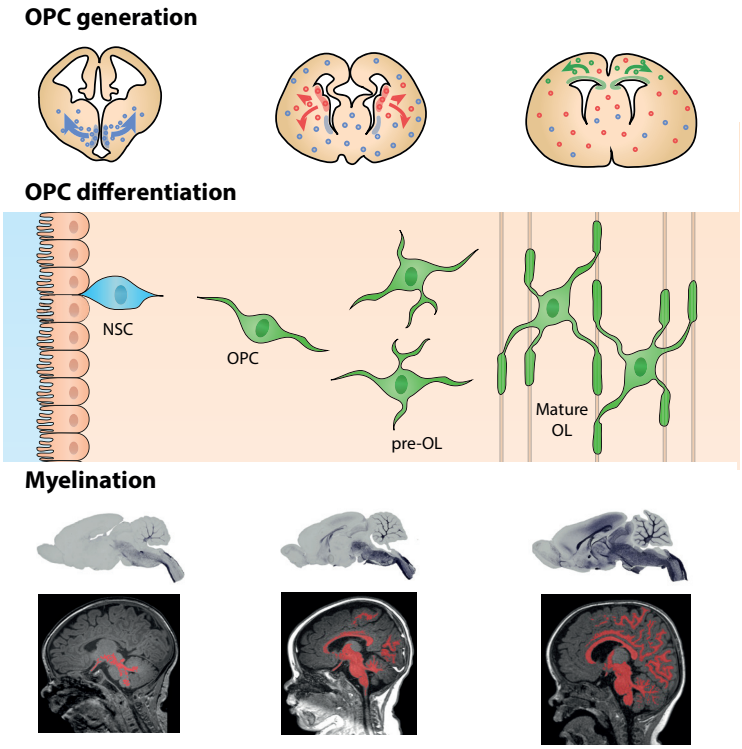

MAIN POINTS:

- Preterm infants are at risk to develop perinatal white matter injury.
- OPCs are generated in multiple waves and form a heterogeneous population throughout the brain.
- Myelination patterns in the rodent and human brain are strikingly similar.

ABSTRACT

Infants born prematurely are at high risk to develop white matter injury (WMI), due to exposure to hypoxic and/or inflammatory insults. Such perinatal insults negatively impact the maturation of oligodendrocytes (OLs), thereby causing deficits in myelination. In order to elucidate the precise pathophysiology underlying perinatal WMI, it is essential to fully understand the cellular mechanisms contributing to healthy/normal white matter development. OLs are responsible for myelination of axons. During brain development OLs are generally derived from neuro-epithelial zones, where neural stem cells committed to the OL lineage differentiate into OL precursor cells (OPCs). OPCs, in turn, develop into premyelinating OLs and finally mature into myelinating OLs. Recent studies revealed that OPCs develop in multiple waves and form potentially heterogeneous populations. Furthermore, it has been shown that myelination is a dynamic and plastic process with an excess of OPCs being generated and then abolished if not integrated into neural circuits. Myelination patterns between rodents and humans show high spatial and temporal similarity. Therefore, experimental studies on OL biology may provide novel insights into the pathophysiology of WMI in the preterm infant and offers new perspectives on potential treatments for these patients.

Keywords
Oligodendrocyte precursor cells, white matter injury, myelination, brain development, preterm birth

TABLE OF CONTENTS

1. WHITE MATTER INJURY IN PRETERM INFANTS

Approximately 10% of all births occur prematurely, i.e. before 37 weeks of gestation (Liu et al., 2012). Preterm infants spend the first weeks of life under suboptimal extra-uterine conditions, during which they often encounter inflammatory and hypoxic insults due to immature organs, vascularization and immune system (Volpe, Kinney, Jensen, & Rosenberg, 2011). Such insults can have severe consequences on brain development, leading to neurological problems later in life (Ancel et al., 2015; Stoll et al., 2015). The white matter in preterm infants is particularly vulnerable to injury due to crucial processes in white matter development taking place during the third trimester of pregnancy (Salmaso, Jablonska, Scafidi, Vaccarino, & Gallo, 2014; Volpe, 2009). Consequently, at present the most commonly observed type of brain injury in preterm infants is white matter injury (WMI) (Back & Miller, 2014).

Over the past decades, the clinical presentation of perinatal WMI has changed. In the 1980s, relatively large cystic lesions in the white matter were observed in 5-10% of extremely preterm infants (born before a gestational age of 28 weeks) and this type of WMI was referred to as cystic periventricular leukomalacia (cPVL). cPVL is defined as localized necrosis forming cystic lesions in the deep periventricular white matter that are well visualized by cranial ultrasound, as well as by MRI (Khwaja & Volpe, 2008) (Figure 1). The cysts resolve over weeks to months, causing ex-vacuo dilatation and periventricular glial scar formation as observed on MRI scans. cPVL is almost invariably related to severe impairment of neurological functioning, including serious disabilities such as cerebral palsy. Over the past decades, cPVL has become less common and the incidence has decreased to below 1% in some centers (Gano et al., 2015; Hamrick et al., 2004; van Haastert et al., 2011). Presently, the term 'WMI' is increasingly being used and rarely refers to cystic WMI, but rather refers to *diffuse* WMI (Back, 2006; Woodward, Anderson, Austin, Howard, & Inder, 2006). In diffuse WMI instead of macroscopic cysts, microscopic cysts (not detected by ultrasound or MRI) develop and evolve into glial scars with marked astrogliosis and microgliosis over the course of several weeks (Volpe, 2009). Furthermore, diffuse WMI is associated with reduced total white matter volumes, thinning of important white matter tracts, ventriculomegaly and altered white matter microstructure as measured by diffusion tensor imaging (Alexandrou et al., 2014; Glass et al., 2008; Mwaniki, Atieno, Lawn, & Newton, 2012; Rutherford et al., 2010; Shankaran et al., 2006; van Vliet, de Kieviet, Oosterlaan, & van Elburg, 2013). Besides atrophy of white matter tracts and diffuse changes in signal intensity in the white matter on MRI, punctate white matter lesions (PWML) are observed in the periventricular white

matter in as many as 20% of extremely preterm infants (Figure 1). PWML appear as focal signal intensity changes on conventional MRI and as restricted diffusion when the MRI is performed within a week after the presumed insult suggestive of ischemic injury (Kersbergen et al., 2014). Compared to cPVL, diffuse WMI and PWML are related to a milder degree of neurological impairment affecting mostly cognitive functioning and increased risk of psychological disorders later in life (Back & Miller, 2014; T. Guo et al., 2017; Keunen et al., 2017; Woodward, Clark, Bora, & Inder, 2012).

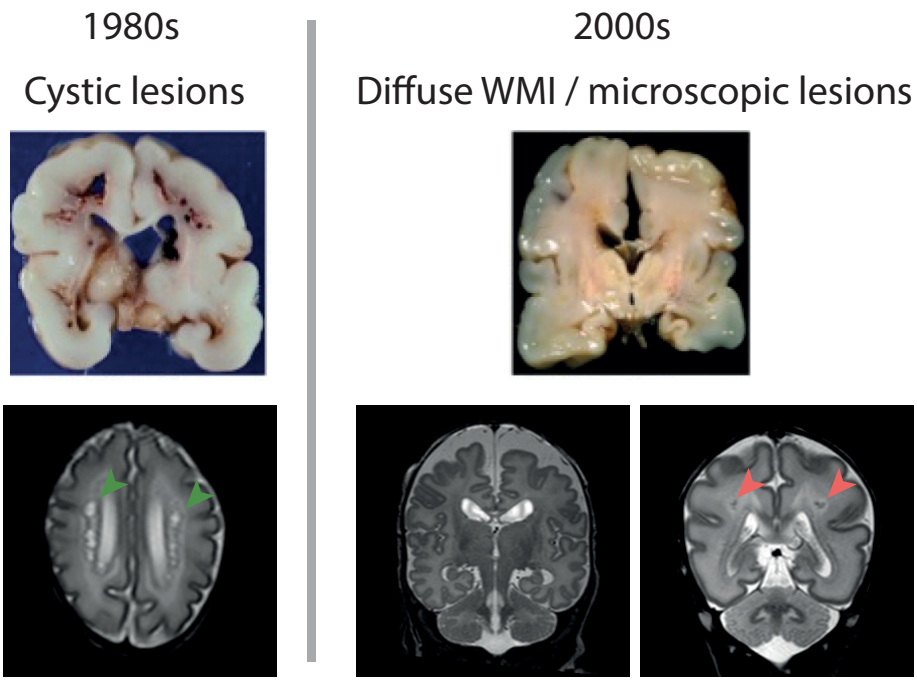

Figure 1 The clinical problem of perinatal white matter injury (WMI) has evolved over time. Upper panels: photographs of post-mortem brain slices of preterm infants with WMI (published with permission from http://neuropathology-web.org/). Lower panels: T2-weighted MR images of preterm infants with WMI. Left part of the figure: in the 1980s, cystic periventricular leukomalacia (cPVL) was often observed in preterm infants. cPVL is associated with large cystic lesions in the white matter clearly present at macroscopic post-mortem tissue and at (transverse) MRI scan (T2 sequence) as indicated by the green arrowheads. cPVL leads to severe disabilities like cerebral palsy. Right part of the figure: at present, diffuse WMI is most often associated with atrophy of white matter causing loss of brain volume (middle (coronal) MRI scan) and punctate white matter lesions (PWML) (right (coronal) MRI scan: red arrowheads). Diffuse types of WMI are mainly associated with impaired cognitive functioning later in life.

Based on pathology studies using human post-mortem brain tissue, the stressful environment that preterm infants reside in during the first weeks of life is thought to negatively affect the developmental programming of oligodendrocytes (OLs), the cells responsible for myelination (Billiards et al., 2008; Buser et al., 2012; Verney et al., 2012). An arrest in OL maturation due to perinatal insults causes myelination deficits associated with perinatal WMI (Volpe et al., 2011). In order to elucidate the precise pathophysiology underlying perinatal WMI, it is crucial to fully understand the mechanisms through which OLs develop and achieve myelination during normal white matter development. In the next sections, we will describe fundamental aspects contributing to white matter development starting with the generation of oligodendrocyte precursor cells (OPCs), followed by the temporal and spatial patterns through which these cells populate and eventually myelinate the central nervous system (CNS). Furthermore, heterogeneity within the OL lineage is discussed. Finally, we translate these findings to the human situation by discussing similarities and differences between myelination patterns in rodents versus humans and we highlight possible implications for WMI in preterm infants.

2. OPC GENERATION

The white matter contains myelinated axons that allow communication between distant brain regions. Myelin sheaths surrounding these axons are essential for proper brain connectivity, as myelination enables rapid and efficient propagation of action potentials, and provides protection and trophic support to axons (Funfschilling et al., 2012; Saab et al., 2016). In the central nervous system (CNS), oligodendrocytes (OLs) are responsible for the production of myelin. Since first being described by Pío del Río Hortega in 1921, much research has been done into OL biology and the developmental regulation and functions of these cells (Bergles & Richardson, 2015; Perez-Cerda, Sanchez-Gomez, & Matute, 2015). OLs originate from neural stem cell (NSC)-derived oligodendrocyte precursor cells (OPCs) that differentiate into immature premyelinating OLs (pre-OLs) and finally differentiate into mature OLs that contact neuronal axons and start the production of myelin (Figure 2) (Emery, 2010).

Throughout the CNS, OPCs generally originate from neuroepithelial zones surrounding the ventricles. Here, proliferative NSCs commit to the OL lineage under the influence of transcription factors such as Olig1, Olig2, Nkx2.2 and Sox10 (Figure 2) (Emery, 2010). During development, OPCs can also derive from radial glial cells (Casper & McCarthy,

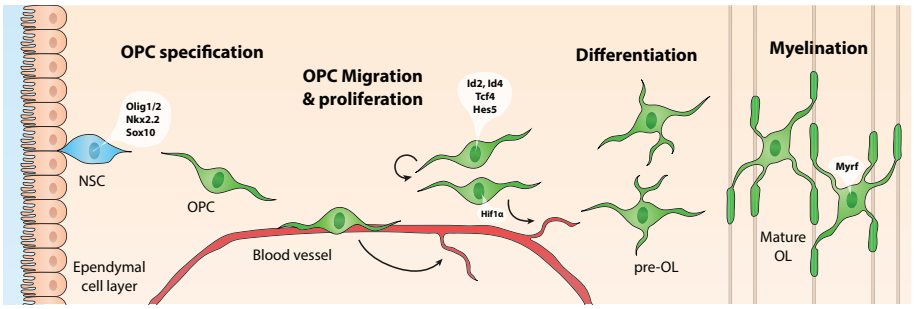

Figure 2 Schematic representation of oligodendrocyte (OL) development and transcription factors that contribute to OL lineage progression at different developmental stages. OL precursor cells (OPCs) originate from neuroepithelial zones surrounding the ventricles, where neural stem cells (NSCs) differentiate into (OPCs) under the influence of OL-specific transcription factors Olig1/2, Nkx2.2 and Sox10. OPCs migrate towards an appropriate site via blood vessels, while at the same time promoting angiogenesis in a HIF1α-dependent manner, in areas requiring more oxygen. At their final destination OPCs proliferate to expand the pool of OPCs, under the regulation of transcription factors such as Id2, Id4, Tcf4 and Hes5. When proliferation is inhibited, OLs differentiate into pre-myelinating OLs (pre-OLs), and finally into mature OLs that enwrap neuronal axons with myelin sheaths, under the influence of e.g. Myrf.

2006; Merkle, Tramontin, Garcia-Verdugo, & Alvarez-Buylla, 2004). The origin and dispersion of OPCs have been extensively studied in the rodent CNS, particularly in the forebrain, the cerebellum and the spinal cord. Interestingly, OPCs are generated in multiple waves, starting with a ventral wave, which shifts towards a more dorsal origin during the second wave (Figure 3) (Fancy et al., 2009; Kessaris et al., 2006).

There are also spatial differences in OPC development. In the murine *forebrain* OPCs originate from the subventricular zone (SVZ) in a ventral wave from the medial ganglionic eminence and the anterior entopeduncular area at embryonic day E12.5 (Figure 3, left panel, blue cells). Gradually, the stream of OPC generation moves dorsally with OPCs being produced from the lateral and caudal ganglionic eminences by the age of E15.5 (Figure 3, left panel, red cells). At birth (E21/P0) a wave of OPCs arises from the dorsal SVZ into the cortex (Kessaris et al., 2006; Rowitch & Kriegstein, 2010) (Figure 3, left panel, green cells). Remarkably, tracing studies revealed that OPCs derived from the first ventral wave are almost completely replaced postnatally, and that the complete population of OPCs in the forebrain at postnatal day P10 is derived from the medial and dorsal streams (Kessaris et al., 2006). However, when generation

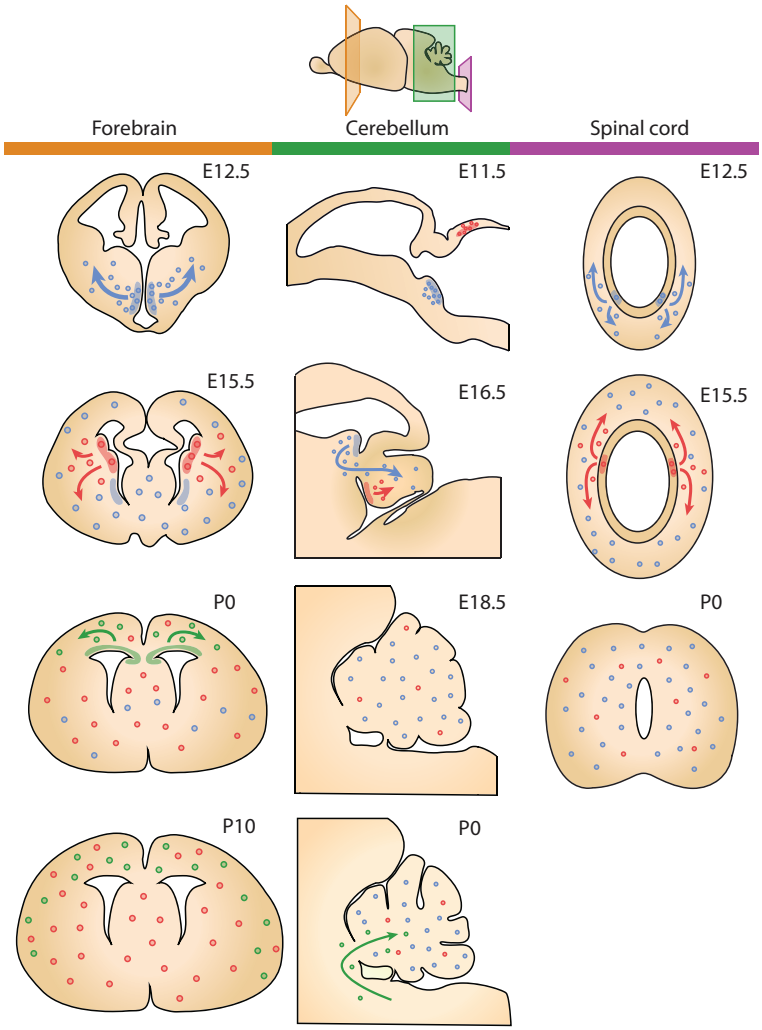

Figure 3 Schematic representation of how different waves of OPC generation populate different regions of the CNS throughout development (panels: left/orange: forebrain; middle/green: cerebellum; right/purple: spinal cord). OPCs originating from different niches are represented by differently colored dots (blue, red, green) (based on data by Fogarty, Richardson, & Kessaris, 2005; Grimaldi, Parras, Guillemot, Rossi, & Wassef, 2009; Hashimoto et al., 2016; Kessaris et al., 2006; Ravanelli & Appel, 2015; Vallstedt, Klos, & Ericson, 2005).

of OPCs during the second or third wave is disturbed, OPCs from the ventral wave do survive and differentiate to eventually myelinate the forebrain (Kessaris et al., 2006). These findings indicate that a competitive mechanism exists, creating a flexible system with the first, ventral wave acting as 'backup' in case later waves are impaired. At P10, the OPCs derived from the medial wave evenly populate the entire forebrain, whereas the destination of OPCs from the dorsal stream is restricted to cortical areas (Kessaris et al., 2006).

Cerebellar OPCs have been shown to mostly originate from extracerebellar brain regions (Grimaldi, Parras, Guillemot, Rossi, & Wassef, 2009). More specifically, at E11.5 OPCs start to arise from the metencephalic ventral rhombomere 1 region and migrate towards the cerebellum where the first OPCs arrive at E16.5 (Figure 3, middle panel, blue cells). At E18.5 a large population of OPCs has reached the cerebellum, where these cells likely proliferate to further expand the cerebellar population of OPCs (Hashimoto et al., 2016). A second stream of OPCs is generated locally from the cerebellar ventricular zone (Figure 3, middle panel, red cells), but these cells comprise only 6% of the cerebellar OPC population at E18.5 (Hashimoto et al., 2016). After birth, neuroepithelial regions surrounding the fourth ventricle likely continue to produce OPCs that migrate towards the cerebellum (Reynolds & Wilkin, 1988; L. Zhang & Goldman, 1996) (Figure 3, middle panel, green cells).

In the *spinal cord*, the first OPCs arise from the ventrally located pre-motor neuron (pMN) domain, which starts to generate OPCs after E12.5 (Ravanelli & Appel, 2015; Richardson et al., 2000) (Figure 3, right panel, blue cells). At E15.5, a later wave of OPCs is generated from more dorsal precursor domains of the spinal cord (Fogarty, Richardson, & Kessaris, 2005; Vallstedt, Klos, & Ericson, 2005) (Figure 3, right panel, red cells). Spinal cord OPCs generated during the first ventral wave rapidly populate the whole plane of the spinal cord. OPCs that originate from the dorsal wave at E15.5 also distribute evenly across the spinal cord, but by the time of birth dorsally derived OPCs make up only 10-20% of the OPCs and the majority of OPCs being derived from the ventral stream (Fogarty, Richardson, & Kessaris, 2005; Vallstedt, Klos, & Ericson, 2005).

The origin of OPCs in deep brain structures has not been studied extensively. Likely, OPCs in these regions are similarly derived from neuroepithelial zones surrounding the ventricles, from where they migrate towards the appropriate location and proliferate to expand the OPC population. For example, in the hypothalamus OPCs are derived from a neuroepithelial niche surrounding the third ventricle from E13.5 onwards with a peak at E17.5 (Marsters et al., 2016). Also, it has recently been demonstrated that OPCs generated at E12.5 in the preoptic area gradually migrate via the optic chiasm to eventually populate and myelinate the optic nerve (Ono et al., 2017).

3. OPC MIGRATION, PROLIFERATION AND DIFFERENTIATION

3.1 OPC migration

OPCs contain growth-cone like structures that sense numerous chemotactic cues to guide them towards their destination (Simpson & Armstrong, 1999). A wide variety of signaling molecules have been implicated in regulating OPC migration. For instance, spatial gradients of bone morphogenic proteins (BMPs), Sonic hedgehog (Shh) and Wnt proteins determine the direction of migrating OPCs. For example, dorsally secreted BMPs repel ventrally derived OPCs, thereby guiding them towards more ventral brain areas (Choe, Huynh, & Pleasure, 2014). In addition, different types of local cues, e.g. growth factors, extracellular matrix proteins, axon guidance molecules and neuronal activity can affect OPC migration as well (de Castro & Bribian, 2005; de Castro, Bribian, & Ortega, 2013). Examples of growth factors that promote OPC migration include platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) (Bribian, Barallobre, Soussi-Yanicostas, & de Castro, 2006; Hayakawa et al., 2011; Hayakawa et al., 2012; Milner et al., 1997; Murcia-Belmonte, Medina-Rodriguez, Bribian, de Castro, & Esteban, 2014; Yan & Rivkees, 2002). Furthermore, extracellular matrix components like laminin, fibronectin, vitronectin, anosmin-1 and tenascin-C have been shown to stimulate migration of OPCs (Bribian et al., 2008; Garcion, Faissner, & ffrench-Constant, 2001; Milner, Edwards, Streuli, & Ffrench-Constant, 1996; Murcia-Belmonte et al., 2016). Moreover, various factors associated with axon guidance, e.g. neural cell adhesion molecules, semaphorins, netrin-1 and the chemokine CXCL1, guide migrating OPCs by attraction or repulsion (Okada, Tominaga, Horiuchi, & Tomooka, 2007; Spassky et al., 2002; Tsai et al., 2002; H. Zhang, Vutskits, Calaora, Durbec, & Kiss, 2004). Interestingly, it has been shown that neural activation mediates OPC migration (Mangin, Li, Scafidi, & Gallo, 2012). By acting on AMPA receptors glutamate enables the formation of an AMPA/α_v integrin/proteolipid protein complex, which promotes motility. In addition, glutamate promotes OPC migration via NMDA receptors by stimulating expression of the polysialic acid-neural cell adhesion molecule and by activating the Tiam1/Rac1/ERK signaling pathway (Gallo et al., 1996; Gudz, Komuro, & Macklin, 2006; C. Wang et al., 1996; Xiao et al., 2013; Yuan, Eisen, McBain, & Gallo, 1998).

In addition to local and neuronal cues, Tsai et al. (2016) recently demonstrated that proper brain vascularization is crucial for OPC migration. More specifically, it was shown that OPCs migrate by 'crawling' along blood vessels (Figure 2), and that OPCs can also 'jump' from one blood vessel to another (Tsai et al., 2016). Wnt-mediated expression

of the chemokine receptor CXCR4 on OPCs enables the coupling to blood vessels expressing the CXCR4 ligand SDF1 (CXCL12). Interestingly, increased Wnt signaling caused clustering of OPCs surrounding the vasculature (Tsai et al., 2016). Earlier reports demonstrated that OPCs can promote angiogenesis by monitoring oxygen tension through hypoxia-inducible factor (HIF) signaling and by secreting Wnt7a/b in response to low oxygen levels, thereby promoting angiogenesis. Presumably, by doing this, OPCs ensure oxygen supply during myelination which requires high oxygen consumption (Yuen et al., 2014). Additionally, active interactions between pericytes and OPCs have been implicated in maintaining blood-brain barrier integrity (Maki et al., 2015; Seo et al., 2014). Collectively, these data highlight the importance of reciprocal interactions between OPCs and the vasculature (Figure 2).

3.2 Proliferation and differentiation

Once OPCs reach their destination, the population is expanded by proliferation. OPCs are highly proliferative cells that continue to divide until a homeostatic balance in the number of OPCs is achieved (Hughes, Kang, Fukaya, & Bergles, 2013). If this balance is disturbed due to cell death, differentiation or migration, local OPCs are triggered to proliferate in order to maintain a consistent pool of OPCs (Hughes, Kang, Fukaya, & Bergles, 2013). During development, OPC expansion is actively stimulated by extracellular signals that promote proliferation, but at the same time inhibit differentiation (for a list of regulatory pathways, see Table 1). One important signal is PDGF, which has been shown to potently drive OPC proliferation (Calver et al., 1998; Richardson, Pringle, Mosley, Westermark, & Dubois-Dalcq, 1988). Other examples of signals that inhibit OPC differentiation include Notch and Wnt (Fancy et al., 2011; Genoud et al., 2002; Givogri et al., 2002; F. Guo et al., 2015; Kremer, Aktas, Hartung, & Kury, 2011; S. Wang et al., 1998). Downstream of these extracellular signaling molecules, transcription factors such as inhibitor of differentiation (Id)2, Id4 and Hes5 are responsible for preventing premature differentiation of OPCs. Downregulation of extracellular differentiation inhibitors during development relieves the "inhibition to differentiate", for instance by promoting expression of microRNAs that prevent transcription of differentiation inhibitors (Dugas et al., 2010; Zhao et al., 2010). The disinhibition allows OPCs to differentiate into pre-OLs and finally into mature, myelinating OLs under the influence of differentiation *promoting* transcription factors like myelin regulatory factor (Myrf) (Bujalka et al., 2013). As OLs mature, they contact neuronal axons and begin the expression of a large amount of myelin genes, including myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and myelin proteolipid protein (PLP), to start the assembly

Table 1

Pathway	Promote/inhibit OPC differentiation	References	Potential clinical intervention
BMP4 signaling	Inhibitor	Dizon et al., 2011; Reid et al., 2012; See et al., 2004	BMP4 inhibition; noggin
Endothelin 2	Promotor	Yuen et al., 2013	Endothelin receptor agonists
GABAergic signaling	Promotor (conflicting data)	Hamilton et al., 2017; Zonouzi et al., 2015	Anti-epileptic drugs, e.g. tiagabine, vigabatrin
Hyaluronan/CD44 signaling	Inhibitor	Back et al., 2005; Buser et al., 2012; Hagen et al., 2014	CD44 inhibition; hyaluronidase
IGF1 signaling	Promotor	Cai et al., 2011; Pang et al., 2007; Pang et al., 2010a; Ye et al. 2007	IGF1 administration; cell based therapy
JAK/STAT signaling	Inhibitor	Raymond et al., 2011	JAK/STAT inhibition
JNK signaling	Inhibitor	Wang et al., 2012; Wang et al., 2014	JNK inhibition
Muscarinic acetylcholine signaling	Inhibitor	Deshmukh et al., 2013; Franklin 2015; Mei et al., 2014	Muscarinic acetylcholine inhibition; benztropine; clemastine
Notch signaling	Inhibitor	Scafidi et al., 2014; Wang et al., 1998	Notch inhibition; EGF
PDGF signaling	Inhibitor	Calver et al., 1998; Richardson et al., 1988	PDGF inhibition
Potassium signaling	Promotor	Fogal, McClaskey, Yan, Yan, & Rivkees, 2010; Zhu, Wendler, Shi, & Rivkees, 2014	K_{ATP} agonists; diazoxide
Pro-inflammatory cytokines	Inhibitor	Favrais et al., 2011; Miron et al., 2013; Pang et al., 2010b; Taylor et al., 2010	Anti-inflammatory treatments; activin A
Prostaglandin E2	Inhibitor	Gano et al., 2015; Shiow et al., 2017	Cyclooxygenase inhibition; indomethacin
Retinoid X receptor γ (RXR-γ)	Promotor	Franklin 2015; Huang et al., 2011	RXR-γ agonists; 9-cis-retinoic acid
Sirt1	Inhibitor	Jablonska et al., 2016	Class III HDAC inhibitors; sirtinol
Wnt/β-catenin signaling	Inhibitor	Fancy et al., 2009, 2011; Lee et al., 2015a, 2015b	Wnt inhibition; Apcdd1 stimulation

Regulators of OPC differentiation are promising therapeutic targets to promote white matter development in perinatal WMI.

of myelin sheaths enwrapping axons (Nawaz et al., 2015). All in all, OL maturation and subsequent myelin assembly are tightly regulated processes that have been widely described earlier, but the exact mechanisms are beyond the scope of this review (for excellent reviews see (Emery, 2010; He & Lu, 2013; Mitew et al., 2014; Simons & Nave, 2015). Interestingly, throughout adulthood OPCs remain present, are required for myelin maintenance, and can be recruited to drive remyelination in case of injury to the myelin sheaths (Gautier et al., 2015). In adult mice, OPCs make up 8-9% of the cell population in the white matter and 2-3% of the population in the gray matter (de Castro et al., 2013; Dimou, Simon, Kirchhoff, Takebayashi, & Gotz, 2008).

4. OPC HETEROGENEITY

Over recent years, it has become clear that OPCs and OLs throughout the brain are not merely a homogeneous population of cells, but that different OPCs can express different markers and exert different functions. It has been proposed that different OPC subtypes can be classified based on various aspects such as their developmental stage, their origin, the expression of specific receptors/ion channels, or the spatial environment they reside in, which will be explained in more detail below.

4.1 Classification based on developmental stage

To explore heterogeneity in the OL lineage in detail, Marques et al. (2016) performed single-cell sequencing on OPCs and OLs from various brain areas of juvenile and adult mice. Based on clustering of genes expression profiles, their data indicate that during the transformation from OPCs to mature OLs, cells progress through a strict program of six distinct developmental stages before maturing into myelinating OLs. Furthermore, they showed that based on their gene expression profile, during their final developmental stage mature OLs can be clustered into six additional subsets, indicating that also mature OLs eventually form a heterogeneous population (Marques et al., 2016). However, it should be noted that such a distinction may not necessarily reflect intrinsic OL differences as many transcripts that define the mature OL subsets are primarily neuronal, therefore these interesting findings should be further validated to exclude the possibility of potential bias by environmental RNA.

4.2 Classification based on site of generation

As described in section 2, during development OPCs arise from different brain areas. This raises the question whether OPCs from different origins represent specific OPC

subpopulations with distinct functional features (Ornelas et al., 2016). Kessaris et al. (2006) revealed that OPCs derived from the three successive waves depicted in Figure 3 are characterized by different transcription factors. Whereas the OPCs from the ventral stream express transcription factor Nkx2.1, OPCs from the medial and dorsal waves express transcription factors Gsh2 and Emx1, respectively (Kessaris et al., 2006). As OPCs derived from distinct waves possess intrinsically different genetic profiles, they may also exert functional differences. Indeed, depending on their origin OPCs have been shown to respond differently to certain stimuli. Whereas the generation and specification of ventrally derived OPCs depend on Shh signaling, generation of dorsal OPCs has been shown to be independent of Shh (Cai et al., 2005; Chandran et al., 2003; Nery, Wichterle, & Fishell, 2001). Additionally, Ortega et al. (2012) showed that migration of the first wave of OPCs, which populate the optic nerve, is dependent on signaling of the epidermal growth factor (EGF)-related ligand neuregulin-1 via its receptor ErbB4. However, migration of OPCs derived from later waves is not affected by neuregulin-1, further indicating that OPCs from distinct waves show differential sensitivity to stimuli (Ortega et al., 2012). Moreover, Crawford et al. (2016) showed that in the mouse spinal cord, dorsally derived OPCs have a greater capacity to contribute to remyelination compared to ventrally derived OPCs. However, dorsal OPCs show a higher susceptibility to age-related functional impairments (Crawford, Tripathi, Richardson, & Franklin, 2016). Together, these studies indicate that different OPC subpopulations do exist and that functionality of OPCs may partly depend on their developmental origin.

4.3 Classification based on expression of receptors/ion channels

Differences between OPC populations may not only be explained by their origin and the genetic factors that drive their specification, but also by the expression of specific proteins, e.g. voltage-gated ion channels (Clarke et al., 2012; Karadottir, Hamilton, Bakiri, & Attwell, 2008). More specifically, OPCs that engage in glutamatergic signaling and show voltage-dependent depolarization have been shown to be more vulnerable to ischemic injury (Karadottir et al., 2008). Additionally, Vigano et al. (2016) identified a subpopulation of OPCs expressing G protein-coupled receptor (GPR)17 present throughout the brain. Activation of GPR17 has been shown to negatively regulate OPC differentiation (Chen et al., 2009; Fumagalli et al., 2011). Indeed, GPR17+ OPCs in adult mice do not differentiate under normal circumstances, but have the ability to differentiate and start myelination in case of injury to other GPR17 negative OPCs, creating a reserve pool of OPCs that can compensate for OPC loss in the event of pathological situations (Bonfanti et al., 2017; Vigano et al., 2016). These GPR17-

expressing OPCs may represent a specific subpopulation of OPCs involved in remyelination. Whether OPCs with similar protein expression profiles (i.e. expression of ion channels, GPR17) share similar origins is currently unknown, but is an interesting issue for further research.

4.4 Classification based on location

Besides their developmental origin, the location that OPCs and OLs eventually reside in may also affect their functionality. This is illustrated by several differences that have been observed between OLs in the gray and white matter. For instance, in the spinal cord white matter OLs show altered localization of gap-junction protein connexin-32, decreased cell-cell coupling and less susceptibility to metabolic disturbances compared to gray matter OLs (Bauer et al., 2002; Pastor, Kremer, Moller, Kettenmann, & Dermietzel, 1998). Furthermore, OLs in the gray matter have a lower differentiation capacity and their iron homeostasis is fully dependent on the ferroxidase hephaestin, whereas in the white matter loss of hephaestin can be compensated for by the ferroxidase ceruloplasmin (Dimou et al., 2008; Schulz, Vulpe, Harris, & David, 2011). It could be speculated that such region-dependent differences are mediated by adaptations of OLs to their environment by reacting to local signaling molecules.

4.5 Intrinsic heterogeneity versus environmentally determined differences

As mentioned above, OPCs from distinct developmental origins are driven by distinct transcription factors. These diverse transcriptional profiles may account for functional differences between various subtypes of OPCs. However, based on data obtained from single cell RNA sequencing it might be more plausible that OPCs from different origins converge into a transcriptionally homogeneous pool of OPCs (Marques et al., 2016). As OPCs migrate and further develop, they may adapt their gene expression profile to the specific needs in the environment they reside in. In that case, OPC heterogeneity is mainly determined by environmental factors. To which extent various OPC subtypes are functionally interchangeable should be further elucidated in the coming years.

To summarize, more and more evidence indicates that OPCs and OLs form heterogeneous cell populations with differences in functions and differentiation capacities that are driven by both intrinsic and extrinsic factors. Likely, the origin and gene expression profile drive OLs to migrate and mature in a specific way that can be affected by local signaling molecules – eventually resulting in differences in expression of specific proteins like receptors (e.g. GPR17) and ion channels. Exactly how differences in origin, location and gene expression profile between OPC populations affect their functions should be further elucidated.

5. RODENT VS. HUMAN OL BIOLOGY

Human white matter development is a time-consuming process. Although the axonal bundles that form the basis of the white matter are mostly in place before the third trimester of pregnancy, myelination of the first axons does not start until 30 weeks gestational age (GA) and predominantly occurs postnatally (Huppi et al., 1998; Inder & Huppi, 2000; Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013) (Figures 4,5). The number of OLs in the human white matter increases until the age of 5 years and remains stable after this age, as determined by nuclear bomb test-derived carbon (^{14}C) dating (Yeung et al., 2014). Most myelination occurs during the first year of life (Aubert-Broche, Fonov, Leppert, Pike, & Collins, 2008), but myelinated white matter volumes, particularly of frontal brain regions, keep growing until the age of approximately 40 years (Bartzokis et al., 2001). Most knowledge regarding the generation and maturation of OPCs that myelinate the white matter is derived from rodent studies, as described above. To which extent findings from rodent studies can be extrapolated to the human situation is an important issue for translational purposes. Below, we discuss several similarities and differences in rodent versus human white matter development.

5.1 Similarities between rodent and human myelination

Careful analysis of human fetal brain tissue revealed that the first OPCs emerge in the developing brain at 10 weeks GA, followed by an expansion of the population before mid-gestation (15-20 weeks GA) (Jakovcevski, Filipovic, Mo, Rakic, & Zecevic, 2009; Jakovcevski & Zecevic, 2005a; Rivkin et al., 1995). Several findings indicate that the ventral-to-dorsal routes of OPC generation are conserved in humans (Jakovcevski et al., 2009; Jakovcevski & Zecevic, 2005b). Similar to the rodent situation, OPCs in the human forebrain are derived from the lateral-medial ganglionic eminence and the SVZ from where they migrate towards the cortical plate (Jakovcevski & Zecevic, 2005b; Rakic & Zecevic, 2003). In addition, transcription factors associated with ventrally derived OPCs in rodents, Dlx2 and Nkx2.1, have also been observed in human cortical OPCs. A subpopulation of OPCs that does not express these transcription factors has also been identified, but whether these cells express factors associated with a dorsal origin was not investigated (Rakic & Zecevic, 2003). Further evidence of OPC heterogeneity in the human brain comes from Leong et al. (2014), who demonstrated presence of different OPC subtypes based on marker expression (A2B5, O4, MOG) in both fetal and adult brain tissue using fluorescence activated cell sorting. Besides differential expression of OPC markers, it was shown that the microRNA expression profile differs between fetal and adult OPCs (Leong et al., 2014). Together, these observations raise the possibility

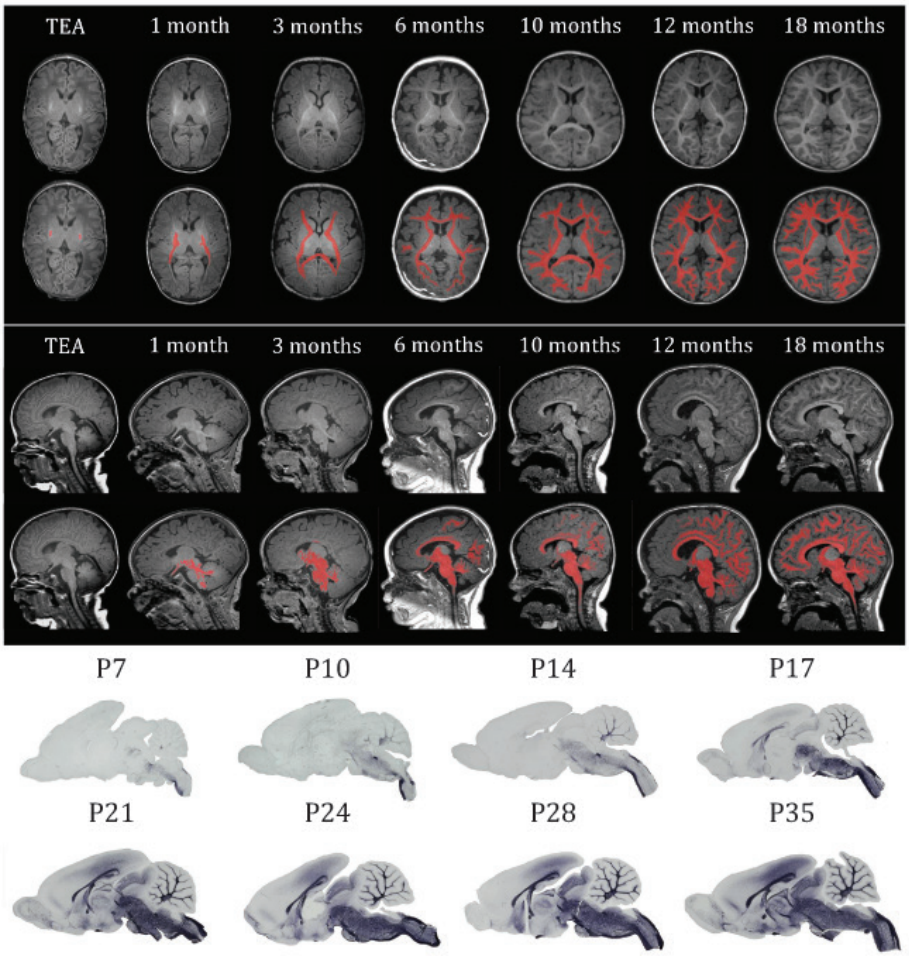

Figure 4 Overview of myelination throughout development from term-equivalent age (TEA) to 18 months in human infants and from postnatal day (P)7 to P35 in rats. The upper panel shows transverse sections of T1-weighted MRI scans at different ages, in the lower sections the myelinated white matter is manually colorized (red). The middle panel shows sagittal sections of T1-weighted MRI scans at different ages, in the lower sections the myelinated white matter is manually colorized (red). The lower panel shows sagittal sections of rat brains at different ages, stained for myelin basic protein (MBP), a myelin marker. The gross spatio-temporal pattern of myelination in humans shows high resemblance with that of rodents.

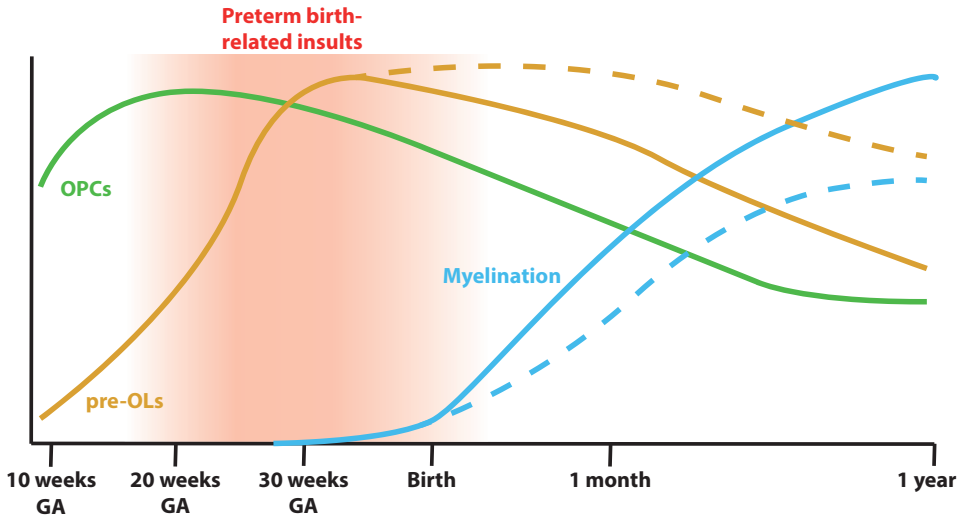

Figure 5 Developmental progression of OPCs, pre-OLs and myelination. The population of OPCs is expanded between 15-20 weeks gestational age (GA) (green line). Pre-OLs peak between 30-40 weeks GA (orange line). Myelination starts before birth, but mostly occurs during the first year of life and continues for several decades (blue line). Children born prematurely are exposed to perinatal insults during the peak of pre-OLs (red window), which hamper their ability to differentiate into myelinating OLs resulting in increased numbers of pre-OLs and reduced myelination as observed in diffuse WMI (dashed orange and blue lines) (based on data by Back et al., 2001; Buser et al., 2012).

that the human brain also contains different subpopulations of OPCs, originating from different brain areas, expressing different transcription factors and showing diverse phenotypes.

At an anatomical level, myelination patterns also show a high degree of similarity between rodents and humans, as illustrated in Figure 4. In the human brain, the caudal-to-rostral order of myelination is conserved with early myelination of the brain stem, midbrain and cerebellum during the first three months of life. Later, myelination of the telencephalon occurs in a caudal-to rostral manner starting from occipital cortical regions towards, lastly, the prefrontal cortex (Inder & Huppi, 2000; Jakovcevski et al., 2009; Jakovcevski & Zecevic, 2005b).

5.2 Differences between rodent and human myelination

Although myelination throughout development is rather conserved between species, differences between rodent and human OL biology have also been described. One obvious difference is brain size; compared to rodents, humans possess a 3000x larger brain volume and the relative proportion of white matter in the brain has greatly increased throughout evolution (Semple et al., 2013; Zhang & Sejnowski, 2000). Whereas myelin proteins have been shown to be extremely long-lived (Savas, Toyama, Xu, Yates, & Hetzer, 2012), several reports indicate that a relatively high turnover of OLs is crucial for myelin maintenance in rodents (Gibson et al., 2014; Young et al., 2013). In contrast, human OLs have been shown to have a low annual turnover rate of 0.3%, indicating that human myelinating OLs survive for a long time (Yeung et al., 2014). These findings indicate that the dynamics by which the pool of OL is maintained may differ between species. However, it should be noted that OPC maintenance has been investigated in much less detail in humans, compared to rodents. Of note, the observed differences may also be explained by regional differences, i.e. whereas in some brain regions OL turnover is low, other brain regions may require higher rates of OL turnover.

Moreover, several differences between rodent and human OLs have been reported at a cellular level. For instance, OPCs cultured from human fetal brain tissue show reduced responsiveness to the mitogens bFGF and CXCL1 compared to rodent OPCs (Filipovic & Zecevic, 2008; Wilson, Onischke, & Raine, 2003). These data indicate that not necessarily all findings in rodent OPCs can be straightforwardly translated to the human situation. Over the past years, the technology of producing human OPCs and mature OLs from induced pluripotent stem cells (iPSCs) has become available (Douvaras et al., 2014; S. Wang et al., 2013). Human iPSC-derived OPCs may be used to more specifically assess the biology of human OLs in vitro and to investigate to which extent findings from rodent OPCs can be translated to the human situation. In studying human OL biology, iPSC-derived OPCs provide an alternative to studies on scarce fetal or post-mortem brain tissue. However, downfalls of iPSC-derived OPC studies are also evident. Studying aspects such as OPC heterogeneity or functional differences remains difficult as iPSC-derived OPCs have not experienced the strict developmental programs of migration, proliferation and maturation in the developing brain. Clearly, it should always be taken into account that artificial settings of in vitro cultures are different from the in vivo situation.

5.3 Myelin plasticity

Myelin plasticity has been extensively studied in rodents, but is difficult to study in humans. More and more evidence indicates that experience-dependent neural activity can drive myelination, thereby promoting neural plasticity. To illustrate, physical exercise has been shown to promote differentiation of OPCs and myelination in mice (Simon, Gotz, & Dimou, 2011). In addition, multiple groups have shown that acute differentiation of OPCs is required for complex motor learning in mice. In more detail, motor learning is thought to depend on OPC-mediated plasticity as neuronal activity of involved motor pathways acutely activates OPC production, differentiation and myelination, thereby improving connectivity (Gibson et al., 2014; McKenzie et al., 2014; Schneider et al., 2016; Xiao et al., 2016). Whether a specific OL subtype is responsible for this type of activity-dependent myelination is an important question for further investigation. One study showed that specifically immature OLs expressing the stage-specific marker Enpp6 are increased during a motor task and are required for proper motor learning (Xiao et al. 2016). Furthermore, $PDGFR^{-}Sox10^{+}$ OLs expressing the intracellular calcium channel ITPR2 are increased in number after motor learning, indicating that $ITPR2^{+}$ OLs may also play an important role in this respect (Marques et al., 2016). Whether similar processes of myelin-mediated motor learning take place in the human CNS is difficult to study at a cellular level. However, imaging studies do provide evidence of experience-dependent changes in white matter microstructure in response to various types of learning (Zatorre, Fields, & Johansen-Berg, 2012). To illustrate, several studies revealed that exercise and skill-learning can improve integrity of white matter tracts (Scholz, Klein, Behrens, & Johansen-Berg, 2009; Svatkova et al., 2015), indicating that indeed OPC-mediated white matter plasticity also occurs in humans.

Another example of experience-dependent myelination was observed in mice; social isolation of juvenile or adult animals negatively affected myelination of the prefrontal cortex (Liu et al., 2012; Makinodan, Rosen, Ito, & Corfas, 2012). Similarly, early social deprivation was associated with reduced white matter integrity of various brain areas, including frontal regions, in human subjects (Eluvathingal et al., 2006; Govindan, Behen, Helder, Makki, & Chugani, 2010). White matter changes after social neglect may therefore contribute to the development of psychiatric disorders in socially deprived individuals (Toritsuka, Makinodan, & Kishimoto, 2015). Together, these findings indicate that activity- or experience-dependent myelination does take place in the human brain. However, whether this type of myelin plasticity requires the production of new OPCs remains unsure considering the stable amount and low turnover rate of OLs

as observed by Yeung et al. (2014). To what extent the cellular basis of myelin plasticity in humans is similar to that of rodents remains an important issue to be elucidated.

To summarize, available evidence indicates that the general spatio-temporal progress of white matter development in rodents closely resembles that of humans, including OPC generation from similar neuroepithelial zones and myelination in a caudal-to-rostral manner (Figure 4). By combining findings from experimental rodent studies and imaging studies in humans, it is possible to speculate on the mechanisms underlying phenomena such as activity-dependent myelination. However, it should be considered that discrepancies between rodents and humans have also been observed in the signaling pathways by which OPC generation, migration and maturation are regulated. Furthermore, the dynamics by which the existing pool of oligodendrocytes is maintained may differ between rodents and humans. Such possible differences should at least be taken into account when developing novel therapies to enhance (re)myelination.

6. IMPLICATIONS FOR PRETERM BIRTH-RELATED WMI

6.1 Preterm birth & diffuse WMI

As discussed above, white matter development is a tightly regulated, intricate process involving many steps. Perinatal insults after preterm birth coincide with the critical window of pre-OLs populating the white matter (Back et al., 2001) (Figure 5). Hypoxic and/or inflammatory insults are thought to create an unfavorable environment for OLs to fully mature and properly myelinate neuronal axons, thereby dysregulating myelination leading to long-term impairments in functional outcome (van Tilborg et al., 2016). Whereas cystic WMI (or cPVL) is associated with necrotic/apoptotic cell death and axonal injury (Silbereis, Huang, Back, & Rowitch, 2010), the nowadays predominant diffuse type of perinatal WMI is not necessarily associated with axonal damage. No overt axonal abnormalities were observed in several animal models, but to which extent impaired myelination leads to axonal defects in the human diffuse WMI remains unsure (Riddle et al., 2012; van Tilborg et al., 2017). Dysregulated OL development can cause alterations in the microstructure of white matter tracts, which is often observed clinically (Alexandrou et al., 2014; Glass et al., 2008; Mwaniki, Atieno, Lawn, & Newton, 2012; Rutherford et al., 2010; Shankaran et al., 2006; van Vliet, de Kieviet, Oosterlaan, & van Elburg, 2013). However, to which extent myelination in other brain areas such

as the neocortex is affected in human children with diffuse WMI, should be further elucidated.

6.2 Implications of novel insights in OL biology

6.2.1 Signaling pathways regulating OPC differentiation

Impaired maturation of pre-OLs causing myelin deficits is an important pathophysiological mechanism of diffuse WMI, making OLs interesting targets for therapeutic strategies. Various pathological mechanisms have been proposed to contribute to impaired OL maturation in diffuse WMI. For instance, inflammatory mediators negatively affect OL development (van Tilborg et al., 2016), but also changes in regulatory pathways such as the Daam2/Wnt/β-catenin and Notch pathways may contribute to impeded OL maturation in preterm infants (Back, 2017; Fancy et al., 2009; Fancy et al., 2011; John et al., 2002; Lee et al., 2015a). Increased activation of JNK signaling in response to perinatal insults has also been implicated in neonatal WMI (Wang, Tu, Huang, & Ho, 2012; Wang et al., 2014). In addition, production of high molecular weight hyaluronan produced by reactive astrocytes has been associated with inhibition of OPC differentiation in MS and perinatal WMI (Back et al., 2005; Buser et al., 2012; Hagen et al., 2014). Inhibiting or reversing the effects of these pathological mediators may be valid therapeutic strategies. However, knowledge on the regulation of healthy OL dynamics may also aid the development of new treatments to combat diffuse WMI in preterm infants. Importantly, the signaling pathways contributing to OPC differentiation may be potential therapeutic targets to overcome the maturational arrest of OLs (see Table 1). Negative regulators of OPC differentiation may be inhibited to allow proper differentiation of OPCs into mature myelinating OLs. For example, BMP4 has been identified as an inhibitor of oligodendrocyte maturation and overexpression of the BMP4 antagonist noggin was shown to protect the white matter from hypoxia-ischemia-induced neonatal brain injury in mice (Dizon, Maa, & Kessler, 2011; See et al., 2004). Additionally, activation of the Notch receptor inhibits differentiation of OPCs into mature OLs (Wang et al., 1998). In line, downregulation of Notch signaling by EGF treatment promotes recovery after neonatal WMI in mice (Scafidi et al., 2014). Also, after hypoxia the histone deacetylase (HDAC) Sirt1 promotes proliferation but inhibits differentiation of OPCs. Consequently, Sirt1 inhibition by the HDAC inhibitor sirtinol induces differentiation of OPCs in vitro and may therefore have therapeutic potential for neonatal WMI (Jablonska et al., 2016). Similarly, inhibition of the Wnt pathway by activation of the protein Apcdd1 promotes myelination in hypoxic cerebellar slice cultures indicating potential of Wnt inhibition as a therapeutic intervention for neonatal WMI.

Conversely, stimulating signals that promote OPC differentiation may improve myelination and functional outcome in preterm infants with diffuse WMI. For instance, reduced GABAergic input was shown to contribute to impaired cerebellar OPC maturation in mice with hypoxia-induced WMI (Zonouzi et al., 2015). Pharmacologically increasing the availability of GABA using anti-epileptic drugs reversed the effects of hypoxia and rescued myelination (Zonouzi et al., 2015). However, the exact mechanism requires further investigation as Hamilton et al. (2017) reported a negative effect of endogenous GABA signaling on myelination in cortical organotypic slice cultures. Insulin-like growth factor (IGF)1 is a trophic factor that has been associated with the positive regulation of OL maturation and IGF1 treatment was shown to protect the white matter in rats with neonatal WMI (Cai, Fan, Lin, Pang, & Rhodes, 2011; Pang et al., 2010) (Table 1).

Of note, treatments that have been shown to promote remyelination in animal models of multiple sclerosis may also have beneficial effects on myelination in the developing brain and are therefore of great interest as potential treatments for perinatal WMI (Franklin, 2015). For example, anti-inflammatory compounds such as activin-A, and activation of retinoid X receptor γ (RXR-γ) have been shown to promote remyelination, and may therefore also promote myelination during development (Huang et al., 2011; Miron et al., 2013) (Table 1). Additional examples of pathways implicated in the regulation of OPC differentiation of which intervention may enhance myelination in WMI are listed in Table 1.

6.2.2 OPC-Vascular interactions

As discussed in section 3.1, experimental data indicate that OPCs interact closely with the brain vasculature. Electron microscopy of human post-mortem brain tissue revealed that similar interactions take place in the human brain (Maki et al., 2015). From these findings, the question rises whether interactions between OPCs and blood vessels may contribute to neonatal WMI. Interestingly, it was demonstrated that vascular OPCs may play a dual role in WMI in adult mice. Initially, OPCs can contribute to injury by inducing blood-brain barrier leakage, but later they can contribute to vascular remodeling during recovery (Pham et al., 2012; Seo et al., 2013). Whether similar mechanisms play a role in WMI in the developing brain remains to be investigated, but the close interaction between OPCs and the vasculature indicates that specifically these OPCs may be targeted through systemic intervention.

6.2.3 Timing of insults

Other recent insights in the biology underlying white matter development also have implications for understanding the pathophysiology of WMI in preterm infants. For example, the developmental stage during which preterm infants are exposed to perinatal insults ranges from early maternal infections (in utero) to postnatal episodes of inflammation and/or hypoxia (Chau et al., 2012; Chau et al., 2009; Fyfe, Yiallourou, Wong, & Horne, 2014; Glass et al., 2008). Considering the different developmental processes taking place in the white matter at these distinct times, it is possible that the timing of an insult impacts the extent and localization of WMI, as well as related outcome (Back et al., 2001; Semple et al., 2013). For instance, different migratory streams of OPCs may be affected by early or late inflammatory or respiratory insults. Combining clinical data of preterm infants with imaging data and follow-up data may reveal the role of the timing of certain insults on the extent and localization of WMI.

6.2.4 Origin of OPCs

As discussed in section 5.1, most evidence indicates that the pool of OPCs in the human brain develops similar to that of rodents, with an initial dorsal stream of OPCs that, over time, shifts to a more ventral stream. Crawford et al. (2016) showed in the rodent spinal cord that OPCs from different origins have variable susceptibility. Whether OPCs from distinct developmental origins or OPCs with distinct transcriptional profiles are differentially susceptible to perinatal insults is an interesting question to further explore in future research. In case certain OPCs are more susceptible than others, targeting receptors specifically expressed by such vulnerable OPC subsets may be an interesting therapeutic strategy.

6.2.5 Compensatory mechanisms

As explained in sections 2 and 4.3, rodent studies revealed that myelination is a relatively robust process with an abundance of OPCs being generated, creating a pool of 'backup' OPCs that can start differentiating in case of injury to surrounding OLs (Kessaris et al., 2006; Vigano et al. 2016). Whether similar compensatory mechanisms take place in the human brain, and whether such mechanisms are active in preterm infants with WMI, are interesting questions to further explore. In case compensatory mechanisms are at play, boosting such regenerative processes may promote the recovery of neonatal WMI.

6.2.6 Myelin plasticity in WMI

Animal and human studies have shown that neural activity stimulates OPC differentiation and myelination during motor learning (Scholz et al., 2009; Svatkova et al., 2015; Zatorre et al., 2012). With this in mind, preterm infants may benefit from neural stimulation to promote white matter development. Physical therapy or tactile/auditive stimulation may have beneficial effects and may complement other therapeutic strategies that are currently being investigated, such as anti-inflammatory treatments or stem cell therapy. Indeed, several studies revealed that music has beneficial effects on preterm infants in terms of cortisol levels, heart rate and pain (Qiu et al., 2017; Schwilling et al., 2015), but due to contradictory results more research is required to accurately assess the effects of sensory stimulation on white matter development and outcome in preterm infants (Bieleninik, Ghetti, & Gold, 2016; Pineda et al., 2017).

In sum, knowledge on the developmental processes underlying healthy white matter development is crucial to come up with novel treatment options for when proper myelination is hampered. More research into the different functionalities and vulnerabilities of specific OPC subsets is essential to gain more insight into white matter development. The different aspects of OL lineage development and dynamics should be taken into account when exploring the pathophysiological mechanisms underlying WMI and pursuing novel therapies for WMI.

7. CONCLUDING REMARKS

Over the past years, experimental research, mostly in rodents, provided much insight in novel concepts regarding OL development and myelination. As depicted in Figure 4, gross white matter development in humans shows high resemblance to that of rodents (Jakovcevski & Zecevic, 2005a; Jakovcevski & Zecevic, 2005b; Rakic & Zecevic, 2003). However, it should be taken into account that some differences between rodent and human OLs have been reported at a cellular level (Filipovic & Zecevic, 2008; Wilson et al., 2003).

During development, OPCs are generated over the course of multiple waves, originating from distinct brain regions. Exactly how the spatial and temporal origins of OPCs determine their functionality remains to be further investigated, but evidence suggests that OPCs converge into a single pool of cells that disperse throughout the CNS and adapt their gene expression profile to the needs of their environment

(Marques et al., 2016). Most OPCs eventually differentiate into mature OLs to myelinate neuronal axons. Rodent studies revealed that myelination is a highly dynamic process with an excess of OPCs being generated in order to compensate for possible injury to developing OLs (Kessaris et al., 2006; Vigano et al., 2016). Furthermore, OPCs can contribute to neural plasticity by inducing activity-dependent myelination, which is required for motor learning (Marques et al., 2016; McKenzie et al., 2014). These insights should be taken into account in order to understand the pathophysiological mechanisms underlying WMI in preterm infants. To date, the precise mechanisms underlying impaired maturation of OLs in perinatal WMI are not fully understood. Therefore, it is crucial to perform more research into white matter development under normal and pathological situations in order to develop novel OL-targeted therapies to combat WMI and its severe consequences in preterm infants.

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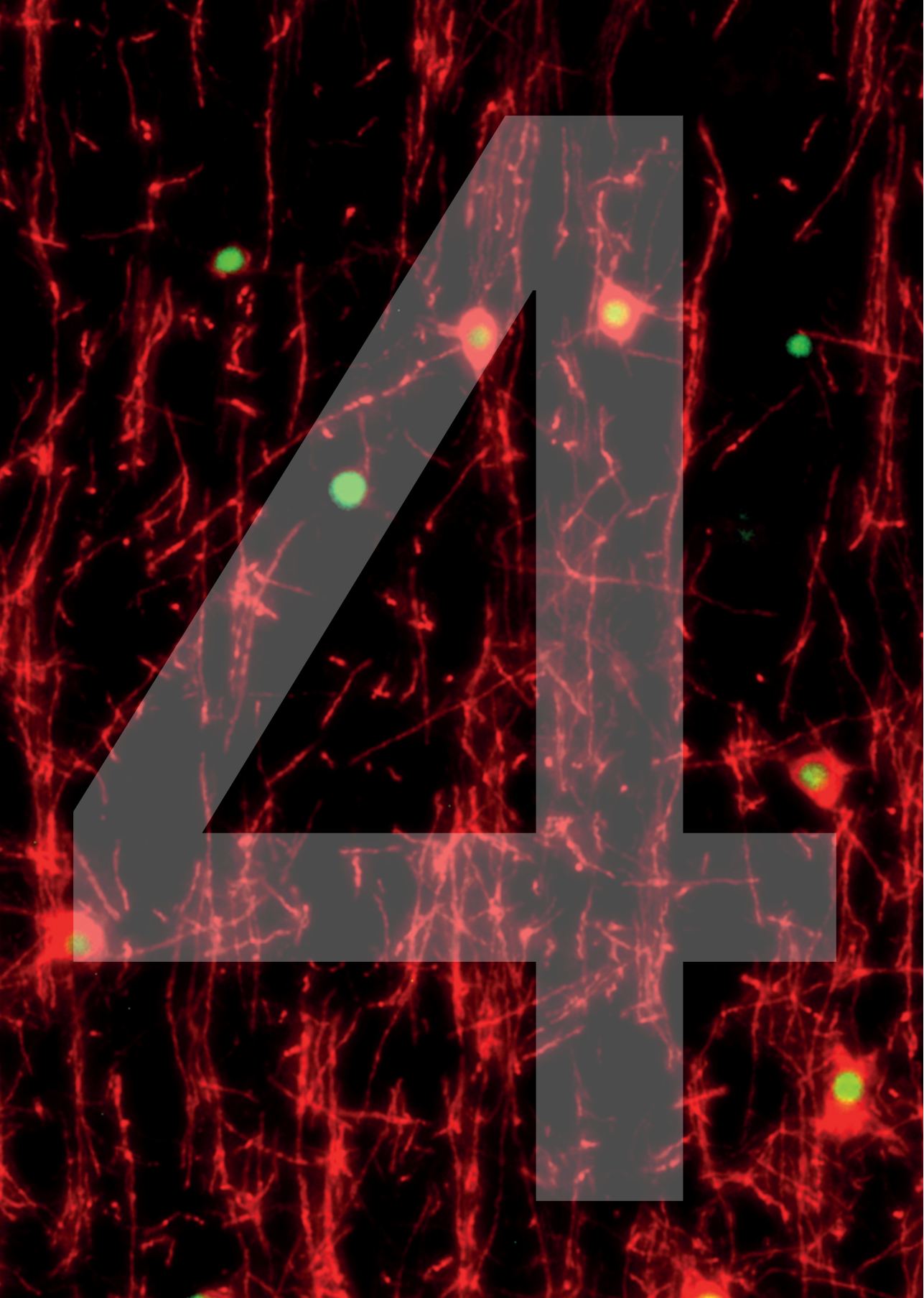
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CHAPTER 4

Combined fetal inflammation and postnatal hypoxia causes myelin deficits and autism-like behavior in a rat model of diffuse white matter injury

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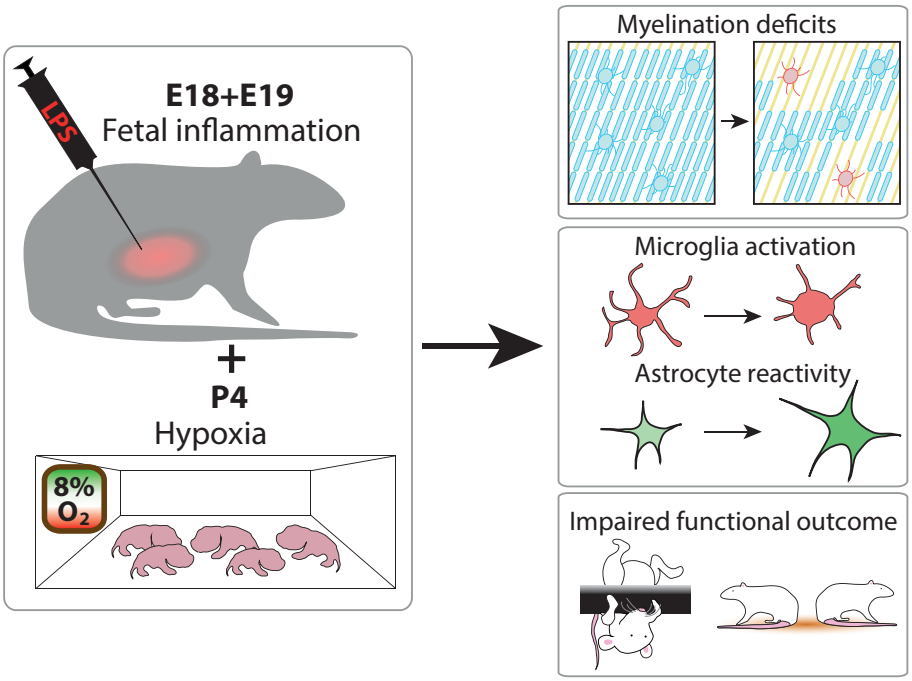


MAIN POINTS:

- Combined inflammation in utero and postnatal hypoxia causes impaired oligodendrocyte maturation, myelin deficits and behavioral problems in rats.

- This model may be used to study treatment options for diffuse white matter injury in preterm infants.

ABSTRACT

Diffuse white matter injury (WMI) is a serious problem in extremely preterm infants, and is associated with adverse neurodevelopmental outcome, including cognitive impairments and an increased risk of autism-spectrum disorders. Important risk factors include fetal or perinatal inflammatory insults and fluctuating cerebral oxygenation. However, the exact mechanisms underlying diffuse WMI are not fully understood and no treatment options are currently available. The use of clinically relevant animal models is crucial to advance knowledge on the pathophysiology of diffuse WMI, allowing the definition of novel therapeutic targets. In the present study, we developed a multiple-hit animal model of diffuse WMI by combining fetal inflammation and postnatal hypoxia in rats. We characterized the effects on white matter development and functional outcome by immunohistochemistry, MRI and behavioral paradigms. Combined fetal inflammation and postnatal hypoxia resulted in delayed cortical myelination, microglia activation and astrogliosis at P18, together with long-term changes in oligodendrocyte maturation as observed in 10 week old animals. Furthermore, rats with WMI showed impaired motor performance, increased anxiety and signs of autism-like behavior, i.e. reduced social play behavior and increased repetitive grooming. In conclusion, the combination of fetal inflammation and postnatal hypoxia in rats induces a pattern of brain injury and functional impairments that closely resembles the clinical situation of diffuse WMI. This animal model provides the opportunity to elucidate pathophysiological mechanisms underlying WMI, and can be used to develop novel treatment options for diffuse WMI in preterm infants.

Key words
Preterm birth, microglia, astrocytes, oligodendrocytes, autism-like behavior

INTRODUCTION

White matter injury (WMI) is a major complication in infants born prematurely. Diffuse, non-cystic WMI as characterized by decreased white matter volumes and low fractional diffusion anisotropy (FA) values detected by MRI, affects the majority of WMI patients in neonatal intensive care units (Back & Miller, 2014). Studies on postmortem brain tissue of preterm infants indicated that myelination deficits in diffuse WMI are mediated by impaired differentiation of immature oligodendrocytes, without specific loss of oligodendrocytes (Billiards et al., 2008; Buser et al., 2012; Verney et al., 2012). In contrast, several other studies found evidence of oligodendrocyte-specific apoptosis in the brains of preterm infants (Haynes et al., 2003; Robinson, Li, Dechant, & Cohen, 2006). Additionally, activation of microglia and astrogliosis are important pathophysiological hallmarks of diffuse WMI (Buser et al., 2012; Haynes et al., 2003; Verney et al., 2012). Functionally, WMI is associated with behavioral, cognitive, motor and psychological problems, like autism spectrum disorders (ASD), later in life (Back & Miller, 2014; Dudova et al., 2014; Guo et al., 2017; Johnson et al., 2010; Joseph et al., 2017; Kuzniewicz et al., 2014; Limperopoulos et al., 2008; Peyton et al., 2016; Peyton et al., 2017; Pritchard et al., 2016; Treyvaud et al., 2013). While antenatal administration of magnesium sulfate in women at risk for preterm delivery reduces the risk of cerebral palsy in the fetus (Crowther et al., 2003; Doyle et al., 2009; Gano et al., 2016 Rouse et al., 2008), limited treatment options are currently available to protect preterm infants against diffuse WMI. Moreover, the mechanisms underlying diffuse WMI are not completely understood (van Tilborg et al., 2016). Therefore, it is crucial that clinically relevant animal models are being developed to allow the investigation of new therapies.

Important risk factors for diffuse WMI include fetal or perinatal inflammatory insults and postnatal hypoxic/hyperoxic events (Chau et al., 2012; Procianoy & Silveira, 2012; Resch et al., 2012; Shah et al., 2008; Shankaran et al., 2006; Tsuji et al., 2000). It has been proposed that exposure to multiple perinatal hits plays a crucial role in the etiology of WMI, with a first insult sensitizing the developing brain to subsequent insults that aggravate injury (Kaindl, Favrais, & Gressens, 2009; Van Steenwinckel et al., 2014). This notion is supported by clinical data indicating that exposure to multiple insults dramatically increases the risk of white matter abnormalities (Korzeniewski et al., 2014; Leviton et al., 2013). In the present study, we investigated the pathology and outcome in a novel rat model of diffuse WMI in preterm infants, in which two clinically relevant perinatal hits, i.e. fetal inflammation and postnatal hypoxia, are combined during

relevant stages of brain development. Neuropathology was studied by histology and MRI. Furthermore, functional outcome on several relevant modalities was investigated by behavioral paradigms for motor coordination, cognitive functioning, anxiety-like behavior and autism-like behavior.

MATERIALS AND METHODS

Animals

All procedures were carried out according to Dutch ("Wet op de dierproeven", 1996) and European regulations (Guideline 86/609/EEC) and were approved by the Animal Ethics Committee of Utrecht University.

Wistar rats (Envigo, Horst, The Netherlands) were kept under standard housing conditions. Timed-pregnant animals received intraperitoneal injections of 100 µg/kg lipopolysaccharide (LPS) (from E. Coli O55:B5, L2880, Sigma, St. Louis, MO) in 1.0 ml/kg saline or saline (vehicle) on E18 and E19. Maternal LPS injections have previously been shown to induce cytokine expression in the fetal brain (Cai, Pan, Pang, Evans, & Rhodes, 2000). Increasing the dosage of LPS (up to 500 µg/kg) resulted in high rates of stillbirth and/or high perinatal mortality in the offspring (data not shown). At P4, offspring was randomly placed in a temperature-controlled hypoxic chamber containing 8% O_2 in N_2 or in a normoxic temperature-controlled environment (i.e. not in the homecage to circumvent potential confounding effects of maternal deprivation) for 140 minutes. Pups of both sexes were used. Specific animal numbers are mentioned in the figure captions.

Immunohistochemistry and analysis

At P5, P18, P30 (post-MRI see below) and P69, animals received an overdose of 300 mg/kg pentobarbital and were transcardially perfused with phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in PBS. Brains were fixed in 4% PFA for 24 hours and embedded in paraffin. 8µm coronal sections were cut, deparaffinized and rehydrated prior to antigen retrieval by heating sections to 95°C in sodiumcitrate buffer (0.01M, pH 6) for 9 minutes. Sections were blocked with 5% normal serum or 2% bovine serum albumin (BSA) and 0.1% saponin in PBS and incubated overnight with primary antibodies diluted in PBS (mouse-anti-CNPase, 1:500, Abcam; mouse-anti-GFAP, 1:250, Cymbus; rabbit-anti-Iba1, 1:500, WAKO; rabbit-anti-Ki67, 1:300, Abcam; mouse-anti-MBP, 1:1000, Sternberger Monoclonals; rabbit-anti-NF200, 1:400, Sigma;

rabbit-anti-Olig2, 1:500, Chemicon; mouse-anti-Olig2, 1:500, Millipore). Sections were then incubated with alexafluor-594 or -488 conjugated secondary antibodies (Life Technologies, Carlsbad, CA) for 1 hour at room temperature, followed by DAPI counterstaining.

For 3,3′-diaminobenzidine (DAB) staining, sections were deparaffinized, incubated in 3% H_2O_2 in methanol, and sections were blocked in 20% normal horse serum and 0.5% TritonX in PBS. After overnight incubation with mouse-anti-MBP primary antibody (see above, 1:1600), sections were incubated with biotinylated horse-anti-mouse secondary antibody (Vector Laboratories, Peterborough, UK) for 45 minutes at room temperature, and revealed by Vectastain ABC kit (Vector Laboratories) and DAB.

For cell death measurements, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) cell death detection kit (Roche, Basel, Switzerland) was used on P5 brain sections according to manufacturer's instructions. Prior to the TUNEL assay, sections were immunofluorescently stained for Olig2, as described above.

For each animal, micrographs from both hemispheres were obtained in a blinded fashion using a Cell Observer microscope (Zeiss, Oberkochen, Germany). For MBP stainings and CNPase/Olig2 stainings, in each hemisphere a 20X picture was taken at a fixed distance from the external capsule in the barrel field of the sensory cortex. For TUNEL/Olig2 and Ki67/Olig2 stainings, two 20X pictures of the corpus callosum were acquired in each hemisphere. Similarly, for Iba1 stainings in each hemisphere two 20X pictures were taken in the corpus callosum and in the motor cortex. For GFAP stainings, a 10X picture of the cingulum and a 20X picture just medial to the cingulum were acquired in each hemisphere. The MBP-DAB staining was photographed on an AxioLab microscope (Zeiss, Oberkochen, Germany). Of each hemisphere, a 10X picture was acquired of the external capsule and sensory cortex and a 40X photograph was acquired at a fixed distance from the external capsule.

Densitometry (controlled for background signal) and threshold analyses were performed using ImageJ software v1.47. Segmentation and structural analysis of MBP stainings were performed by ImageJ plugin DiameterJ (Hotaling, Bharti, Kriel, & Simon, 2015). For each animal, values of all acquired pictures were averaged. Coherency of $NF200^+$ axons was measured using the OrientationJ plugin for ImageJ (Fonck et al., 2009). Cell counting was performed manually using ImageJ or Axiovision (Zeiss, Oberkochen, Germany) software and controlled for measured area. Criteria for

cell inclusion were clear presence of a DAPI$^+$ nucleus, together with clear Ki67$^+$/Olig2$^+$, CNPase$^+$/Olig2$^+$, CNPase$^-$/Olig2$^+$ or Iba1$^+$ staining. The particle analysis function of ImageJ software was used to assess different morphological aspects of microglia, as described earlier (Zanier, Fumagalli, Perego, Pischiutta, & De Simoni, 2015). Manually, correctly identified microglia were selected and shape descriptor parameters were measured. For each animal, the values of all measured microglia were averaged.

Quantitative real time reverse transcriptase (RT)-PCR

At P5, rats were euthanized by an overdose of 300 mg/kg pentobarbital. The cerebrum was flash frozen in liquid nitrogen and pulverized. Total RNA was isolated using TRIzol (Invitrogen, Paisley, UK). cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen). The PCR reaction was performed with iQ5 Real-Time PCR Detection System (Bio-Rad) using primers for PDGF-A (forward: CCCA TGTGTGGAGGTGAAG; reverse: TGGCTTCTTCCTGACATACTCC), PDGF-B (forward: ATCCGCTCCTTTGATGACCT; reverse: TCAGCCCCATCTTCGTCTAC), BMP4 (forward: TGAGGAGTTTCCATCACGAA; reverse: CACCTGCTCCCGAAATAGC), Jagged1 (forward: CTGCGTGGTCAATGGAGACT; reverse: CAAAACCAGGGGC ACATTCG), GLT-1 (forward: GAGCATTGGTGCAGCCAGTATT; reverse: GTTCTCA TTCTATCCAGCAGCCAG; Hammad et al., 2017) and GLAST (forward: AATGAAG CCATCATGAGATTGGT; reverse: CCCTGCGATCAAGAAGAGGAT; Zschocke et al., 2005). Data were normalized to the relative expression of β-actin (forward: CACTAT CGGCAATGAGCGGTTCC; reverse: CAGCACTGTGTTGGCATAGAGGTC).

Postmortem MRI

At P30, animals received transcardial perfusion with PBS, followed by 4% PFA in PBS. The head of the animal was removed and post-fixed for 3 days in 4% PFA, followed by incubation in PBS with 0.05% sodium azide for a minimum of 10 days. Prior to scanning, all skin and muscle tissue was removed from the skull. Brains were scanned overnight in a 9.4T MR system (Varian, Palo Alto, CA, USA) to obtain anatomical images at 100 µm isotropic resolution using a balanced steady state free precession (BSSFP) sequence (repetition time (TR)/echo time (TE) 15.4/7.7 ms, flip angle 40°, 320x160x190 matrix, field-of-view (FOV) 32x16x19 mm^3, 6 averages, pulse angle shift 0°, 90°, 180° and 270°), and diffusion parameter maps at 150 µm isotropic resolution using diffusion tensor imaging (DTI) with b = 3842 s/mm2 (TR/TE 500/32.4 ms, 220x128x108 matrix, FOV 33x19.2x16 mm^3, Δ/δ 15/4 ms, 60 diffusion-weighted images in non-collinear directions and 5 images without diffusion weighting (b=0), number of averages 1, total number of images 65). Data were analyzed using the FMRIB (Oxford Center for Functional MRI

of the Brain) Software Library (FSL) (Jenkinson, Beckmann, Behrens, Woolrich, & Smith, 2012) and Matlab software. Following registration to an anatomical rat brain template, we measured mean diffusivity and FA in atlas-based regions of interest (ROIs) (Figure S1). The size of the ROIs was calculated from the anatomical images and are expressed relative to the cerebrum size. Relative white matter volumes were calculated by setting a threshold on BSSFP scans: signal intensities between 0.91 and 1.24 were considered white matter. After MRI, brains were used for further immunohistochemical analyses as described above.

Behavioral assessment

All behavioral experiments and analyses were performed by experienced researchers blinded to experimental conditions. In between testing trials, testing apparatuses were thoroughly cleaned. Except for the rotarod task, all behavioral tests were carried out under red light conditions.

Rotarod

Gross motor skills and coordination were determined using the rotarod (RTR) task . At the age of 5 weeks, rats were trained on two consecutive days to remain on a rotating rod (5 rotations per minute (rpm)) for 150 seconds. On days 3 and 4, animals were placed on the RTR, with the rotation speed increasing from 5-40 rpm over the course of a 300 second trial. The time spent on the rod (averaged over the two test trials) was recorded, which is considered a measure of motor performance.

Novel object recognition task

Memory function was assessed using the novel object recognition task (NORT) at an age of 9 weeks . Animals were placed in a test cage without bedding containing two identical objects (white/transparent spherical glass globe or upside-down dark-gray ceramic bowl) for 10 minutes, after which the animal was placed back into its homecage. One hour later, animals were placed back in the test cage for 5 minutes with one object being replaced with a novel object. Exploration behavior was videotaped and the time that the animal spent exploring the familiar and novel objects was recorded. Object types (globe/bowl) on a randomly assigned side (left/right) of the test cage were randomly designated as familiar/novel objects, to exclude a potential bias of side- or object preference. Data are presented as percentage of the time spent exploring the novel object relative to the total exploration time on both objects.

Delayed spontaneous alteration (T-maze)

Working memory was assessed at the age of 6 weeks by measuring delayed spontaneous alteration in a T-maze paradigm. Each trial in this test consisted of two runs in a T-shaped maze: a sample run and a choice run. During the sample run, the animal was placed in the starting arm of the T-maze. The investigator waited until the animal entered one of either goal arms (max. 2 minutes) and closed the arm, forcing the animal to remain in the same goal arm for 30 seconds. Immediately after the sample run, the animal was placed back into the starting arm for the choice run and it was recorded whether the animal first started exploring the previously explored arm or the unexplored arm. If the animal started exploring the unexplored arm first, this was recorded as correct alteration. For 4 consecutive days, animals performed 2 trials per day. Between each run, feces and urine were removed from the maze. Preference for the alternating arm is expressed as percentage of the total number of choice runs choosing the alternate arm, relative to the previously explored arm.

Repetitive grooming

Repetitive grooming behavior was assessed at the age of 5 weeks by placing the rats in a transparent cage containing only fresh bedding material for 20 minutes. The animals were videotaped and time spent grooming was recorded .

Anxiety-related behavior

We used two paradigms to investigate anxiety-related behavior at the age of 7 weeks. In the open field test , animals were placed in a circular arena with a diameter of 1 m for 5 minutes and locomotion behavior was automatically tracked using Ethovision software (Noldus, Wageningen, The Netherlands). The arena was divided into two sections: the outer rim of the arena (12.5 cm) and the inner zone (diameter of 75 cm). The number of times that animals left the outer zone (sheltered by the walls of the arena) and entered the inner zone was recorded. This is considered a measure of non-anxious behavior.

For the elevated plus maze (EPM), animals were placed in the middle of a plus-shaped maze with a 10 cm x 10 cm center (light intensity: 8-10 lux) connecting two opposite open arms (length: 50 cm; 16 lux) and two opposite arms closed with 30 cm high walls (length: 50 cm; 0 lux), 1 m above a dimly lit floor for five minutes. Animal movement was tracked using Ethovision software (Noldus, Wageningen, The Netherlands). The time spent in the open arms is considered a measure of non-anxious behavior.

Social play behavior

Impaired social play behavior is associated with ASD (Jordan, 2003). At the age of 4 weeks, social play behavior was assessed as previously described (Achterberg, van Kerkhof, Damsteegt, Trezza, & Vanderschuren, 2015). Briefly, animals were habituated to the 40 cm x 40 cm test chamber for 10 minutes on 2 consecutive days. On the third day, animals were socially isolated for 2.5 hours before placing them into the test chamber together with a non-familiar, weight- and sex-matched animal of the same experimental group. For 15 minutes, social play behavior was videotaped and later scored for pouncing (attempting to nose or rub the nape of the other animals neck), pinning (standing over the other animal while the other is lying on the floor with its dorsal surface) and general social exploration (e.g. sniffing) using Observer software (Noldus, Wageningen, The Netherlands).

Statistics

Statistics were performed using Graphpad Prism v6.02. For all parameters within this study, the presence of sex-specific effects were first investigated by two-way ANOVA with sex and experimental group as dependent variables. In case of a significant effect of sex or an interaction between experimental group and sex, data were further analyzed by multiple comparisons with Bonferroni correction and data are presented for males and females separately. In case there was no significant effect of sex, males and females were pooled. To compare control vs. WMI groups, independent samples T-tests were used and in case of unequal variances Welch's corrections were applied. When comparing more than 2 groups, one-way ANOVA was performed, followed by post-hoc multiple comparisons with Bonferroni correction. Correlations were calculated by linear regression. Overall, $p<0.05$ was considered statistically significant and data are presented as mean with SEM.

RESULTS

The combination of fetal inflammation and postnatal hypoxia causes delayed myelination in rats.

To assess the effects of fetal inflammation plus postnatal hypoxia on myelination, brain slices were stained for myelin basic protein (MBP). Compared to control animals, we observed no differences in MBP^+ area in animals that were exposed to only postnatal hypoxia ($p>0.99$) or only fetal LPS ($p>0.99$) at P18 (Figure 1a,b). However, rat pups exposed to the combination of fetal inflammation and postnatal hypoxia showed a significant decrease in MBP^+ area in the sensory cortex ($p=0.044$), indicating that the combination of both insults is required to induce myelination deficits. Since no myelination deficits were observed in animals exposed to only fetal inflammation or only hypoxia, further experiments were restricted to animals exposed to neither insult or combined fetal inflammation and postnatal hypoxia (from here onwards referred to as control and WMI animals, respectively). A more detailed analysis of cortical myelination was performed using segmentation of MBP images, which revealed a less complex organization of myelinated axons, comprising a reduced number of intersections between myelinated axons ($p=0.042$) and reduced fiber lengths ($p=0.020$) in WMI animals compared to control animals at P18 (Figure 1c-e). MBP stainings were also performed on P30 and P69 rats in order to assess the effects of fetal inflammation and postnatal hypoxia on cortical myelination over time. Compared to control animals, WMI rats showed reduced MBP staining in the sensory cortex at the ages of P18 ($p=0.006$) and P30 ($p=0.021$), which had returned almost to control levels at P69 ($p=0.869$) (Figure 1f,g). Collectively, these data demonstrate that the combination of fetal inflammation and postnatal hypoxia induces reduced myelination until past P30.

To study the size of the lateral ventricles and the hippocampus, the thickness of the corpus callosum and microstructural axonal integrity, additional HE, MBP and NF200 stainings were performed on sections of P18 animals. The experimental rat model induced subtle diffuse WMI, as we did not observe differences in gross neuroanatomy as indicated by ventricle size ($p=0.691$), hippocampus size ($p=0.499$) and corpus callosum thickness ($p=0.871$) (Supporting information Figure S2). Furthermore, WMI animals showed impaired myelination of axons as indicated by a decreased MBP:NF200 ratio ($p=0.016$) (Supporting Information Figure S3a,d), whereas we did not observe any signs of axonal damage as assessed by NF200 stainings ($p=0.911$) (Supporting Information Figure S3a-c).

Fetal inflammation and postnatal hypoxia induce impaired oligodendrocyte maturation

It has been suggested that arrested oligodendrocyte maturation underlies impaired myelination in diffuse WMI in preterm infants (Back & Miller, 2014). In order to assess oligodendrocyte maturation in animals exposed to combined fetal inflammation and postnatal hypoxia, we performed double-stainings for developmental stage-specific markers Ki67 (proliferation marker – oligodendrocytes lose their proliferative capacity during differentiation) or CNPase (more mature oligodendrocytes), together with nuclear oligodendrocyte marker Olig2. Compared to control animals, WMI animals showed no changes in oligodendrocyte maturation at P5 (Ki67: $p=0.117$; CNPase: $p=0.259$) (Figure 2a-d). Interestingly, at P18 oligodendrocyte maturation was impaired in WMI animals compared to control animals, as illustrated by increased numbers of immature $Ki67^{+}Olig2^{+}$ cells in the corpus callosum ($p=0.027$) and decreased numbers of $CNPase^{+}Olig2^{+}$ cells in cortical areas ($p < 0.001$) (Figure 2a-d). By P69, the number of $Ki67^{+}Olig2^{+}$ cells in the corpus callosum was reduced in WMI animals compared to controls, indicating reduced proliferative capacity of oligodendrocytes after fetal inflammation plus postnatal hypoxia ($p=0.003$). Furthermore, the proportion of $CNPase^{+}$ oligodendrocytes in cortical areas remained lower in WMI rats at P69 compared to control animals ($p=0.029$) (Figure 2a-d).

In order to elucidate whether fetal inflammation plus postnatal hypoxia induced oligodendrocyte cell death, we performed a TUNEL assay on sections of P5 rats that were stained for oligodendrocyte marker Olig2. In the corpus callosum, we sporadically observed $TUNEL^{+}Olig2^{+}$ cells in both control and WMI animals, but no differences were observed between the groups ($p=0.246$) (Figure 3). Taken together, these results indicate that impaired myelination in WMI animals results from arrested oligodendrocyte maturation, rather than oligodendrocyte-specific cell death.

WMI is associated with increased activation of microglia and astrocytes

To investigate the activation of different glia populations, sections of P5 and P18 animals were stained for the microglia marker Iba1 and the astrocyte marker GFAP. In the corpus callosum of WMI animals, a significant increase in the number of microglia was observed ($p=0.008$) (Figure 4a,b). A more detailed analysis of microglia morphology, including measures for circularity and perimeter, revealed a more proinflammatory, amoeboid morphology of these cells in WMI animals compared to controls (Figure 4c-f). Similar observations were made in the cortex of WMI animals in comparison to control rats, albeit somewhat less pronounced (Supporting Information Figure

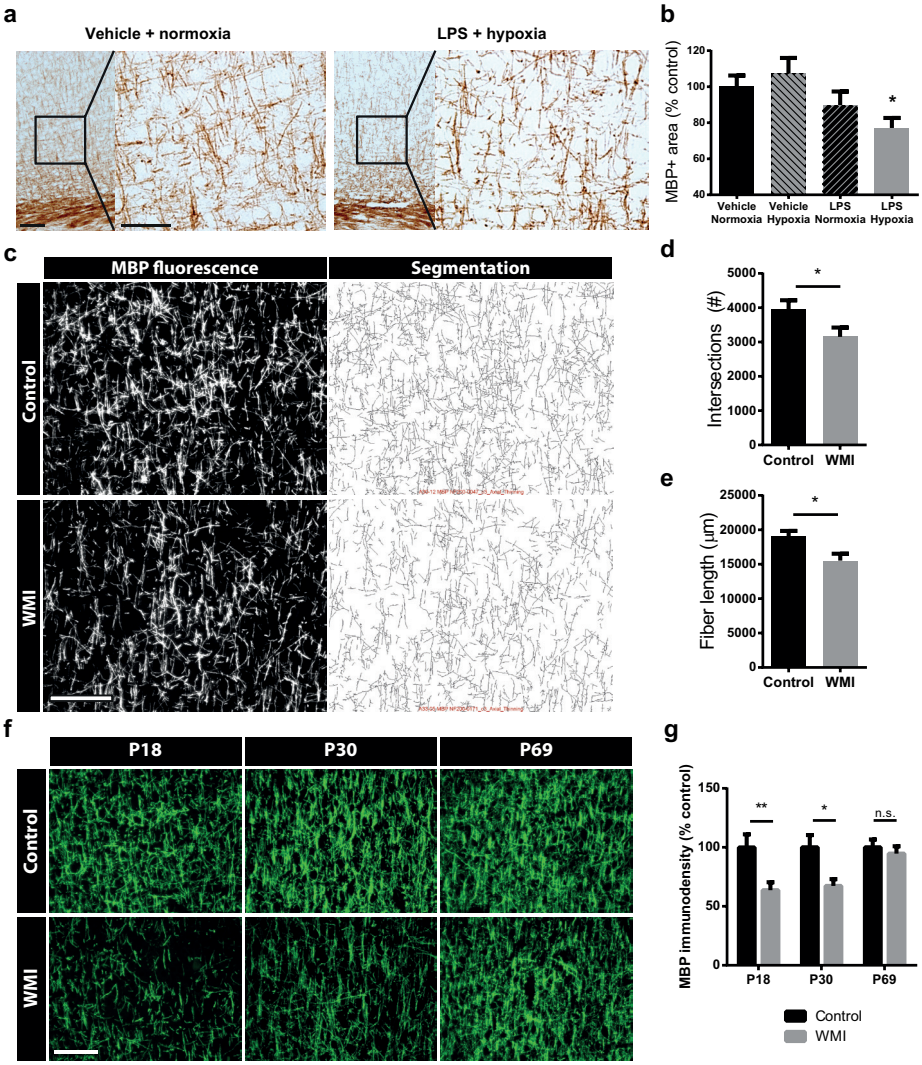


Figure 1 The combination of fetal LPS exposure and postnatal hypoxia causes delayed myelination in neonatal rats. (a) Representative images of MBP (DAB) stainings of animals from mothers injected with vehicle that were exposed to postnatal normoxia at P4 or animals from mothers injected with LPS (fetal inflammation) that were exposed to postnatal hypoxia at P4. Scale bars: 100 µm (left), 50 µm (inset). (b) Quantification of MBP^+ area in animals from mothers injected with vehicle that were expose to postnatal normoxia (n=9) or to postnatal hypoxia at P4 (n=7), and animals from mothers injected with LPS (fetal inflammation) that were exposed to postnatal normoxia (n=8) or postnatal hypoxia (n=16) at P4. (c) Fluorescent stainings for MBP (left panels) and automated segmentation (right panels) of the sensory cortex in P18 control and WMI animals. Scale bar: 100 µm. (d-e) Quantification of intersections between myelinated axons (d) and fiber length (e) to assess the complexity of cortical myelination. These measures were calculated from automated segmentation images from MBP fluorescent stainings in the sensory cortex of P18 rats. Animal numbers: control n=10; WMI n=16. (f) Fluorescent stainings for MBP in the cortex of control and WMI animals at P18, P30 and P69 reveal delayed myelination. Scale bar: 100 µm. (g) MBP immunodensity of control vs. WMI animals at P18 (n=10; n=17), P30 (n=6; n=6) and P69 (n=10; n=10), normalized to control values. *: $p < 0.05$; **: $p < 0.01$; n.s.: not significant.

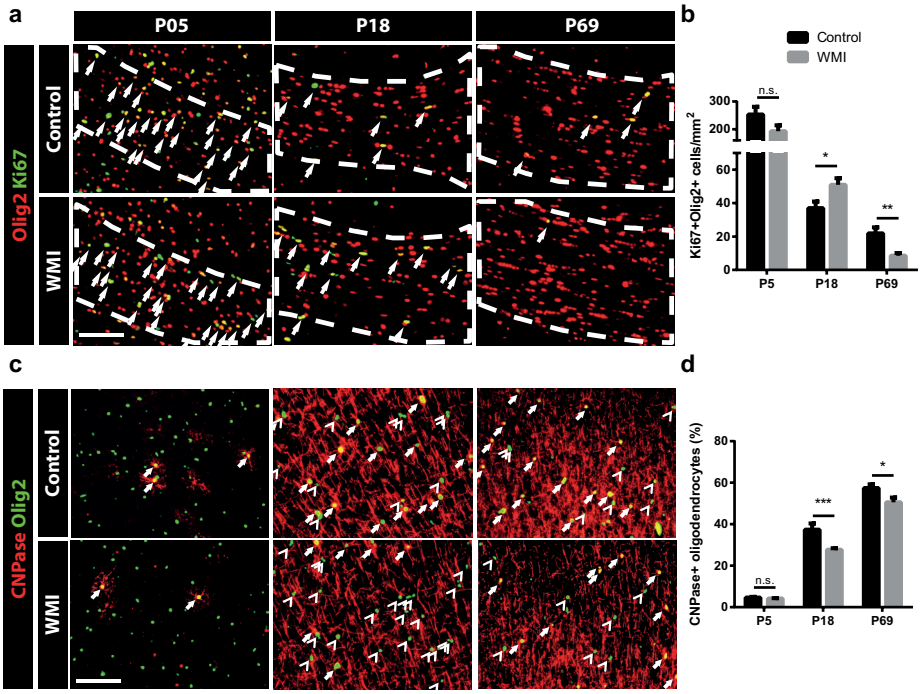


Figure 2 Impaired oligodendrocyte maturation in WMI animals. (a) Corpus callosum (outlined) of control vs. WMI animals stained for oligodendrocyte marker Olig2 (red) and proliferation marker Ki67 (green). $Ki67^{+}Olig2^{+}$ cells indicate immature proliferative oligodendrocytes (arrows). Scale bar: 100 µm. (b) Quantification of $Ki67^{+}Olig2^{+}$ cells in the corpus callosum at P5 (control: n=5; WMI: n=6), P18 (control: n=10; WMI: n=17) and P69 (control: n=10; WMI: n=10). (c) Cortical areas of control vs. WMI animals stained for oligodendrocyte differentiation marker CNPase and oligodendrocyte marker Olig2 at P5, P18 and P69. Differentiated $CNPase^{+}Olig2^{+}$ oligodendrocytes (arrows) have matured further compared to undifferentiated $CNPase^{-}Olig2^{+}$ oligodendrocytes (arrowheads). Scale bar: 100 µm. (d) Quantification of the percentage of $CNPase^{+}Olig2^{+}$ cells in the sensory cortex of control and WMI animals at P5 (n=5; n=6), P18 (n=7; n=17) and P69 (n=10; n=10). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; n.s.: not significant.

S4). In addition to microglia activity, we assessed astrocyte reactivity at P18, medial to the cingulum. We observed a significant increase in $GFAP^{+}$ area in WMI animals compared to control pups (p=0.041) (Figure 4g,h). Interestingly, at P5 we observed pathological $GFAP^{+}$ gliosis patches in varying cortical areas, in 30% of the WMI animals (Figure 4i). $GFAP^{+}$ gliosis patches were not observed in control animals at P5, nor were they observed in WMI animals at P18 or later, indicating that these $GFAP^{+}$ patches disappear over time.

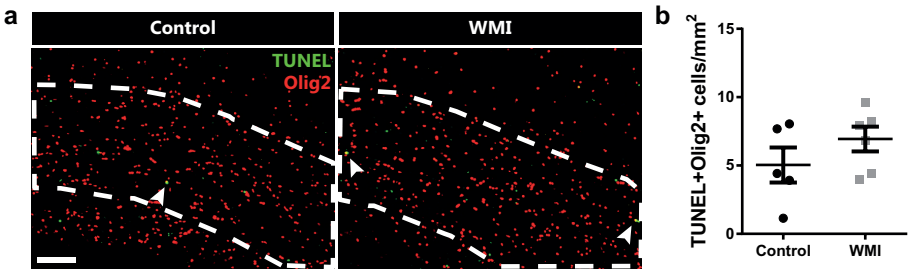
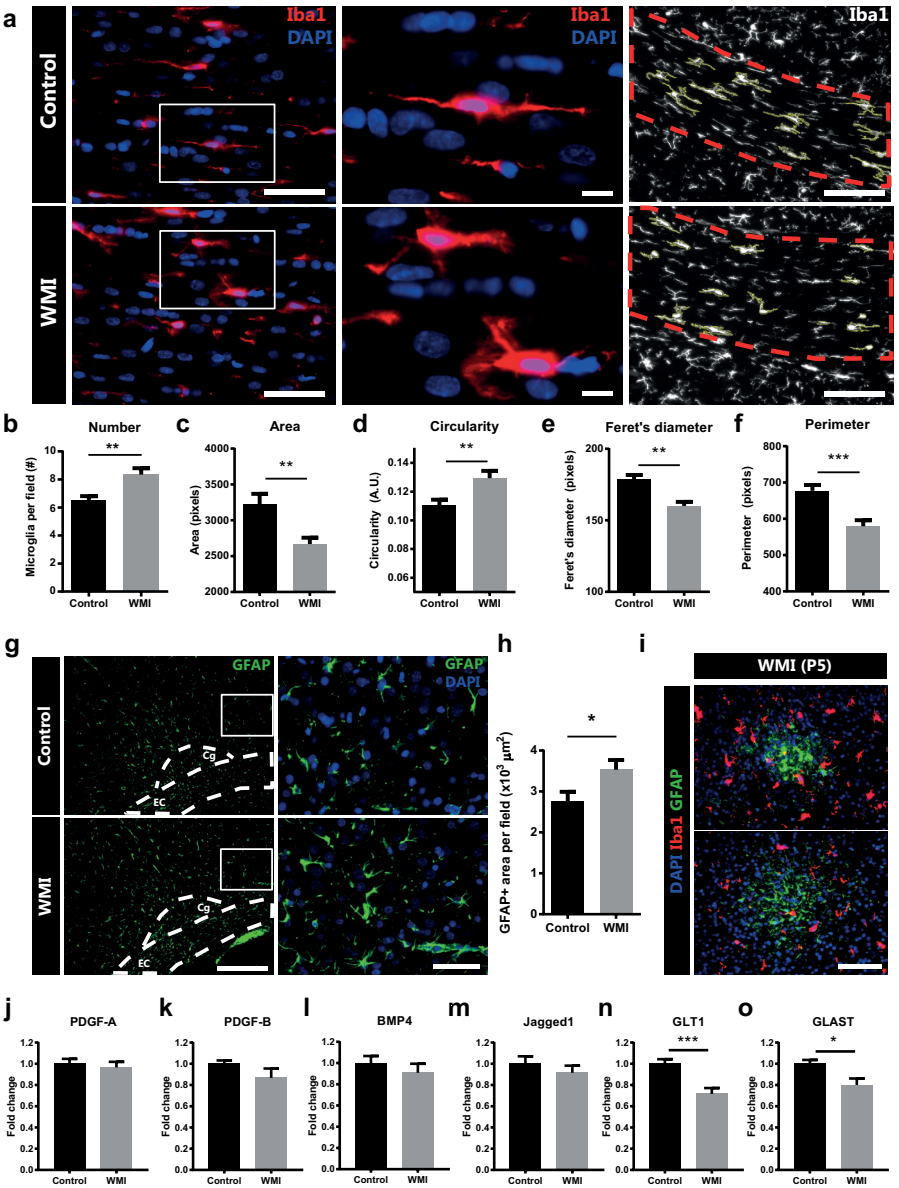


Figure 3 WMI is not associated with increased oligodendrocyte cell death at P5. (a) Corpus callosum (outlined) of control or WMI animals at P5 double-stained for DNA fragmentation marker TUNEL (green) and oligodendrocyte marker Olig2 (red) (arrowheads). Scale bar: 100 µm. (b) No significant changes in the number of $TUNEL^{+}Olig2^{+}$ cells were observed in the corpus callosum of control (n=5) vs. WMI animals (n=6).

To investigate in what way reactive astrocytes may contribute to the pathology observed in WMI animals, one day after hypoxia we measured cerebral mRNA expression levels of genes that were previously associated with astrocyte-mediated myelination impairments. Expression levels of platelet derived growth factor (PDGF)-A, PDGF-B, bone morphogenic protein (BMP)4 and Jagged1 (p=0.631; p=0.176; p=0.413; p=0.393, respectively) were not affected by WMI as compared to control animals (Figure 4j-m). Interestingly, WMI animals showed significantly reduced mRNA expression levels of both glutamate transporter (GLT)-1 (p=0.0005) and glutamate aspartate transporter (GLAST) (p=0.012) compared to control rats (Figure 4n,o). These findings provide an interesting lead regarding the mechanisms underlying WMI resulting from fetal inflammation plus postnatal hypoxia.

A negative correlation between cortical FA values and MBP staining

Diffuse WMI in preterm infants is associated with reduced FA values, which are predictive of impaired cognitive outcome (Ment, Hirtz, & Huppi, 2009; Thompson et al., 2016; van Kooij et al., 2012). Using postmortem MRI, we assessed the size, as well as diffusivity and FA parameters of various brain regions at P30. WMI animals did not show any gross neuroanatomical changes (Figure 5a). Furthermore, no differences between WMI animals and controls were observed in the size of predefined ROIs, nor in the FA values and mean diffusivity values of these regions, including the corpus callosum (Supporting Information Table S1; Figure 5b,c). No significant changes were observed in relative white matter volumes (control: 20.4% +/- 1.1%; WMI: 18.7% +/- 0.9%), however we observed a negative correlation between FA values and myelination as determined by MBP immunodensity in several cortical areas (Figure 5d-f).


a
Control
WMI
Iba1
DAPI
Iba1
DAPI
Iba1
b
Number
Microglia per field (#)
Control
WMI
c
Area
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d
Circularity
Circularity (A.U.)
e
Feret's diameter
Feret's diameter (pixels)
f
Perimeter
Perimeter (pixels)
g
GFAP
GFAP
DAPI
Cg
EC
h
GFAP+ area per field (x10³ µm²)
i
WMI (P5)
DAPI Iba1 GFAP
j
PDGF-A
k
PDGF-B
l
BMP4
m
Jagged1
n
GLT1
o
GLAST
Fold change

Figure 4. Microglia and astrocyte activity in WMI animals. (a) Left and middle: corpus callosum of P18 control vs. WMI animals stained for microglia marker Iba1 (red) and nuclear marker DAPI (blue). Middle panels show higher magnification of left panels. Right: the right panel demonstrates how the morphology of microglia (Iba1 staining of P18 animals in white) in the corpus callosum was assessed. The red line marks the corpus callosum. Yellow lines highlight included microglia. Scale bars: 50 µm (left), 10 µm (middle), 100 µm (right). (b) The number of $Iba1^+$ microglia per field was increased in WMI animals compared to control animals at P18. (c-f) For each animal, several morphological aspects of all measured microglia in the corpus callosum were averaged and recorded. Increased circularity and decreased area, Feret's diameter and perimeter indicate a more pro-inflammatory phenotype in WMI animals at P18 (Zanier, Fumagalli, Perego, Pischiutta, & De Simoni, 2015). (g) GFAP staining along the white matter of P18 control and WMI rats (GFAP: green; DAPI: blue; Cg: cingulum; EC: external capsule). Scale bars: 200 µm (left), 50 µm (right). (h) Quantification of $GFAP^+$ area reveals increased GFAP reactivity in WMI animals compared to control animals at P18. (a)-(h): control (n=10) vs. WMI animals (n=17). (i) Staining for microglia marker Iba1 (red), astrocyte marker GFAP (green) and DAPI (blue) reveals cortical $GFAP^+$ patches in P5 animals. Scale bar: 100 µm. (j-o) Compared to control rats, WMI animals do not show changes in cerebral mRNA expression of PDGF-A, PDGF-B, BMP4 or Jagged1 at P5. WMI animals show a decrease in cerebral mRNA expression of GLT-1 and GLAST, indicating that reactive astrocytes may contribute to WMI pathology by attenuating their glutamate reuptake (control: n=12; WMI: n=13). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Functional consequences of WMI include impaired motor skills and autism-like behavior.

In order to assess functional consequences of WMI in rats, animals were subjected to a number of behavioral tests. First of all, WMI animals demonstrated impaired motor performance on the rotarod task (p=0.009) (Figure 6a). Secondly, no changes were observed in object recognition memory (p=0.736) or working memory (p=0.568) (Figure 6b,c), as assessed by performance on the NORT and delayed spontaneous alteration on the T-maze, respectively. Thirdly, anxiety-related behavior was assessed on the open field task and the EPM task. In the open field task, WMI animals entered the inner zone of the arena less frequently compared to control animals (p=0.035), which is a sign of anxiety-like behavior (Figure 6d). On the EPM, we observed a significant main effect of sex, indicating that female animals spent more time in the open arms compared to males (p=0.031). However, there was no significant main effect of WMI, nor was there a significant interaction between sex and WMI (Figure 6e). On both the open field task and the EPM, no differences in locomotor activity were observed between control and WMI animals (data not shown).

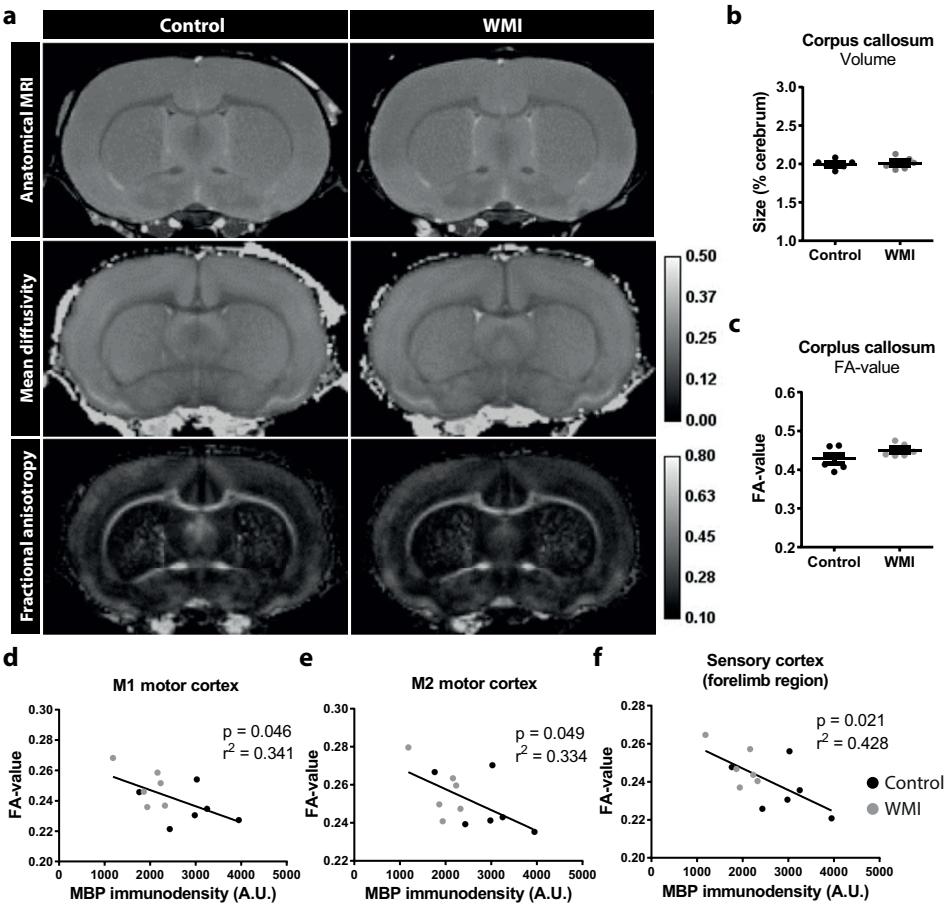

Figure 5. MRI scans reveal no gross neuroanatomical changes in WMI animals. (a) Representative anatomical images (top), mean diffusivity (middle) and FA (bottom) maps from control and WMI animals reveal no gross changes in neuroanatomy, nor in diffusion parameters. (b,c) WMI animals did not show a reduction in the relative size or FA values of the corpus callosum compared to controls (p=0.771, p=0.139, respectively) (n=6 per group). (d-f) Negative correlations were observed between cortical MBP immunostaining and FA values in the M1 cortex (R^2=0.341; p=0.046), the M2 cortex (R^2=0.334; p=0.049) and the forelimb region of the sensory cortex (R^2=0.428; p=0.021).

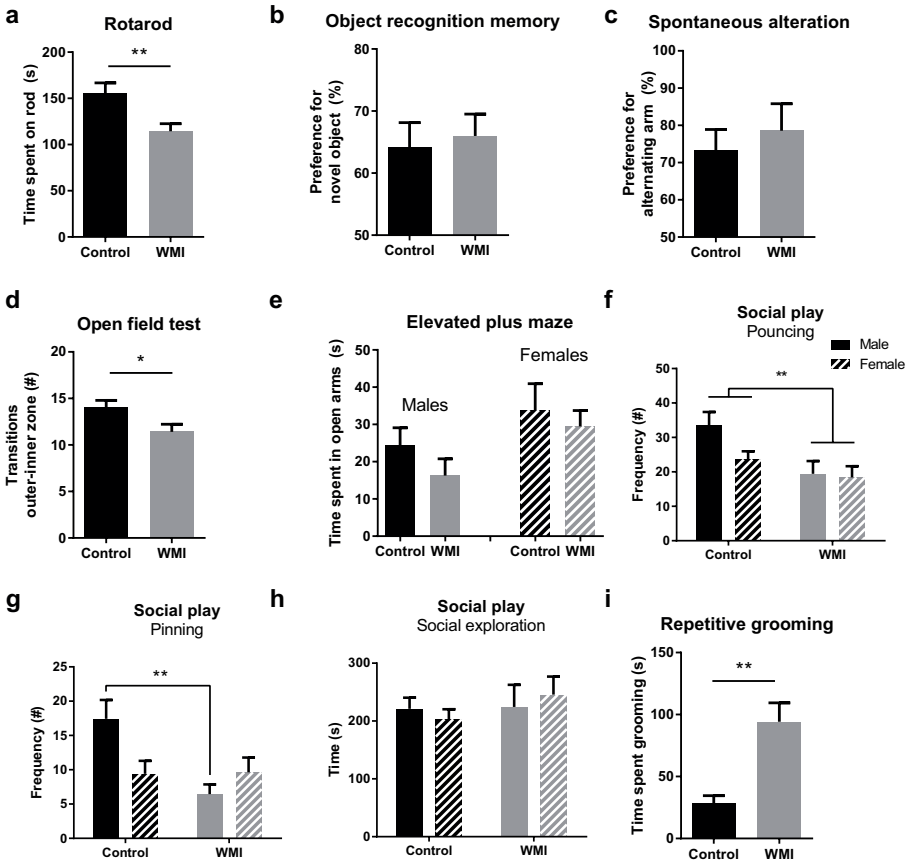

Figure 6. Functional consequences of combined fetal inflammation and postnatal hypoxia. (a) WMI animals (n=12) performed worse compared to control animals (n=11) on the rotarod (RTR) task, indicating impaired motor coordination. (b) No changes between control (n=13) and WMI animals (n=15) in object recognition memory were observed on the novel object recognition task. (c) No differences were observed between control (n=9) and WMI animals (n=9) on delayed spontaneous alteration in a T-maze. (d) Compared to control animals (n=12), WMI animals (n=15) entered the inner zone of the open field less frequently. (e) On the elevated plus maze, we observed significant sex differences. However, no significant differences were found in male control (n=14) vs WMI (n=12) animals or in female control (n=15) vs WMI (n=7) animals in time spent in the open arms of the EPM. (f) During analysis of social play behavior, we observed a significant main effect of WMI on pouncing behavior. There was no significant effect of sex, nor was there an interaction between sex and WMI (males: n=14, n=11; females: n=15, n=8). (g) No significant differences were observed between males and females on pinning behavior. However, we observed a significant interaction between sex and WMI. Multiple comparisons revealed that WMI reduced pinning behavior in males (control n=14; WMI n=11), but not in females (control n=15; WMI n=8). (h) Social exploration was not affected by WMI in both males (n=8 couples; n=6 couples) and females (n=8 couples; n=4 couples). (i) WMI animals (n=10) spent more time grooming, compared to control animals (n=9). *: $p < 0.05$; **: $p < 0.01$.

Preterm infants are at risk to develop ASD (Dudova et al., 2014; Johnson et al., 2010; Joseph et al., 2017; Kuzniewicz et al., 2014; Limperopoulos et al., 2008; Pritchard et al., 2016; Treyvaud et al., 2013). To assess autism-like behavior, we studied social play behavior and grooming behavior of the animals. Social functioning was assessed at the age of 4 weeks by analyzing social play behavior after 2.5 hours of social isolation. Several differences were observed in play behavior. First, WMI animals demonstrated reduced pouncing behavior (main effect WMI: $p=0.009$), regardless of sex (main effect sex: $p=0.124$; interaction: 0.214) (Figure 6f). For pinning behavior, a statistically significant interaction was observed between sex and WMI ($p=0.019$): compared to control animals, males with WMI showed reduced pinning behavior ($p=0.002$), whereas this effect was not observed in females ($p>0.999$) (Figure 6g). General social exploration (e.g. sniffing) was not affected by WMI in both sexes ($p=0.399$) (Figure 6h), indicating a specific effect on play behavior. Additionally, WMI animals showed a 3-fold increase in repetitive grooming behavior compared to control animals ($p=0.002$) (Figure 6i), which is associated with autism-like behavior (Kalueff et al., 2016).

DISCUSSION

In the present study, we assessed the functional and neuroanatomical consequences of a multiple hit model of diffuse WMI in rats, by behavioral paradigms, MRI and immunostainings. Whereas exposure to a single hit did not induce myelination deficits, rats exposed to both fetal inflammation and postnatal hypoxia displayed a pathology closely resembling that of preterm infants with diffuse WMI, including delayed myelination and impaired oligodendrocyte differentiation. At P5, 30% of the WMI animals showed cortical gliosis patches that disappear over time and might originate from microscopic ischemic or hemorrhagic events, or from blood-brain barrier leakage. Furthermore, a more amoeboid morphology of microglia was observed in both the white and gray matter, indicating a primed or pro-inflammatory phenotype of these cells, which may contribute to the hampered maturation of oligodendrocytes (Pang et al., 2007). Additionally, we observed a widespread increase in microglia numbers. Considering microglia are highly proliferative cells that undergo clonal expansion after injury (Askew et al., 2017; Tay et al., 2017), it is likely that increased numbers of Iba1+ cells are a consequence of increased proliferation (Umekawa et al., 2015), although the possibilities of microglia migrating from distant brain regions or infiltrating macrophages cannot be excluded. At P18 WMI animals showed increased immunoreactivity of GFAP in comparison to control brains, indicating the presence

of reactive astrocytes. To investigate how astrocyte reactivity may contribute to WMI pathology, we analyzed expression levels of several genes that previously have been associated with astrocyte-mediated myelination impairments. Astrocytes are an important source of PDGF, which inhibits OPC differentiation (Gard et al., 1995; Silberstein et al., 1996). Furthermore, reactive astrocytes have been associated with increased expression of BMP4 and Jagged1, two factors that inhibit OPC differentiation (Hammond et al., 2014; Reid et al., 2012; Wang et al., 2011). No changes were observed in the expression of PDGF, BMP4 or Jagged1 in brains of WMI animals versus controls. Another important function of astrocytes is glutamate reuptake through glutamate transporters GLT-1 and GLAST, which we showed to be downregulated in WMI animals. These results are in line with earlier findings of transiently reduced expression of GLT-1 and GLAST after chronic hypoxia (Raymond et al., 2011). Deficiency in glutamate uptake causes increased availability of glutamate, hampers proper development OPCs through AMPA/NMDA receptor-mediated excitotoxicity (Volpe et al., 2011). These findings provide an interesting lead regarding the mechanisms through which reactive astrocyte may contribute to WMI, but more detailed analyses are needed to directly relate these findings to astrogliosis and to definitively assess the contribution of excitotoxicity to WMI pathology.

In WMI rats, cortical myelination was reduced up to P30. Impeded maturation of oligodendrocytes likely underlies the impaired myelination in WMI animals, as indicated by increased numbers of proliferating OPCs and reduced proportion of more mature CNPase-expressing oligodendrocytes at P18. Also, we did not find evidence of increased oligodendrocyte cell death at P5. In addition, our data indicate that at least to the extent of the sensory cortex, white matter development is delayed rather than permanently damaged since myelination deficits seem largely restored by the age of P69. Interestingly, long-term changes in oligodendrocyte maturation remained present at P69, as indicated by the lower number of proliferating OPCs in the corpus callosum and the percentage of $CNPase^{+}Olig2^{+}$ cells in the cortex. An explanation for the reduced number of proliferating OPCs at P69 might be that due to increased proliferation during development (P18) the OPC pool is expanded, resulting in a downregulation of proliferation at later ages. This would be in line with findings of increased numbers of $Olig2^{+}$ cells in postmortem WMI brain tissue (Buser et al., 2012). Despite partially restored MBP levels at P69, the possibilities cannot be excluded that myelination in brain regions other than the sensory cortex do not fully restore to control levels, or that physiological properties of cortical myelin (e.g. myelin sheath thickness) remain affected at later ages. Alterations in these physiological properties

may account for the impaired functional outcome that remains present in older rats, despite restored MBP levels. Myelin-independent mechanisms may also contribute to impaired functional outcome at later ages. For example, reductions in gamma-aminobutyric acid (GABA)-expressing neurons were observed in post-mortem tissue of preterm infants with perinatal brain injury (Robinson, Li, Dechant, & Cohen, 2006) and damage to GABAergic interneurons has been implicated in the pathophysiology of hypoxia-induced diffuse WMI in mice (Komitova et al., 2013; Zonouzi et al., 2015).

On a behavioral level, WMI animals displayed impaired motor performance, anxiety-like behavior and signs of autism-like behavior, i.e. repetitive self-grooming and a reduction in social play behavior. In line with these findings, recent clinical studies revealed an association between diffuse WMI and motor abnormalities in preterm infants (Guo et al., 2017; Peyton et al., 2016; Peyton et al., 2017). Also, multiple studies have demonstrated that preterm infants are at risk to develop ASD later in life (Dudova et al., 2014; Johnson et al., 2010; Joseph et al., 2017; Kuzniewicz et al., 2014; Limperopoulos et al., 2008; Pritchard et al., 2016; Treyvaud et al., 2013). Although a clear association exists between preterm birth and ASD, and between white matter integrity and ASD (Ameis & Catani, 2015; Ameis et al., 2016; Catani et al., 2016; Fitzgerald, Gallagher & McGrath, 2016; Vogan et al., 2016), the relationship between preterm birth-related WMI and ASD remains poorly investigated. One study showed an association between *cystic* white matter lesions and ASD in preterm infants (Ure et al., 2016). However, the link between ASD and non-cystic, diffuse white matter abnormalities in preterm infants has, to our knowledge, not been studied. Furthermore, both clinical and experimental evidence indicate that inflammation *in utero* is associated with ASD (reviewed in (Estes & McAllister, 2016)). Considering our current data showing signs of autism-like behavior in rats with diffuse WMI, it would be interesting to further investigate whether ASD in preterm infants is directly related to diffuse changes in white matter microstructure. We observed that social play behavior was affected in a more pronounced manner in male WMI rats, compared to females (i.e. males showed attenuated pinning and pouncing, females showed reduced pouncing). This observation seems to reflect the clinical situation, where male gender is an important predictor for ASD in the preterm population (Johnson et al., 2010; Limperopoulos et al., 2008; Stephens et al., 2012). No signs of severe impairments in object recognition memory and working memory were detected. This is in line with the clinical situation, in which preterm birth is generally associated with subtle cognitive impairments, rather than severe mental retardation (Burnett, Scratch, & Anderson, 2013; Nosarti & Froudist-Walsh, 2016). WMI rats show impaired cognitive abilities, more complex behavioral tasks should be performed.

Postmortem MRI revealed no significant differences in MD or FA values in WMI animals compared to control animals at P30. These data are similar to observations made in a rat model of fetal growth restriction, where reductions in FA values were observed at P10, but which returned to normal, or even regionally increased FA values at P21 (Rideau Batista Novais et al., 2016). When interpreting data from these rodent models of neonatal brain injury, it should be considered that such findings are in contrast with the general view that WMI in preterm infants is associated with reduced FA values in white matter tracts, which predict adverse outcome (Allin et al., 2011; Ment et al., 2009; van Kooij et al., 2012). However, various imaging studies demonstrated that preterm birth can also cause regionally increased FA values (Brossard-Racine et al., 2017; Padilla et al., 2014; Travis, Adams, Ben-Shachar, & Feldman, 2015). Together, these data indicate that FA findings in developing mammals are highly time- and region-dependent. This should be taken into account when interpreting MRI data, especially in rodent models considering the high restorative capacity of white matter microstructure that has been observed in various models of neonatal brain injury (Rideau Batista Novais et al., 2016). Additionally, we observed a negative correlation between FA values and myelination as indicated by MBP immunostaining in several cortical areas. Although a negative correlation seems in contrast with expected lower FA values in WMI, increased FA values in the cortex might be explained by reduced arborization of myelinated axons, i.e. less complex myelination of axons in the cortex.

We demonstrated that combined fetal inflammation and postnatal hypoxia induces a relevant pattern of diffuse WMI. As in the clinical situation, the resulting pattern of injury is relatively subtle. A possible limitation of the model is that in contrast to the clinical situation, we did not observe gross neuroanatomical changes such as increased ventricle size and thinning of the corpus callosum. Despite this limitation, we propose that the combination of fetal inflammation and postnatal hypoxia can be used as a novel translational model to investigate underlying mechanisms, as well as effects of novel treatment options on oligodendrocyte maturation, myelination, glial cell activation and related behavioral abnormalities. Previously, various rodent models have been proposed to study perinatal diffuse WMI (Back, & Rosenberg, 2014; Silbereis, Huang, Back, & Rowitch, 2010; van Tilborg et al., 2016)). Hypoxic rearing (Ganat et al., 2002; Ment et al., 1998; Scafidi et al., 2014), transient hyperoxia (Gerstner et al., 2008), maternal inflammation (Rousset et al., 2006), and postnatal systemic inflammation (Favrais et al., 2011) have been demonstrated to affect myelination without causing cystic lesions. These models mimic certain aspects of the distress encountered by preterm infants and can be used as a model for specific populations of preterm infants

that are exposed to e.g. respiratory deficits, ventilation therapy or maternal/postnatal infections. Our multiple-hit model distinguishes itself from the previously mentioned models, as it takes in to account the multifactorial etiology of diffuse WMI in preterm infants (Korzeniewski et al., 2014; Leviton et al., 2013), by applying two subtle insults during a peak of immature oligodendrocytes residing in the developing brain (Salmaso et al., 2014; Semple et al., 2013). Similarly, it was shown that the combination of LPS exposure at P3 plus hyperoxia for 24 hours at P6 negatively affects white matter development in mice (Brehmer et al., 2012). Our data show that the combination of both the inflammatory and the hypoxic insult is required to induce white matter deficits. It has been suggested previously that early inflammatory insults sensitize the brain to subsequent injurious events (Kaindl et al., 2009; Van Steenwinckel et al., 2014). Interestingly, the intrauterine environment is relatively hypoxic compared to extra-uterine conditions. Yet, due to immature and often diseased respiratory, vascular and immune systems, preterm infants are often exposed to unstable oxygen saturation levels (Van Tilborg et al., 2016). Although optimal oxygen saturation levels in preterm infants are still an important topic of debate (Castillo et al., 2008; Lakshminrusimha et al. ,2015), in case of hypoxia infants are often treated with ventilation therapy, receiving supplemental oxygen which further contributes to large fluctuations in oxygen levels and which has been associated with increased levels of harmful oxidative stress (Perrone et al., 2017). Oxygen instability has been used in a variety of neonatal rodent models to mimic preterm birth-related brain injury. For example, hyperoxic and intermittent hypoxic periods in neonatal rodents have been shown to cause brain damage (Darnall et al. 2017; Gerstner et al., 2008). Furthermore, hypoxic rearing by placing complete litters with nursing mothers at 9-11% O_2 for >7 days starting at P3 causes myelination deficits (Ment et al., 1998; Scafidi et al., 2014). In comparison, the hypoxic insult (140 minutes at 8% O_2) used in the present study is relatively subtle and short-lived but due to an earlier sensitizing inflammatory insult, the detrimental effects may be aggravated. Considering the various types of inflammatory insults and oxygen fluctuations that preterm infants often encounter, the combination of two (relatively mild) hits is highly relevant for clinical translation.

Taken together, combined fetal inflammation and postnatal hypoxia induces clinically relevant diffuse white matter pathology in neonatal rats in terms of neuroanatomy and functional outcome. This model can contribute to the investigation of novel treatment strategies aimed at attenuating neuroinflammation and promoting oligodendrocyte differentiation and myelination, which can lead to development of new, desperately needed therapies to combat diffuse WMI in preterm infants.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

All supplementary data can be found in the online version of this article. Doi: *10.1002/glia.23216*

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5

CHAPTER 5

A quantitative method for microstructural analysis of myelinated axons in the injured rodent brain

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ABSTRACT

MRI studies (e.g. using diffusion tensor imaging) revealed that injury to white matter tracts, as observed in for instance perinatal white matter injury and multiple sclerosis, leads to compromised microstructure of myelinated axonal tracts. Alterations in white matter microstructure are also present in a wide range of neurological disorders including autism-spectrum disorders, schizophrenia and ADHD. Whereas currently myelin quantity measures are often used in translational animal models of white matter disease, it can be an important valuable addition to study the microstructural organization of myelination patterns in greater detail. Here, we describe methods to extensively study the microstructure of cortical myelination by immunostaining for myelin. To validate these methods, we carefully analyzed the organization of myelinated axons running from the external capsule towards the outer layers of the cortex in three rodent models of neonatal brain injury and in an adult stroke model, that have all been associated with myelination impairments. This unique, relatively easy and sensitive methodology can be applied to study subtle differences in myelination patterns in animal models in which aberrations in myelination integrity are suspected. Importantly, the described methods can be applied to determine efficacy of novel experimental treatments on microstructural organization of cortical myelination.

INTRODUCTION

Around 40% of the human brain consists of white matter, which mainly contains myelinated axons that allow rapid transmission of action potentials to distant brain areas[1, 2]. Oligodendrocytes are specialized cells in the central nervous system responsible for the myelination of neuronal axons. Proper myelination not only allows efficient axonal signal transduction, but also provides protection and nutritional support for neurons[3, 4]. Injury to the myelinated white matter has devastating consequences for neural connectivity, causing (severe) impaired neurological performance and reduced quality of life, as observed in for instance multiple sclerosis (MS), stroke and neonatal white matter injury (WMI)[5-7]. Diffusion tensor imaging (DTI) studies revealed that in various white matter diseases the white matter ***microstructure*** is affected as indicated by reduced fractional anisotropy (FA) measurements[8-12]. Moreover, recent studies show that similar changes in white matter microstructure are associated with a wide array of psychological disorders, including autism spectrum disorders[13, 14], schizophrenia[15, 16], bipolar disorder[17] and ADHD[13, 18]. As a result, an increased interest has emerged to study myelination in animal models of neurological disease, with the aim of elucidating the mechanisms underlying impaired white matter integrity and to develop novel treatment options to restore myelination under pathological conditions.

Here, we demonstrate using four different rodent models of both neonatal brain injury and adult stroke that staining brain sections for myelin-basic-protein (MBP) enables highly detailed analysis of the microstructural integrity of myelinated axons running from the external capsule in the deep white matter towards the outer cortical layers. In a rat model of neonatal diffuse WMI, a mouse model of neonatal WMI, and in a mouse model of neonatal asphyxia (comprising both neuronal injury and WMI), we show reduced cortical myelination, reduced immunodensity of MBP staining, reduced MBP-positive (MBP+) area and increased coherency of myelinated axons by conventional methods. Moreover, we introduce and validate a novel sensitive method for the detailed quantification of microstructural complexity (i.e. fiber length, number of intersections) of cortical myelination in these models. The method was also reliably applied in an adult rodent model of stroke. In addition to immunofluorescent stainings for MBP, the method can also be applied using DAB stainings and/or other myelin markers, like PLP.

MATERIALS AND METHODS

Animals

All procedures were carried out in accordance with Dutch and European regulations and were approved by the Animal Ethics Committee of Utrecht University. All efforts were made to minimize animal suffering. Animals of both sexes were used in all neonatal models and experimental conditions were divided among all different litters. No sex differences were observed in this study. For the adult MCAO model of stroke, male rats were used (see below).

Rat model of neonatal diffuse WMI

We used a rat model of neonatal diffuse WMI that was recently introduced[19]. Timed-pregnant Wistar rats (Envigo, Horst, The Netherlands) received intraperitoneal (i.p.) injections of 100 µg/kg LPS (from E. Coli O55:B5; L2880, Sigma-Aldrich, St. Louis, MO) in saline or vehicle on embryonic days (E)18 and E19 of pregnancy. At postnatal day 4 (P4), offspring was placed in a temperature-controlled hypoxic chamber containing 8% O_2 or in a normoxic environment away from the mother for 140 minutes. Afterwards, animals were returned to their dams. Rats that received fetal saline and postnatal normoxia (referred to as 'control', n=8), rats that received fetal saline and postnatal hypoxia ('hypoxia only'; n=7), rats that received fetal inflammation and postnatal normoxia ('LPS only'; n=7), and animals that received both fetal inflammation and postnatal hypoxia ('FIPH', n=8) were euthanized at P18 (i.e. 2 weeks post-hypoxia).

Mouse model of neonatal WMI

Neonatal unilateral WMI was induced in C57BL/6 mice (Envigo) by inducing a hypoxic-ischemic insult, directly followed by an inflammatory stimulus, as adapted from Shen et al.[20, 21]. Briefly, at P5 'HI+LPS' mouse pups (n=6) underwent permanent occlusion of the right common carotid artery under isoflurane anesthesia, followed by exposure to 35 minutes of hypoxia (6% oxygen). Immediately after hypoxia, pups received 1 mg/kg LPS i.p. (List Biological Laboratories, Campbell, CA). Sham 'control' animals (n=6) underwent surgical incision only, without occlusion of the carotid artery and without hypoxia. Procedures were carried out under temperature-controlled conditions and afterwards animals returned to their dams. Mice were euthanized at P26 (i.e. 3 weeks post-HI+LPS).

Mouse model of neonatal asphyxia

Unilateral neonatal hypoxic-ischemic brain injury was induced in C57BL/6 mouse

pups (Envigo,) as described earlier[22]. Briefly, at P9 'HI' mouse pups (n=8) underwent permanent occlusion of the right common carotid artery under isoflurane anesthesia, followed by 45 minutes of hypoxia (10% oxygen). Sham 'control' animals (n=8) underwent surgical incision only, without occlusion of the carotid artery and without hypoxia. All procedures were carried out under temperature-controlled conditions and afterwards animals returned to their dams. Mice were euthanized at P37 (i.e. 4 weeks post-HI). Animals with severe cystic lesions in the cortex (<10%) were not included in the analysis.

Rat model of adult stroke

Adult male Sprague Dawley rats (280–320 g) underwent unilateral middle cerebral artery occlusion (MCAO) (n=3) as described earlier[23, 24]. Briefly, while anesthetized with isoflurane the right middle cerebral artery was occluded by an intraluminal filament for 90 minutes. Sham-operated 'control' animals (n=3) received a surgical incision under isoflurane anesthesia, without MCAO. Animals were euthanized at 70 days post-MCAO.

Histology

All animals were sacrificed by an overdose of 250 mg/kg pentobarbital, followed by transcardial perfusion with PBS and 4% PFA in PBS. Brains were post-fixed for 24 hours in 4% PFA and dehydrated by incubation in increasing concentrations of ethanol, before being embedded in paraffin. 8 µm coronal sections were cut at the level of bregma (rats) or at the hippocampal level (corresponding to -1.80 mm from bregma in adult mice (Paxinos brain atlas)).

Immunofluorescent stainings

Sections were deparaffinized in xylene, followed by rehydration in decreasing ethanol concentrations. For antigen retrieval, sections were heated to 95°C in 10 mM sodium citrate buffer (pH=6.0) for 20 minutes. After cooling down and 3 washes in PBS, sections were blocked with 0.1% saponin (Sigma-Aldrich) and 2% BSA (Sigma-Aldrich) for 30 minutes at room temperature, followed by overnight incubation with primary antibodies against MBP (SMI-94; Sternberger Monoclonals Incorporated, Lutherville, MD; mouse; 1:1000), NF200 (N4142; Sigma-Aldrich; rabbit; 1:400) or PLP (ab105784, Abcam, Cambridge, UK; rabbit; 1:400) diluted in PBS. The next day, sections were washed 3 times in PBS and incubated with alexa-fluor conjugated secondary antibodies goat-anti-mouse AF488 and goat-anti-rabbit AF594 (both Life Technologies, Carlsbad, CA, 1:200) for 2 hours at 37°C. Sections were counterstained with DAPI (1:5000).

Fluorescent stainings were visualized using a Cell Observer microscope, equipped with an AxioCam MRm camera, and using an Endow GFP filter (Filter set 38) (all: Zeiss, Oberkochen, Germany) in a blinded fashion. For measuring thickness of myelinated cortex, one picture of each hemisphere in the rat model was acquired at a 2.5X magnification. In the mouse models, one picture of the hemisphere ipsilateral to the injury was taken at a 2.5X magnification. For all other analyses, in the rat FIPH model one picture of each brain hemisphere was taken using a 20X objective at a fixed distance from the external capsule towards the cortical plate; at the level of the most dorsal part of the striatum adjacent to the external capsule (Fig. 2a). Both pictures were analyzed and values were averaged. In the mouse P9 HI and P5 HI+LPS models, 3 adjacent 40X pictures were taken at a fixed distance from the external capsule into the cortex superimposing the ipsi- and contralateral hippocampal areas (Fig. 2b). Pictures were analyzed and values were averaged. In the rat MCAO model, 20X pictures were taken in the perilesional area, and in control animals at a fixed distance from the external capsule into the cortex.

DAB staining

For 3,3'-Diaminobenzidine (DAB) staining, sections were deparaffinized in xylene, followed by 100% ethanol. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 in methanol and sections were then hydrated by decreasing ethanol concentrations. Sections were blocked in 10% normal horse serum in PBS with 0.5% triton X-100, followed by overnight incubation with the primary antibody against MBP (SMI-94, see above; 1:1000). Sections were then washed and incubated in secondary antibody (biotinylated horse-anti-mouse; Vector Laboratories, Peterborough, UK; 1:400), followed by washing in PBS. Biotin was HRP-labeled using a Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions, followed by a washing step in 0.05M Tris-HCl (pH: 7.6). Staining was performed by incubation in 0.5 mg/ml DAB (Sigma) in 0.05M Tris-HCl with 0.009% H_2O_2. Finally, sections were dehydrated in ethanol and embedded. Stained sections were photographed using an AxioLab.A1 microscope equipped with an AxioCam ICc 5 camera (Zeiss) at 2.5X and 40X magnification at similar brain regions as fluorescent stainings.

Image analyses

In order to quantify different aspects of white matter microstructure, ImageJ was used[25], combined with several publically available plugins: OrientationJ[26] and DiameterJ[27]. Image analyses were performed by researchers blinded to experimental conditions.

Thickness of myelinated cortex

The extent of cortical myelination was assessed by measuring the area of the cortical tissue above the external capsule on 2.5X images and by measuring the area of myelinated cortex (Fig. 1a,c,e). The proportion of myelinated cortex was calculated by (myelinated cortex / total cortex) x 100%.

MBP immunodensity and MBP+ area

MBP immunodensity was assessed by measuring the mean grey value, subtracted by the mean value of the background staining. Levels of the animals with brain injury are normalized to levels in the model-specific control animals, which were put at 100%. MBP+ area was determined by manually setting a threshold to include all MBP+ stained tissue, followed by measuring the proportion of the field that was positive for MBP staining.

Coherency of myelinated axons

Coherency of myelinated axons, as an inverse measure of complexity of white matter microstructure, was assessed by measuring the coherency using the 'OrientationJ measure' function of ImageJ plugin OrientationJ[26] in the selected whole micrograph.

Microstructural complexity

Microstructural complexity of cortical myelination (i.e. fiber length, number of intersections) was assessed using a 2-step approach with the ImageJ plugin DiameterJ[27]. The first step is image segmentation, which is necessary for proper tracing of the myelinated axons and can be achieved by removing the background from the image (Process > Subtract background), applying a threshold to include all MBP+ staining and exclude the background. Images were then saved in 8-bit. Noise/small particles were removed by running the macro listed in supplementary file S1 (Process > Batch > Macro: select input and output folders and paste the macro code) (Fig. 4, segmented). Parameters in the batch code depend on aspects such as microscope objective and camera resolution, and should therefore be optimized by the user. As a second step, various parameters regarding the microstructural organization of myelinated axons are measured by the DiameterJ plugin (Plugins > DiameterJ > DiameterJ 1-018: insert correct scaling, no specific radius), with quantification of the parameters (e.g. fiber length, fiber thickness, intersections, etc.) as output. The software will automatically create maps of the MBP+ fiber tracing (e.g. axial thinning).

Statistics

Statistics were performed with Graphpad Prism 6.02. When comparing two groups, model-specific control animals were compared to FIPH, HI, HI+LPS or MCAO animals by unpaired t-tests. In case of unequal variances, t-tests with Welch's correction were used. When comparing more than two groups, one-way ANOVA followed by multiple comparisons with Bonferroni correction was performed.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Impaired myelination in rodent models of neonatal brain injury

In order to study cortical myelination after neonatal brain injury in great detail, brain sections were stained for MBP and various analyses were performed. Myelination was investigated in three rodent models: a rat 2-hit model of neonatal diffuse WMI (fetal inflammation and postnatal hypoxia (FIPH)), a P5 mouse 2-hit model of neonatal unilateral WMI (hypoxia-ischemia (HI)+LPS), and a P9 mouse model of neonatal unilateral asphyxia comprising both grey and white matter injury (HI). In the developing rodent brain, cortical myelination forms a gradient from densely myelinated axons near inner white matter structures such as the external capsule, towards sparsely myelinated outer layers of the cortex. In order to study whether the extent of cortical myelination is affected in rodent models of neonatal brain injury, we measured the proportion of cortical tissue that contains myelinated axons (Fig. 1a,c,e). We observed a reduced percentage of myelinated cortex in all three models of neonatal brain injury (FIPH: $p=0.007$; P5 HI+LPS: $p=0.017$; P9 HI: $p<0.0001$) (Fig. 1).

To investigate whether differences exist in the quantity of myelination, we measured the immunodensity of the MBP fluorescent signal in the cortex (Fig. 2a,b) for neonatal rats and mice respectively). Furthermore, the MBP+ area of each picture was quantified. In the rat model of diffuse WMI, we observed a reduction in both the MBP immunodensity ($p=0.029$) and in the MBP+ area ($p=0.002$) compared to control animals (Fig. 2c-e). Compared to sham-operated controls, mice exposed to HI+LPS at P5 showed no changes in MBP immunodensity ($p=0.523$), but did show a reduction in MBP+ area ($p=0.001$) (Fig. 2f-h). Furthermore, the mouse model of neonatal asphyxia at P9 caused reductions in both MBP immunodensity ($p=0.026$) and MBP+ area ($p=0.001$) (Fig. 2i-k).

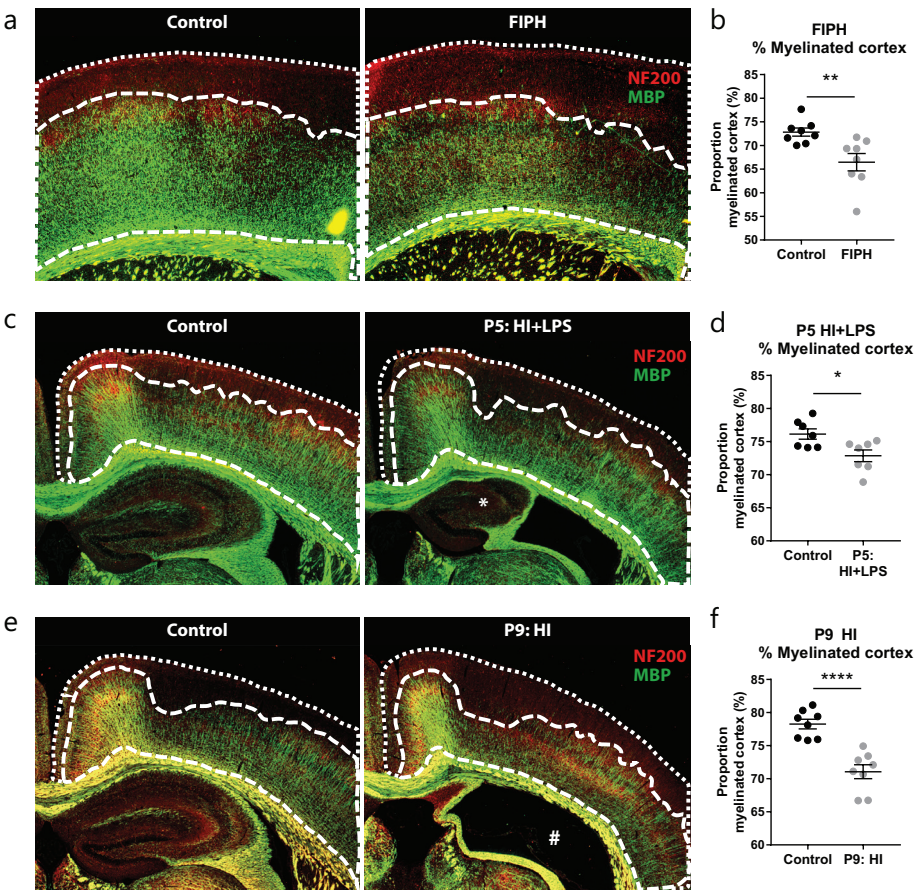

Figure 1 Neonatal brain injury is associated with reduced myelination in outer cortical layers. (**a**) Representative micrographs of a control rat (upper panel) and a rat exposed to fetal inflammation (LPS at E18+E19) and postnatal hypoxia at P4 (FIPH model; lower panel) stained for axonal marker NF200 (red) and myelin marker MBP (green). (**b**) Bar graph showing that rats exposed to FIPH display reduced extent of cortical myelination (n=8 per group). (**c**) Representative images of sham-operated control mice (left) or mice exposed to HI+LPS at P5 (right) stained for MBP (green) and NF200 (red). *: enlarged ventricle and reduced ipsilateral hippocampal volume which is typically observed in this model (**d**) Bar graph illustrating that HI+LPS mice show reduced extent of cortical myelination (n=6 per group). (**e**) Representative images of sham-operated control mice (left) or mice exposed to HI on P9 (right) stained for MBP (green) and NF200 (red). #: severely injured hippocampal area characteristic for this model (**f**) Bar graph illustrating that HI mice show reduced extent of cortical myelination (n=8 per group). Data represent mean ± SEM; *p<0.05; **p<0.01; ****p<0.0001.

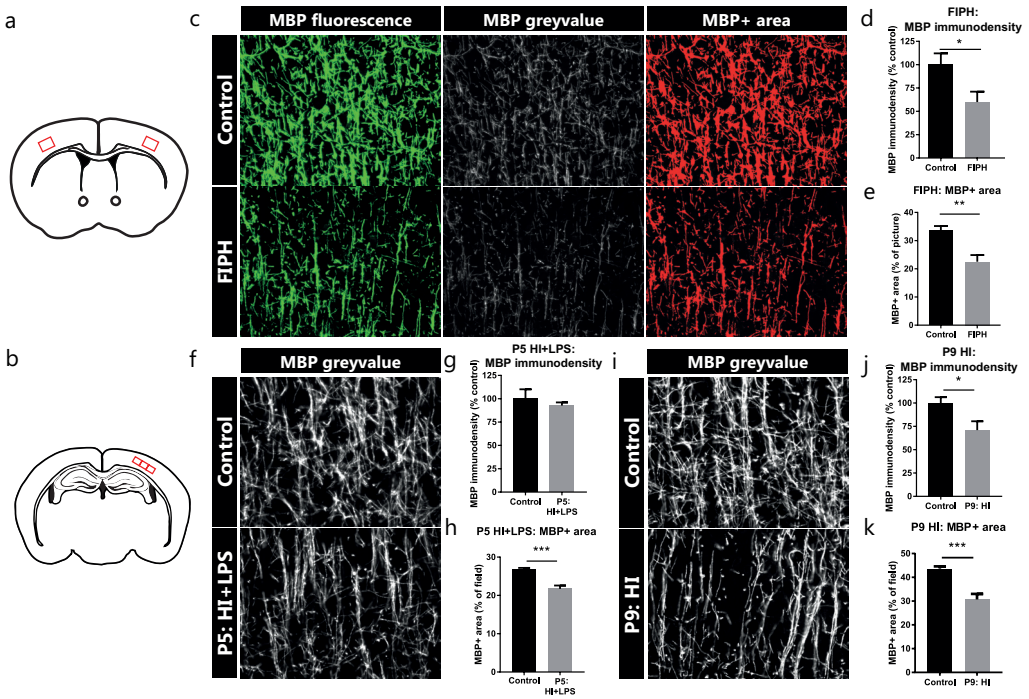

Figure 2. MBP immunodensity and MBP+ area in rodent models of neonatal brain injury. (**a**) Illustration of coronal section of rodent brain with the cortical locations (red squares) of obtained micrographs for the rat FIPH model. (**b**) Illustration of coronal section of rodent brain with cortical locations (red squares) of obtained micrographs for both mouse models of neonatal unilateral brain injury. (**c**) Representative pictures of MBP+ axons in the cortex of control rats (upper panels) and rats exposed to fetal inflammation (LPS at E18+E19) and postnatal hypoxia (P4) (FIPH; lower panels). Left: original fluorescent signal of MBP staining (green); middle: grey-scale image of MBP fluorescent signal (white) from which immunodensity is measured; right: threshold set to include MBP+ signal (red) and exclude background to measure MBP+ area. (**d,e**) Bar graphs showing that rats exposed to FIPH display reduced MBP immunodensity as well as a decrease in MBP+ area (n=8 per group). (**f,i**) MBP fluorescent signal (white) in the cortex of control mice exposed to sham-operation (upper panel) or mice exposed to HI+LPS at P5 (lower panel, **f**) or to HI at P9 (lower panel, **i**). (**g**) No change was observed in MBP immunodensity in mice exposed to HI+LPS at P5 compared to control mice (n=6 per group). (**h**) P5 HI+LPS animals showed a decrease in MBP+ area compared to sham-operated controls (n=6 per group). (**j,k**) Compared to controls, mice exposed to HI at P9 showed reduced MBP immunodensity (n=8 per group) as well as a decrease in MBP+ area (n=8 per group). Data represent mean ± SEM; *p<0.05; **p<0.01; ***p<0.001.

Coherency as a measure of complexity in the organization of myelination patterns

In order to investigate changes in the organization of cortical myelination in greater detail, we measured the coherency of myelinated axons as an inverse measure of arborization/complexity of myelination patterns (Fig. 3a). Compared to control animals, rats exposed to FIPH showed an increased coherency in axonal myelination, indicative of reduced complexity ($p=0.041$) (Fig. 3b,c). These results indicate that axons running from the deep white matter towards the outer cortex (vertically oriented) remained relatively spared, whereas myelination of perpendicular oriented intersecting axons (horizontally oriented) are most severely affected by exposure to FIPH. Furthermore, whereas mice exposed to HI+LPS at P5 did not show a statistically significant increase in coherency of MBP+ axons ($p=0.105$) (Fig. 3d,f), mice exposed to HI at P9 did show an increased coherency ($p=0.029$) versus their sham-operated controls (Fig. 3e,g).

Reduced microstructural complexity of myelination patterns after neonatal brain injury

In order to get more detailed measures of the microstructural complexity of myelination patterns in the rodent cortex, myelinated axons were digitally tracked and analyzed. Various parameters regarding the organization of the white matter microstructure were calculated, including fiber length and the number of intersections. Compared to controls, only rats exposed to both FI+PH hits showed a reduction in fiber length ($p=0.008$) and a reduction in the number of intersections of MBP+ axons ($p=0.007$) (Fig. 4a-c). Single insults (i.e. PH only or FI only) did not affect microstructural organization of myelination: PH only did not affect fiber length ($p=0.182$) or the number of intersections ($p>0.999$), nor did only FI negatively affect either parameter ($p=0.116$; $p>0.999$, respectively) (Fig. 4b,c). Similarly, the injured (ipsilateral) hemisphere of mice exposed to HI+LPS at P5 showed reductions in fiber length and the number of intersections compared to the contralateral hemisphere or compared to the ipsilateral hemisphere of control littermates (fiber length: $p=0.020$, $p=0.013$; intersections: $p=0.022$, $p=0.002$, respectively) (Fig. 4d-f). Also, mice exposed to HI at P9 displayed statistically significant (or a trends towards) reductions in both fiber length and the number of intersections compared to the contralateral hemisphere (fiber length: $p=0.014$; intersections: $p=0.004$) or compared to either hemisphere of control animals (contra: $p=0.011$, $p=0.058$; ipsi: $p=0.002$, $p=0.005$, respectively) (Fig. 4g-i). Altogether, these results indicate that measures like fiber length and the number of intersections provide a sensitive and specific method for quantifying complexity of myelination in the rodent cortex.

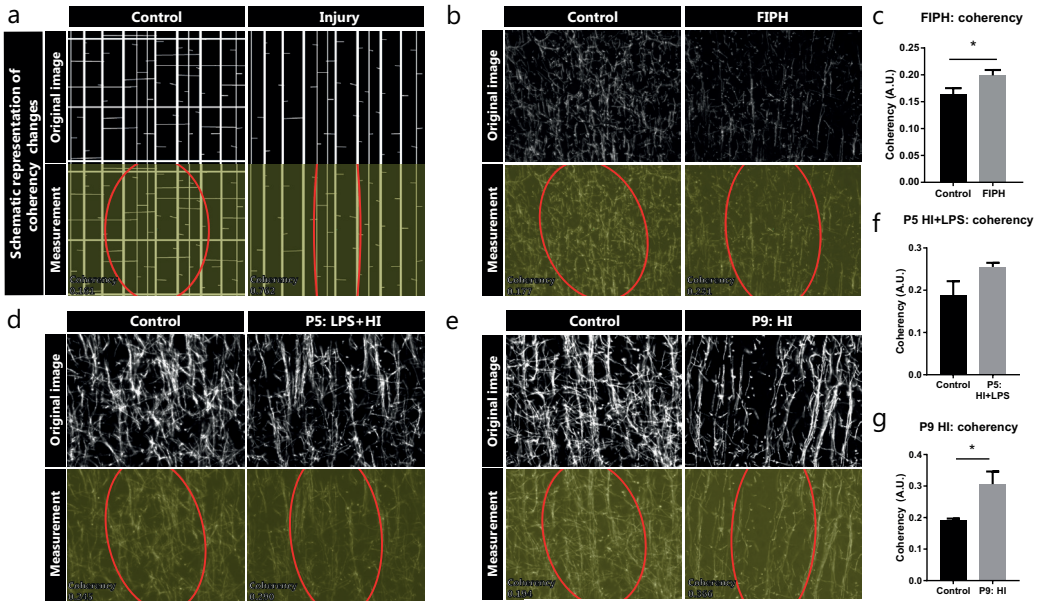

Figure 3. Coherency as an inverse measure of complexity in the organization of myelinated axons. (**a**) Schematic representation of how a less complex organization causes increased coherency between myelinated axons. (**b, d, e**) MBP fluorescent signal (upper panels) and coherency measurement (lower panels) with coherency ellipse (red) of model-specific control animals (left) and rats exposed to fetal inflammation (LPS at E18+E19) and postnatal hypoxia (P4) (FIPH; right **b**), or mice exposed to HI+LPS at P5 (right **d**), or mice exposed to HI at P9 (right **e**). (**c**) Bar graph showing increased coherency of myelinated axons in rats exposed to FIPH (n=8 per group). (**f**) Mice exposed to HI+LPS at P5 do not show statistically significant changes in coherency of myelinated axons in the cortex (n=6 per group). (**g**) Bar graph illustrating increased coherency of myelinated axons in mice exposed to HI at P9 (n=8 per group). Data represent mean ± SEM; *p<0.05.

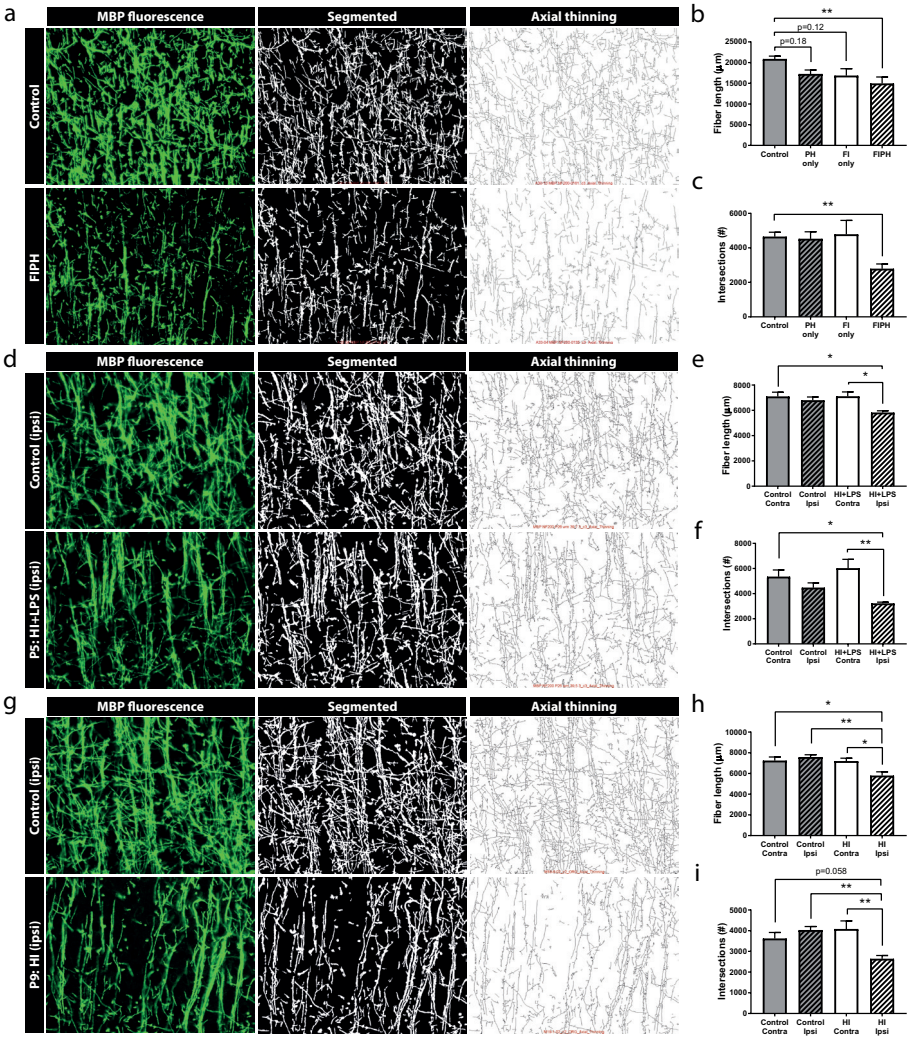

Figure 4. Segmentation of MBP staining allows detailed analysis of microstructural integrity of myelinated cortex. (**a**,**d**,**g**) The original image of MBP fluorescent signal (left), the segmented image extracted from the original image (middle), and the axial thinning map as calculated from the segmented image (right). Upper panels show model-specific control animals and lower panels show (**a**) rats exposed to fetal inflammation (LPS at E18+E19) and postnatal hypoxia (P4) (FIPH); (**d**) mice exposed to HI+LPS at P5 or (**g**) mice exposed to HI at P9. Different parameters of fiber organization can be calculated from these maps. (**b**,**c**) Single insults (postnatal hypoxia (PH) only, n=7; fetal inflammation (FI) only, n=7) do not significantly affect the fiber length or the number of intersections, but the combination of fetal inflammation and postnatal hypoxia (FIPH) (n=8) causes a reduction in both fiber length and intersections compared to control animals (n=8). (**e**,**f**) The ipsilateral (injured; ipsi) hemisphere of mice exposed to HI+LPS at P5 shows decreased length and reduced number of intersections of myelinated axons compared to the contralateral (contra) hemisphere of both control and HI+LPS animals (n=6 per group). (**h**,**i**) The ipsilateral (injured) hemisphere of mice exposed to HI at P9 shows decreased length and reduced number of intersections of myelinated axons compared to the contralateral hemisphere and the hemispheres of the control animals (a trend is observed vs. contralateral hemisphere of control for number of intersections) (n=8 per group). Data represent mean ± SEM; *p<0.05; **p<0.01.

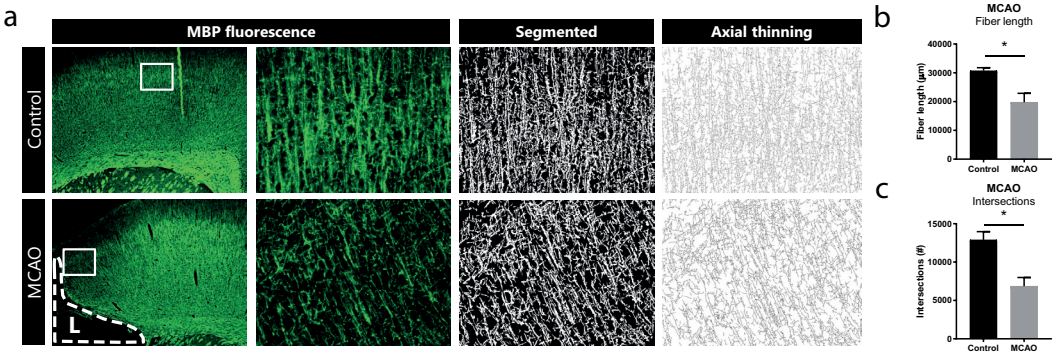

Figure 5. Segmentation of MBP staining allows detailed analysis of the microstructural organization in an adult rat model of stroke by MCAO. (**a**) In brain sections of adult rats, MBP staining allows segmentation and proper tracing of myelinated axons. (**b,c**) At 70 days post-MCAO reduced fiber length and reduced number of intersections are observed in the perilesional area of MCAO animals (n=3), compared to controls (n=3). L= cortical lesion caused by MCAO. Data represent mean ± SEM; *p<0.05.

Reduced microstructural complexity of myelination patterns in a stroke model in adult rats

To find out whether this same methodology to quantify detailed aspects of myelin organization can be applied in adult rodent models of brain injury, we quantified MBP+ fiber length and the number of intersections in adult rats at 70 days post-MCAO and compared them with sham-operated control animals. Also in this adult stroke model, segmentation and tracing of myelinated axons in the perilesional area (MCAO animals) or cortical areas (in control animals) was reliably performed (Fig. 5a). We observed that at 70 days post-MCAO, myelination was still impaired on a microstructural level in the perilesional area: both fiber length and the number of intersections were reduced in MCAO rats compared to control animals (p=0.029; p=0.018, respectively) (Fig. 5b,c).

Quantification of microstructural organization of cortical myelination using other stainings

To study the applicability of the proposed methodology using other types of stainings in comparison to immunofluorescent stainings or to other myelin markers than MBP, we applied the same analysis protocol on a DAB-staining for MBP (Fig. 6a) and on a fluorescent staining for myelin proteolipid protein (PLP) (Fig. 6b), respectively. Using

brain sections of mice exposed to HI at P9, the segmentation and tracing of myelinated axons was successful for both the MBP-DAB staining and the fluorescent PLP staining (Fig. 6). These results highlight the versatility of the proposed methodology and indicate that the analysis procedure can be applied in other (myelin) stainings.

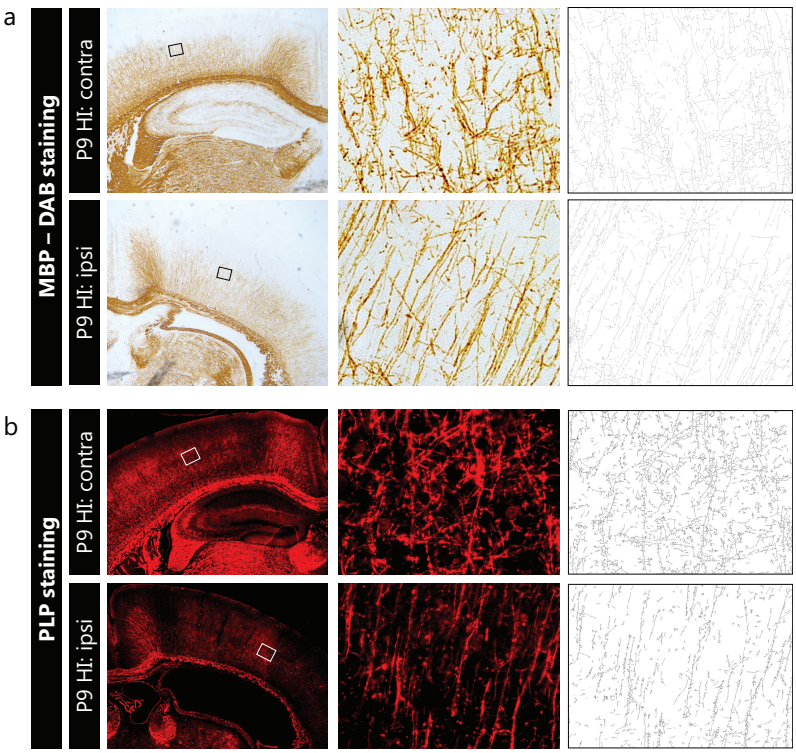

Figure 6. Segmentation of myelinated axons is also applicable on MBP-DAB staining and immunofluorescent PLP staining. (**a**) Contralateral hemisphere (upper panels) and ipsilateral hemisphere (lower panels) of mice exposed to unilateral HI at P9, at 2.5X (left) and 40X (middle) magnification. MBP-DAB staining allowed reliable segmentation and tracing of myelinated axons (right). (**b**) Contralateral hemisphere (upper panels) and ipsilateral hemisphere (lower panels) of mice exposed to unilateral HI at P9, at 2.5X (left) and 40X (middle) magnification. A fluorescent staining for myelin component PLP allowed segmentation and tracing of myelinated axons (right). Squares in 2.5X photograph represent the location of higher magnification images (middle).

DISCUSSION

In the present study, we describe a novel methodology to assess various aspects of white matter microstructure in great detail. The described procedures allow sensitive assessment of several parameters regarding the extent and complexity of cortical myelination patterns. The procedures were applied and validated using four different rodent models of neonatal and adult brain injury, all of which have been reported to cause myelination deficits in rats or mice[19-23]. More specifically, in the neonatal models brain sections stained for myelin marker MBP were analyzed to quantify the percentage of myelinated cortex, MBP immunodensity and MBP+ area, as commonly used measures for the extent of myelination. Furthermore, detailed image analysis allowed assessment of the coherency of myelinated axons. Additionally, a novel sensitive methodology was applied to assess fiber length and number of intersections between MBP+ axons as measures of *microstructural organization*. Even in the most subtle model (HI+LPS in P5 mice), in which no significant differences in MBP immunodensity or coherency of myelinated axons were shown, a clear reduction was observed in fiber length and number of intersections, further highlighting the sensitivity of the described methods. In addition, we confirmed the applicability of the methodology in an adult rat model of stroke. Moreover, we show that the described methodology to quantify fiber length and the number of intersections can also be applied to non-fluorescent stainings (e.g. MBP-DAB immunostaining) and to other myelin markers (e.g. PLP).

Currently, immunodensity and area measurements of myelin markers are considered the 'gold standard' to quantify myelin deviations in various experimental rodent models[28-34]. Although these measures accurately reflect the amount of myelination, relatively subtle deviations in the microstructural integrity of myelinated axons may be overlooked when only assessing immunodensity and/or area measurements of myelin markers (as illustrated in the P5 HI+LPS mouse model). Here, we show that assessment of microstructural integrity is a highly sensitive method to quantify deviations in cortical myelination and organization, thereby providing valuable additional information to immunodensity and area measurements. Considering the high relevance of white matter microstructure for understanding a wide variety of neurological disorders, it will be crucial to take into account these more detailed analyses of myelination patterns in experimental models of brain disease, making these procedures of high translational value. Fiber length and number of intersections of MBP+ axons were the most robust measures in the evaluated rodent models, but the described methodology can provide information on various additional parameters regarding microstructural organization.

In contrast to stereology and MRI techniques, the here described method allows assessment of myelination in a single coronal (2D) plane. The strength of detailed analysis of myelin staining is that it provides additional insight in the quantity and microstructural organization of myelinated axons. A downside can be that conclusions regarding volumetric changes in white matter anatomy or myelination will be difficult; although our analyses can be performed on sections throughout the rodent brain and in various brain regions, as long as the myelinated axons are not too densely organized such as in the corpus callosum. Stereology, a more suitable method to assess volume changes, has previously been performed on electron microscopy (EM) images throughout the brain to accurately assess myelin sheath thickness[35, 36]. However, stereology at a cellular level to assess volumetric changes in the brain would be more challenging, due to the many different brain regions that are functionally, structurally and anatomically distinct. Total gray vs. white matter volume changes can be more straightforwardly determined by whole-brain MRI. Furthermore, MRI-based DTI studies over the past years revealed changes in white matter microstructure, as reflected by altered FA values, in patients suffering from a wide range of neurological disorders, including autism-spectrum disorders, schizophrenia and bipolar disorder[13-18]. MRI can also be applied on rodents. However, the relatively low spatial resolution of MRI makes this technique less appropriate for detection in small (sub)regions as compared to microscopic analysis of MBP immunostaining. In an earlier study performed by our group no FA changes were observed in the FIPH rat model at P30 using MRI, whereas MBP staining did reveal a significant reduction in cortical myelination[19]. In addition, DTI can yield ambiguous results: whereas lowered FA values in white matter are usually interpreted as a reduction in structural integrity, low FA values in *cortical areas* typically reflect a more complex and less coherent microstructural organization. In line, regionally increased FA values as observed in human preterm infants and patients with autism spectrum disorders[37-40] may be explained by increased coherency of myelinated axons as observed in animal models of brain injury[19]. The more advanced diffusion kurtosis imaging (DKI) has been suggested to be more suitable for measuring (changes in) microstructural integrity in complex, rather than coherent fiber arrangements[41], although the relationship between diffusion behavior and tissue microarchitecture appears highly multifaceted[42]. Compared to immunostaining, MRI studies are expensive, require a highly specialized infrastructure and provide indirect measures of microstructural organization. Nevertheless, for clinical translation, MRI techniques remain highly valuable.

The here described method is relatively fast and easy-to-apply as it relies on basic immunofluorescent staining techniques, widely used epifluorescence microscopy and open source image analysis software. The described procedures on microstructural analysis can also be used on non-fluorescent (e.g. DAB) stainings, although it should be noted that standard immunodensity measures are not accurate on DAB stainings. Additionally, in the present study we focused on cortical myelination, but the described methodology may also be applied to other brain regions such as the *cerebellar* cortex. Furthermore, the proposed methodology can also be used on other myelin markers (e.g. PLP), or on markers of other types cell types that form networks. It should be taken into account that in order to successfully apply the described methodology in practice, it is crucial that all procedures (e.g. cutting sections) are carried out as standardized as possible for all samples. For instance, the angle of sectioning and the coronal plane of which sections are used may affect the measurements of microstructural integrity of myelinated cortical axons. It should be noted that based on the proposed image analysis procedures, conclusions on *qualitative* aspects of myelination such as myelin sheath length, paranodal length and myelin thickness cannot be drawn. In order to accurately examine such parameters, in particular myelin sheath thickness, EM remains the method of choice. EM allows the measurement of G-ratios, a measure of myelin thickness relative to axon diameter which is the most accurate way of estimating myelin integrity at a molecular level. However, the microstructural organization of cortical myelination is a measure independent of myelin sheath thickness, i.e. there may be a reduction in the number of myelinated axons without affecting the thickness of the existing myelin sheaths, and conversely, the overall myelin sheath thickness may be reduced without affecting the number and organization of myelinated axons. The described methodology is proposed as a sensitive addition to existing methods to quantify myelination at more microscopical level than MRI, but at a more macroscopical level compared to EM, adding more depth to commonly used techniques such as MBP immunodensity or area measurements. To gain a total understanding of myelination in a particular animal model, different techniques should be combined: MRI could be used to estimate total myelinated white matter volumes, histology allows assessment of myelin quantity and myelin organization in specific brain areas, and EM enables assessment of myelin quality at a molecular level.

To conclude, immunostainings for myelin markers followed by different methods of image analysis yield highly detailed information regarding the extent and microstructural integrity of cortical myelination in rodents. The detailed analysis of microstructural integrity of cortical myelination may be used to study myelination in

white matter diseases, such as neonatal WMI or multiple sclerosis. Additionally, it may be used in other models of neurological impairments. For example, if a specific gene has been implicated in a neurological disorder, e.g. autism-spectrum disorders, a detailed investigation of myelination in a knockout animal may reveal whether symptoms are mediated by white matter changes. Microstructural investigation of cortical myelination may aid the development of novel treatment options aimed at protecting myelination or promoting remyelination[43, 44]. Furthermore, adverse effects of novel treatments for other diseases (e.g. chemotherapy[45], radiation therapy[46]) on myelination may be sensitively characterized. Overall, the detailed analysis of myelin staining in the rodent brain may significantly contribute to preclinical white matter research.

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AUTHOR CONTRIBUTIONS

All authors performed the animal experiments. EvT, CvK and CdT performed histological stainings and analyzed the data. EvT prepared the figures and wrote the manuscript. CvK, CdT, MvM, RD and CN revised the manuscript. CN coordinated and directed the project.

ADDITIONAL INFORMATION

The authors declare no conflict of interest. Supplementary material can be found in the online version of this article. Doi: *10.1038/s41598-017-16797-1*

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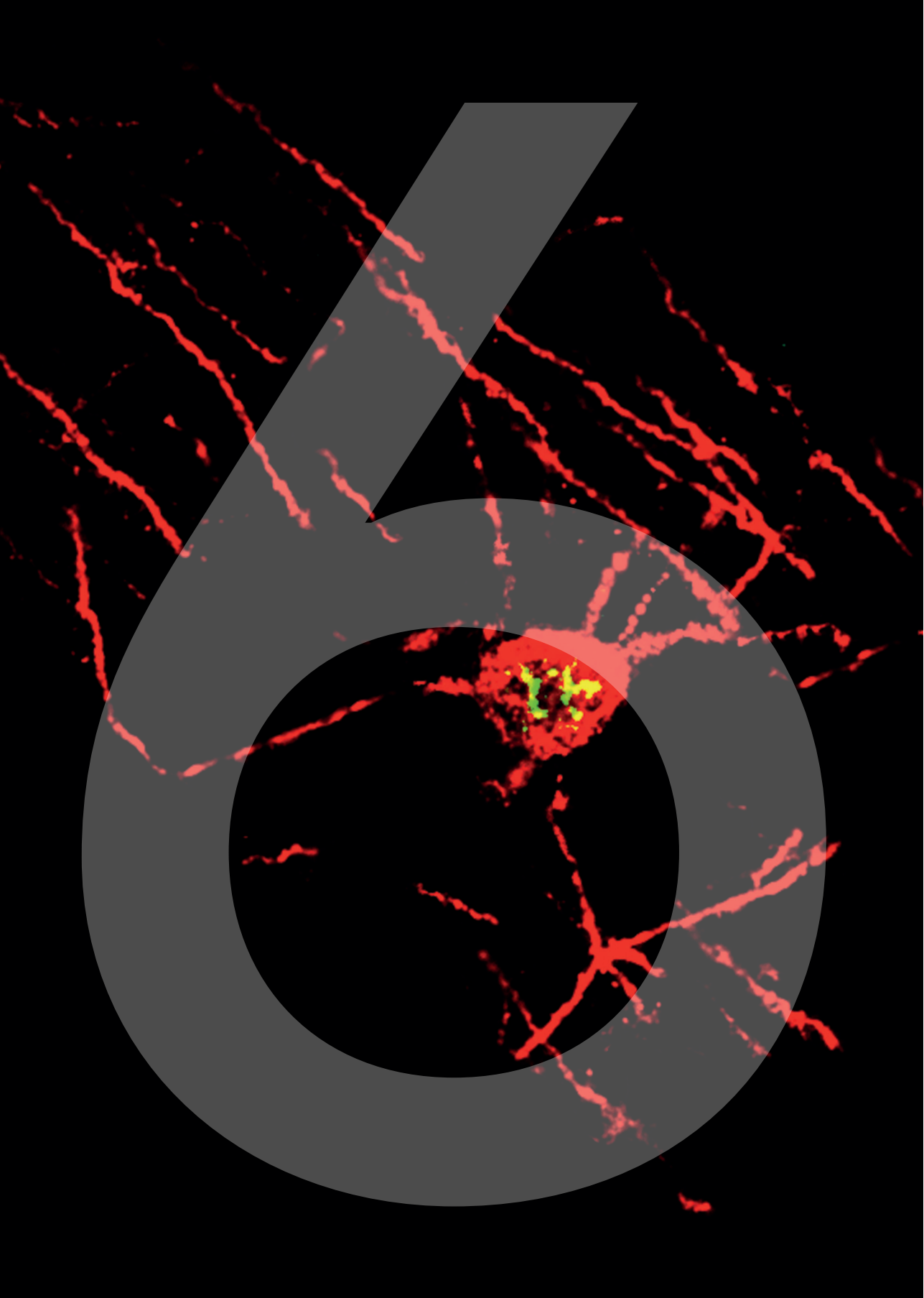
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CHAPTER 6

Cognitive performance during adulthood in a rat model of neonatal diffuse white matter injury

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ABSTRACT

Infants born prematurely are at high risk to develop neonatal diffuse white matter injury (WMI), which is associated with increased risk of neurodevelopmental disorders and impaired cognitive functioning that persists throughout adulthood. We recently introduced a rat model of neonatal diffuse WMI induced by combined fetal inflammation and postnatal hypoxia, in which we observed impaired motor performance, anxiety-like behavior and signs of autism-like behavior in juvenile rats. However, whether long-term cognitive deficits are present in this two-hit rat model of WMI remains unknown. Here, we studied the effects of fetal inflammation (100 µg/kg maternal LPS at E18+E19) and postnatal hypoxia (140 minutes 8% O_2 at P4) on impulsivity, attention and cognitive flexibility in 7-10 months old adult rats using the 5-choice serial reaction time task (5CSRTT) and a probabilistic reversal learning task. Importantly, no major changes in cognitive performance during adulthood were observed after combined fetal inflammation and postnatal hypoxia in the fetal/neonatal period. These findings may be explained by delayed rather than permanently affected myelination, or by the absence of injury to the prefrontal cortex.

INTRODUCTION

Diffuse white matter injury (WMI) is the most common type of brain injury in infants born prematurely[1]. Diffuse WMI is associated with myelination deficits, reduced white matter volumes, punctate white matter lesions and active gliosis, causing sensory, behavioral and cognitive problems that persist throughout adulthood[1-5]. Additionally, preterm birth is associated with increased risk of neurodevelopmental disorders, including autism-spectrum disorders, schizophrenia and attention deficits[6-9]. However, to what extent the increased risk of neurodevelopmental disorders in preterm infants is mediated by white matter changes remains unsure.

Important risk factors for white matter abnormalities in preterm infants are inflammatory insults and disturbed cerebral oxygenation. Inflammation in the preterm brain is often the result of maternal complications leading to fetal exposure to inflammation, postnatal infections or other diseases of prematurity (e.g. bronchopulmonary dysplasia, necrotizing enterocolitis). In addition, disrupted oxygenation of the developing brain results from underdeveloped cerebral vascularization, lung disease and ventilation therapy[10-12]. Moreover, exposure to *multiple* preterm birth-related insults has been suggested to be an important etiological factor, with early hits sensitizing the brain to become more vulnerable to subsequent injurious events[13, 14]. Indeed, clinical data indicate that exposure of the developing brain to multiple hits dramatically increases the risk of WMI[15-17].

Currently, no treatment options are available to protect the neonatal white matter from preterm birth-related insults. Therefore, translational studies are crucial to aid the development of novel therapeutic strategies. Recently, we introduced a novel two-hit rat model of preterm diffuse WMI in which the combination of fetal inflammation and postnatal hypoxia causes impaired oligodendrocyte maturation, delayed cortical myelination, astrogliosis and microglia activation[18]. Moreover, we observed impaired motor performance, anxiety-like behavior, reduced social play behavior and increased repetitive grooming in juvenile rats with WMI[18]. The increased risk of preterm infants to develop neurodevelopmental disorders associated with cognitive problems, impulsivity and attention deficits later in life, raises the question whether rats exposed to combined fetal inflammation and postnatal hypoxia display similar deficits in executive functioning. In the present study, we investigated the consequences of fetal inflammation and postnatal hypoxia on long-term executive functioning at the age of 7-9 months. More specifically, we used the 5-choice serial reaction time task

(5CSRTT) to assess impulsivity, attention and cognitive flexibility during adulthood and we used the probabilistic reversal learning task as an additional measure of cognitive flexibility. It was hypothesized that adult rats exposed to combined fetal inflammation and postnatal hypoxia would display increased impulsivity, attention problems and impaired cognitive flexibility.

METHODS

Animals

All procedures were carried out according to Dutch ("Wet op de dierproeven", 1996) and European regulations (Guideline 86/609/EEC) and were approved by the Animal Ethics Committee of Utrecht University.

WMI was induced as described previously[18]. Briefly, 'WMI' litters (Wistar rats, Harlan, Horst, The Netherlands) received maternal i.p. injections with 100 µg/kg LPS on embryonic days (E)18 and E19 plus were subjected to hypoxia (8% O_2) for 140 minutes in a hypoxic chamber at postnatal day (P)4. 'Control' rats pups were derived from saline-treated mothers and were kept away from the mother for 140 minutes under normoxic conditions at P4. In total, 48 animals were used in this study (control animals: n=28 (13 males, 15 females); WMI animals: n=20 (12 males, 8 females)).

Animals were housed in groups at a 12/12h light/dark cycle (lights on at 7.00AM) and had *ad libitum* access to water. Beginning at one week prior to starting the test of each task, animals were food deprived receiving only 35-40 (males) or 40-45 (females) grams of standard chow per kilogram of bodyweight. During the behavioral paradigms, 45 mg sucrose pellets (TestDiet, St. Louis, MO) were used as food rewards. Training and testing took place at the age of 7-9 months.

5 choice serial reaction time task (5CSRTT)

The 5CSRTT[19-21] took place in operant conditioning chambers (Med Associates, St. Albans, VT, USA) with an array of five response holes located on one wall and a food tray on the opposite wall, 2 cm above a metal bar floor. A stimulus light was located within each hole, and a horizontal infrared beam detected nose-poke responses into these apertures. The entire chamber could be illuminated by a light at the top of the chamber.

Rats were trained to detect and respond to a brief visual stimulus presented randomly in one of the five nose poke units to obtain a food reward. A trial started with an inter trial interval (ITI) of 5s, followed by 1s illumination of one of the five apertures and 2s limited hold. Following a nose poke in the illuminated aperture, i.e. a correct response, animals were rewarded with the delivery of one food pellet in the food magazine. During the training session, stimulus duration was set at 32s and was gradually decreased over sessions to 1s until animals reached stable baseline performance (accuracy >80% correct choice; <20 omissions) (Fig. S1). Each daily session consisted of 100 discrete trials or 30 min, whichever occurred first. A nose-poke response into a non-illuminated aperture, i.e. an incorrect response, as well as a failure to respond within 5s after the onset of the stimulus, i.e. an omission, resulted in no food delivery and a time-out period with the house light extinguished for 5s. Nose pokes made during the ITI, i.e. before the onset of the stimulus (premature responses) were recorded as a measure of impulsivity, and resulted in a 5s time-out and no food reward. Perseverative responses, i.e. repeated responding during the presentation of the stimulus, were measured but did not have any programmed consequences. The following behavioral measures were recorded: (1) premature responses, i.e. number of responses into any of the holes during the ITI preceding stimulus presentation; (2) accuracy, i.e. percentage of correct responses [(number correct responses)/(correct + incorrect responses) x 100]; (3) latency of correct responses, i.e. the mean time between stimulus onset and nose poke in the illuminated unit; (4) omissions, i.e. the total number of omitted trials during a session and; (5) perseverative responses after correct choice[21].

Several parameters of the 5CSRTT were manipulated in order to determine impulsivity, attention and cognitive flexibility of the animals. To assess impulsivity and self-control, a long (7s) ITI, a long variable ITI (between 5s-13s), a mean variable ITI (between 3s-7s) or a short variable ITI (between 1s-5s) were used. To assess visual attention, a short 0.5s stimulus duration was used. To study sustained attention, we used a short ITI of 2s or we introduced a fixed or loose block in the testing chamber as distractors. Finally, to determine performance on the 5CSRTT with a variable stimulus duration or ITI, we used a short variable stimulus duration (alternating between 1s and 0.5s), a mean variable ITI (between 3s-7s), and a short variable ITI (between 1-5s). Between every test, animals were subjected to daily training sessions as previously described until a stable baseline performance was reached (accuracy: >80%; omissions: <20). The day after, animals were tested. Tests measuring the same construct (e.g. impulsivity) were not tested consecutively. The exact order of test manipulations was: long ITI, short stimulus duration, short ITI, fixed block, short variable ITI, short variable stimulus

duration, mean variable ITI, loose block, long variable stimulus duration, *ad libitum* food access. Data are presented as baseline performance preceding the test vs. protocol mutation.

Probabilistic reversal learning

After the 5CSRTT, 9 months old animals were subjected to the probabilistic reversal learning task[22, 23] which took place in standard operant conditioning chambers equipped with two retractable levers and a food magazine in between the levers. After a 5-day period that consisted of habituation and training, animals were subjected to the reversal learning task for 5 consecutive daily sessions of 120 trials each. In this task, both levers were presented, one of which was randomly assigned as the initially active lever, while the other lever was assigned the initially inactive lever. Whereas a press on the active lever resulted in presentation of a food reward with 80% probability, a press on the inactive lever resulted in food reward with a 20% probability. Thus, adopting a strategy to continuously press the active lever would yield the maximum number of rewards to the animal. After 40 trials, the reward scheme switched: the initially active lever became inactive (20% probability of reward) and the initially inactive lever became active (80% probability of reward). After another 40 trials, the reward scheme switched back to the initially active lever becoming active and the initially inactive lever becoming inactive again. The ability of animals to change their strategy in response to a switch in the active vs. inactive levers is considered a measure of cognitive flexibility. Presented are the number of trials that animals pressed the active lever, as well as the number of presses on the initially active lever using a shifting window of 8 trials.

Statistics

For parameters in the 5CSRTT, the effect of sex was determined using repeated measures ANOVA, with test (baseline vs. manipulation) as within subjects factor; and group (control vs. WMI) and sex (male vs. female) as between subjects factors. In case a significant effect or interaction with sex was observed, both sexes were further analyzed and plotted separately. In case no significant effect of sex was observed, both sexes were pooled to be analyzed by repeated measures ANOVA to determine the effects of the test (within subjects factor: baseline vs. manipulation, performance during different sessions) and group (between subjects factor: control vs. WMI). In case of significant interactions between test and group or a significant effect of group, multiple comparisons t-tests with Bonferroni corrections were performed. Data are presented as mean + SEM.

RESULTS

5CSRTT

Training and motivation

Although on the first two training days female control animals demonstrated lower accuracy compared to WMI animals ($t(1029)=6.40$, $p<0.0001$; $t(1029)=4.69$, $p=0.0002$), control females caught up quickly and no differences were observed on the following training days between the groups. Other than these observations, no major differences between control and WMI animals were observed in accuracy, omissions or premature responses during the training phase (Fig. S1). To study whether any motivational differences were present between control and WMI animals, animals were subjected to the task after having *ad libitum* access to food. Both males and females demonstrated a decrease in motivation to perform the task after *ad libitum* access to food as reflected by increased numbers of omissions (no effect of sex: $F(1,44)=0.52$, $p=0.474$; no differences between control and WMI animals: $F(1,46)=0.07$, $p=0.793$; effect of manipulation (*ad libitum* food access): $F(1,46)=60.62$, $p<0.0001$) (Fig. S2).

Long 7s ITI and variable ITIs

The ITI was increased to a fixed time of 7s or a long variable time (between 5s-13s) in order to assess the animals' ability to withhold a response. Using both manipulations, no sex differences were observed (7s ITI: $F(1,44)=1.31$, $p=0.259$; long variable ITI: $F(1,44)=0.05$, $p=0.816$). As expected, increasing the ITI elicited an increase in premature responses in both manipulations of the 5CSRTT ($F(1,46)=77.62$, $p<0.0001$ and $F(1,46)=276.2$, $p<0.0001$ in the 7s ITI and long variable ITI tests, respectively). However, no changes between control and WMI animals were observed (7s ITI: $F(1,46)=0.13$, $p=0.719$; long variable ITI: $F(1,46)=0.50$, $p=0.483$) (Fig. 1A,B). Increasing the ITI caused a reduction in accuracy (7s ITI: $F(1,46)=15.68$, $p=0.0003$; long variable ITI: $F(1,46)=14.99$, $p=0.0003$) and using the long variable ITI we observed an increase in the number of omissions ($F(1,46)=21.25$, $p<0.0001$), but no differences between control and WMI were observed in terms of accuracy (7s ITI: $F(1,46)=2.60$, $p=0.613$; long variable ITI: $F(1,46)=2.01$, $p=0.163$) or omissions (7s ITI: $F(1,46)=2.98$, $p=0.091$; long variable ITI: $F(1,46)=2.33$, $p=0.134$) (Fig. S3A-D).

To further study performance on the 5CSRTT under challenging conditions, we used a mean variable ITI (between 3s-7s) and a short variable ITI (between 1s-5s). Accuracy after introduction of a mean variable ITI (no effect of sex: $F(1,44)=0.003$, $p=0.958$) or a short variable ITI (no effect of sex: $F(1,44)=0.17$, $p=0.679$) remained unchanged (mean variable ITI: $F(1,46)=0.538$, $p=0.467$; short variable ITI: $F(1,46)=3.04$, $p=0.088$)

and no differences between control and WMI animals were observed (mean variable ITI: F(1,46)=0.113, p=0.739; short variable ITI: F(1,46)=0.182, p=0.671) (Fig. 1C,D). A significant interaction was observed between group and sex in the number of omissions during the mean variable ITI test (F(1,44)=5.48, p=0.024) and the short variable ITI test (F(1,44)=9.52, p=0.004). During the mean variable ITI test, no changes in omissions were observed compared to baseline conditions (males: F(1,23)=0.01. p=0.916; females: F(1,21)=3.55, p=0.073) (Fig. S3E,F). Additionally, control males displayed more omissions compared to WMI males (F(1,23)=4.37, p=0.048), but this effect was not specific to the test manipulation (t(46)=1.95, p=0.115) (Fig. S3E). A

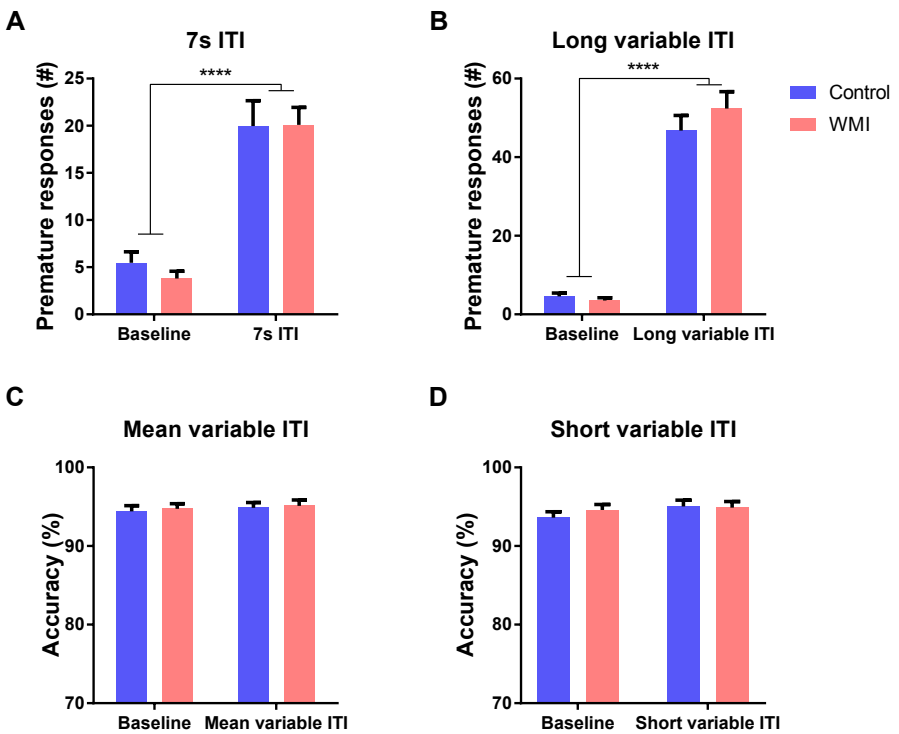

Figure 1 Performance on the 5CSRTT using a long or variable ITI. A: increasing the ITI to 7s induces a comparable increase in premature responses in control and WMI animals. B: testing under a long variable ITI increased the number of premature responses in both control and WMI animals to a comparable degree. C: an ITI varying between 3s-7s did not affect accuracy in any of the exaperimental groups. D: a short ITI varying between 1s-5s did not cause any changes in accuracy in either control or WMI animals. Baseline vs. manipulation: ****: p<0.0001.

short variable ITI increased the number of omissions in male animals ($F(1,23)=34.11$, $p<0.0001$) and during the test manipulation male WMI rats showed less omissions compared to control males ($t(46)=2.69$, $p=0.020$) (Fig. S3G). Additionally, WMI females showed an increase in omissions in the short variable ITI task (interaction group vs. test: $F(1,21)=4.53$, $p=0.045$; $t(42)=2.90$, $p=0.012$) (Fig. S3H).

Short stimulus duration, short ITI, distractors and short variable stimulus duration
To further study the functional consequences of fetal inflammation and early life postnatal hypoxia in adult rats, we studied performance on the 5CSRTT using a short 0.5s stimulus duration, a short 2s ITI, in the presence of a distractor (a fixed or a loose block in the test apparatus), and using a stimulus duration alternating between 1s and 0.5s every 20 trials.

The stimulus duration in the 5CSRTT was reduced from 1s to 0.5s. During this test, no sex differences were observed in terms of accuracy ($F(1,44)=0.15$, $p=0.697$). A short 0.5s stimulus duration impaired accuracy of the rats ($F(1,46)=110.1$, $p<0.0001$), but no differences were observed between control and WMI animals ($F(1,46)=0.40$, $p=0.532$) (Fig. 2A). There was a significant interaction between group and sex in terms of omissions ($F(1,44)=4.98$, $p=0.031$). A short stimulus duration caused an increase in the number of omissions in both males and females (males: $F(1,21)=62.64$, $p<0.0001$; females: $F(1,21)=41.75$, $p<0.0001$), and during the 0.5s stimulus duration manipulation male WMI rats showed significantly more omissions compared to control males ($t(42)=2.64$, $p=0.023$) (this effect was not observed in females: $t(42)=1.50$, $p=0.281$) ((Fig. S4A,B).

To assess performance on the 5CSRTT with a high event rate, we used a short ITI of 2s. A short ITI caused an increase in response accuracy compared to baseline conditions ($F(1,46)=38.13$, $p<0.0001$; no effect of sex: $F(1,44)=0.002$, $p=0.961$), but no differences in accuracy were observed between control and WMI rats ($F(1,46)=0.176$, $p=0.677$) (Fig. 2B). A significant interaction was observed between group and sex in the number of omissions during the short ITI test ($F(1,44)=9.72$, $p=0.003$). A short ITI caused an increase in omissions in both males ($F(1,23)=30.62$, $p<0.0001$) and females ($F(1,21)=23.29$, $p<0.0001$). In males, WMI rats showed a decrease in omissions compared to controls during the 2s ITI manipulation ($t(46)=3.08$, $p=0.007$). In females, the effect was reversed with WMI females showing an increase in omissions during the test manipulation ($t(42)=2.45$, $p=0.037$) (Fig. S4C,D).
To study to what extent animals were easily distracted, we introduced one of two types

of distractors in the testing apparatus of the 5CSRTT: a fixed block and or a loose block. Introduction of either a fixed or loose block distracted the animals as reflected by increased numbers of omissions (no effects of sex, fixed block: $F(1,44)=0.05$, $p=0.833$; loose block: $F(1,44)=0.60$, $p=0.443$) (increased omissions, fixed block: $F(1,46)=26.5$, $p<0.0001$; loose block: $F(1,46)=47.13$, $p<0.0001$) (Fig. 2C,D). However, in the presence of a distractor no differences were observed between control and WMI animals regarding omissions (fixed block: $F(1,46)=4.02$, $p=0.051$; loose block: $F(1,46)=1.89$, $p=0.176$). Also, the response accuracy was decreased after introducing a distractor in the test (fixed block: $F(1,46)=10.75$, $p=0.002$; loose block: $F(1,46)=13.0$, $p=0.001$) (Fig. S4E,F). During the fixed block test, WMI animals showed increased accuracy compared to control rats, as indicated by a main-effect of experimental group ($F(1,46)=5.76$, $p=0.021$), but this effect was not specific to the manipulation ($t(92)=1.66$, $p=0.062$) (Fig. S4E). No differences in accuracy between the experimental groups were observed in the loose block test ($F(1,46)=0.07$, $p=0.788$) (Fig. S4F).

To study flexibility of animals, we used an alternating variable stimulus duration. Alternating the stimulus duration between 1s and 0.5s every 20 trials caused decreases in the accuracy during the 0.5s stimulus duration (no effect of sex: $F(1,44)$, $p=3.74$; effect of alternation: $F(4,184)=9.12$, $p<0.0001$), but no changes were observed between control and WMI animals ($F(4,184)=0.01$, $p=0.957$) (Fig. 2E). During the short variable stimulus duration test a significant interaction between group and sex was observed in the number of omissions ($F(1,44)=4.92$, $p=0.032$). Male WMI animals showed less omissions compared to control males ($F(1,23)=14.72$, $p=0.001$). No differences in omissions were observed in female control vs. WMI animals ($F(1,21)=0.173$, $p=0.682$) (Fig. S4G,H).

Probabilistic reversal learning test

To assess cognitive flexibility in control and WMI animals, a probabilistic reversal learning test was used. Animals were trained to press levers to earn a food reward. Whereas pressing one 'active' lever was rewarded in 80% of the trials, pressing the 'inactive' lever resulted in a reward in 20% of the trials. After 40 trials, the reward scheme switched to pressing the initially 'active' lever resulting in a 20% probability of a reward and pressing the initially 'inactive' lever resulting in 80% probability of a reward. Over the course of 5 consecutive 120-trial sessions the preference of animals for pressing the high-probability lever increased, as indicated by the increased proportion of trials that animals pressed the active lever ($F(4,184)=12.84$, $p<0.0001$) (Fig. 3A, Fig. S5). However, no changes in preference for the high-probability lever

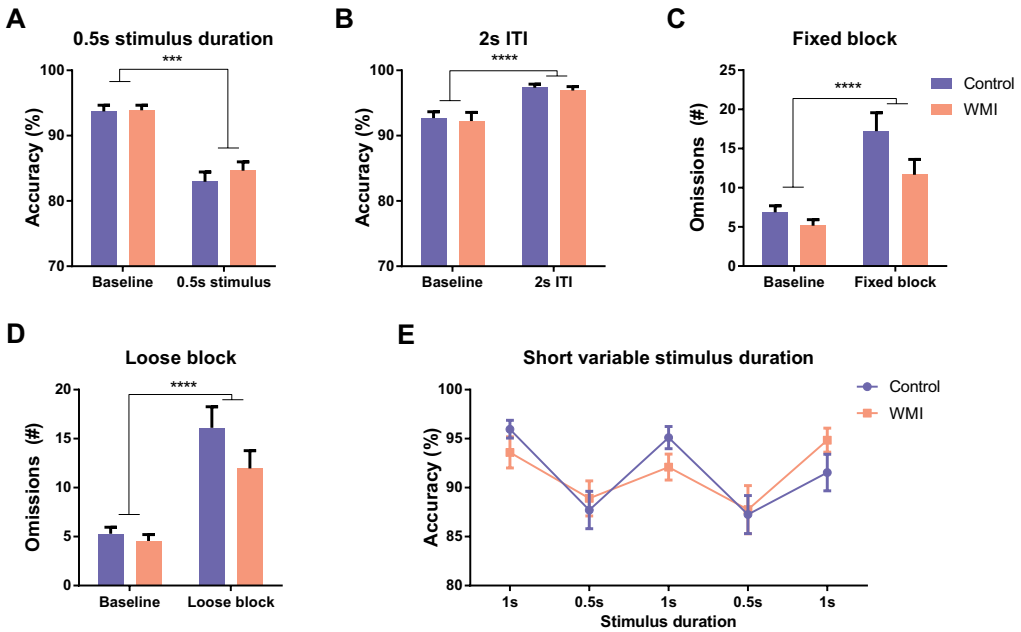

Figure 2 Adult WMI animals do not display major differences in performance on various manipulations of the 5CSRTT compared to control animals. A: a short stimulus duration of 0.5s reduced accuracy of control and WMI animals equally. B: a short ITI of 2s caused an increase in the accuracy of control and WMI animals equally. C: introducing a fixed block as a distractor caused an increase in the number of omissions, but no differences between control and WMI animals were observed. D: introducing a loose block as a distractor caused an increase in the number of omissions, but no differences between control and WMI animals were observed. E: alternating the stimulus duration between 0.5s and 1s within a test run caused temporary reductions in accuracy during short stimulus durations in both control and WMI animals. Baseline vs. manipulation: ***: $p<0.001$; ****: $p<0.0001$

were observed between control and WMI animals ($F(1,46)=2.86$, $p=0.098$) (Fig. 3A, Fig. S5). Animals of both groups successfully developed a strategy during the first 40 trials by pressing the initially 'active' lever >50% of the trials (Fig. 3B). After 40 trials, the reward scheme switched and animals successfully adapted their strategy by pressing the initially 'inactive' (but now high-probability reward) lever >50% of the trials (Fig. 3B). After another 40 trials, the reward scheme switched back to the original scheme and both control and WMI animals adapted their strategy back to pressing the initially 'active' lever >50% of the trials (Fig. 3B). Detailed data on the performance of animals during trials 1-40, 41-80 and 81-120 during sessions 1-5 are shown in Fig. S5.

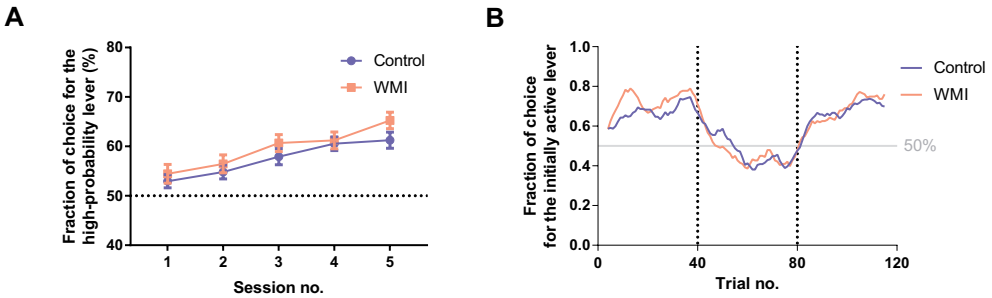

Figure 3 Performance of control and WMI animals on the probabilistic reversal learning test. A: over the course of 5 sessions that animals performed the probabilistic reversal learning task animals showed a gradual increase in performance, as indicated by the proportion of trials that animals pressed the lever with high probability of a reward. B: the fraction of choice for the initially active lever, based on a shifting window of 8 consecutive trials (i.e. for each time point the fraction of choice for the active lever of 4 trials before and 4 trials after that time point was calculated). Both control and WMI animals deployed a successful strategy during the final probabilistic reversal learning task: animals pressed the initially active lever more frequently during trials 0-40, successfully adapted their strategy to pressing the initially inactive lever during trials 41-80, and changed back to the initially active lever during trials 81-120. No differences were observed between control and WMI animals.

DISCUSSION

In the present study, we investigated the long-term consequences of WMI induced early in life by combined fetal inflammation and early postnatal hypoxia on various aspects of executive functioning in adulthood (7-9 months of age), including impulsivity, attention and cognitive flexibility. WMI animals did not show any signs of increased *impulsivity*, as indicated by their ability to withhold a response during the long and variable long ITI manipulations of the 5CSRTT (Fig. 1A,B). Moreover, WMI animals displayed an ability of *self-control* equal to that of control animals as indicated by their response accuracy during the mean or short variable ITI manipulations of the 5CSRTT (Fig. 1C,D). *Visual attention* was not impaired in rats exposed to fetal inflammation and postnatal inflammation, as no changes in response accuracy were observed using the short stimulus duration during the 5CSRTT (Fig. 2A). Furthermore, no deficits in *sustained attention* were observed in WMI animals compared to controls as indicated by similar response accuracy during the short ITI test and equal numbers of omissions

after introduction of a distractor (Fig. 2B-D). Moreover, WMI animals did not show changes in *cognitive flexibility* as indicated by performance using a short variable ITI during the 5CSRTT (Fig. 2E) and by performance on the probabilistic reversal learning test (Fig. 3). To conclude, based on performance on the 5CSRTT and probabilistic reversal learning task, no major deficits in functional outcome were observed in adult WMI rats.

In our previous study, WMI animals were shown to exhibit impaired motor performance, anxiety-like behavior and signs of autism-like behavior, including reduced social play behavior and repetitive grooming[18]. No changes were observed in working memory at the age of 6 weeks or object recognition memory at 9 weeks , indicating no severe memory deficits[18]. Whereas cortical myelination was impaired until P30, myelination seemed largely restored by the age of 10 weeks, raising the question whether WMI animals display subtle functional impairments in adulthood. The lack of differences in the behavioral paradigms between WMI and control animals in this study may be explained by restored cortical myelination in WMI animals at the time of testing and may indicate that any possible neuronal deficits may also have been restored in adult WMI rats. An alternative explanation could be that the prefrontal cortex (PFC), which is essential for executive functions, remains relatively spared following perinatal insults as PFC maturation occurs later compared to more caudal brain regions[24]. Further characterization of the myelination patterns throughout the brains of control vs. WMI animals may reveal whether myelination has been restored throughout the brain, or whether specifically myelination of the PFC was spared following perinatal insults.

The transiency of white matter deficits following neonatal insults in rodents has also been observed in other animal models of neonatal diffuse WMI. In a rat model of maternal inflammation LPS injections at E19 and E20 caused transient motor deficits in offspring which were restored to control levels after P23[25]. Functional recovery observed in these rodent models could be explained by delayed, rather than permanently damaged white matter as indicated by restored white matter integrity[18, 25, 26]. Alternatively, early postnatal rodent brains may respond to inflammatory and/or hypoxic conditions by promoting proliferation and differentiation of neural stem cells to compensate for injurious events. To illustrate, Fagel et al. demonstrated in a mouse model that chronic perinatal hypoxia enhanced cortical neurogenesis and that 45% of newly generated cells became oligodendrocytes[27]. Similarly, following combined fetal inflammation and postnatal hypoxia we observed increased proliferation of oligodendrocyte precursor cells (OPCs) in the white matter of WMI rats[18]. The

increased generation of OPCs following perinatal insults may eventually compensate for the initial lack of myelination. Whether similar processes take place in the human brain following perinatal insults remains unknown.

On a cognitive level, adults (age: 20-36 years) who were born prematurely show lower IQ scores, lower academic achievements and impaired executive functioning[3, 4, 28]. Interestingly, in terms of personality young (19-26 years old) adults born prematurely show more internalizing, avoidant and social problems (i.e. anxiety, withdrawn, lack of self-confidence) and less externalizing (i.e. aggressive, intrusive, rule-breaking behaviors) compared to adults born at term[29]. Together, these studies indicate that functional outcome of preterm infants remains affected during adulthood. Our results in the rat model of diffuse WMI do not reflect these clinical observations regarding long-term impaired cognitive performance that persists throughout adulthood. These findings may be interpreted as a limitation of the currently used rat model of diffuse WMI. However, so far no alternative rodent model has been developed that causes non-cystic diffuse WMI together with subtle cognitive impairment throughout adulthood. Whether the absence of long-term cognitive deficits in rodents exposes an inherent difference between the rodent vs. human brain, or whether alternative perinatal insults (e.g. more severe or systemic perinatal hits) may cause diffuse WMI in combination with long-term cognitive deficits to accurately reflect the clinical situation should be further elucidated. Nonetheless, due to delayed myelination and similar pathophysiological hallmarks observed in our WMI rat model compared to post-mortem human tissue[2, 18, 30], the current WMI rodent model may still be used to investigate cellular mechanisms underlying diffuse WMI and to study novel treatment options within the time window of at least 30 days during which glial activation, myelination deficits and impaired functional outcome are evident.

To summarize, combined fetal inflammation and early postnatal hypoxia does not substantially affect cognitive functioning in rats during adulthood. Recovery in functional outcome may be explained by restored myelination, as observed earlier[18]. Our future studies will focus on detailed spatiotemporal characterization of myelination deficits in these animals during adulthood. The currently available diffuse WMI models can be used to study and develop novel treatment options aimed at relative early recovery after perinatal insults, which may improve myelination in specific brain regions, and may improve negatively affected aspects of functional outcome, like motor performance and autism-like behavior.

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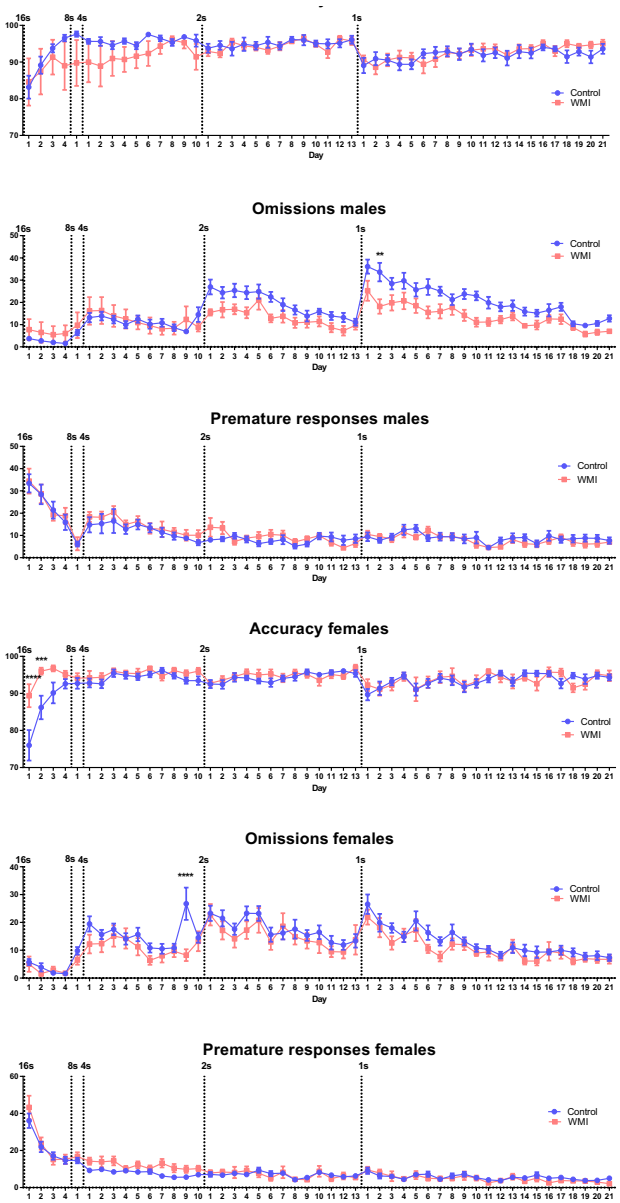


Figure S1 Accuracy (A,D), number of omissions (B,E) and premature responses (C,F) during the training phase of the 5CSRTT of male (A-C) and female (D-F) animals. The initial stimulus duration was 16s, but after all animals reached the criteria of >80% accuracy and <20 omissions, the stimulus duration was halved until it reached 1s. After reaching the same criteria using a 1s stimulus duration, animals were ready to be tested. Multiple comparisons t-test with Bonferroni correction: **: p<0.01; ***: p<0.001; ****: p<0.0001.

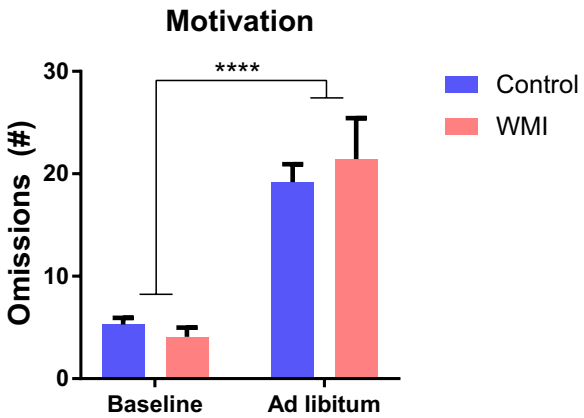


Figure S2 No changes in motivation were observed between control and WMI animals, as indicated by the number of omissions during testing after ad libitum access to food. Baseline vs. ad libitum access: ****: p<0.0001.

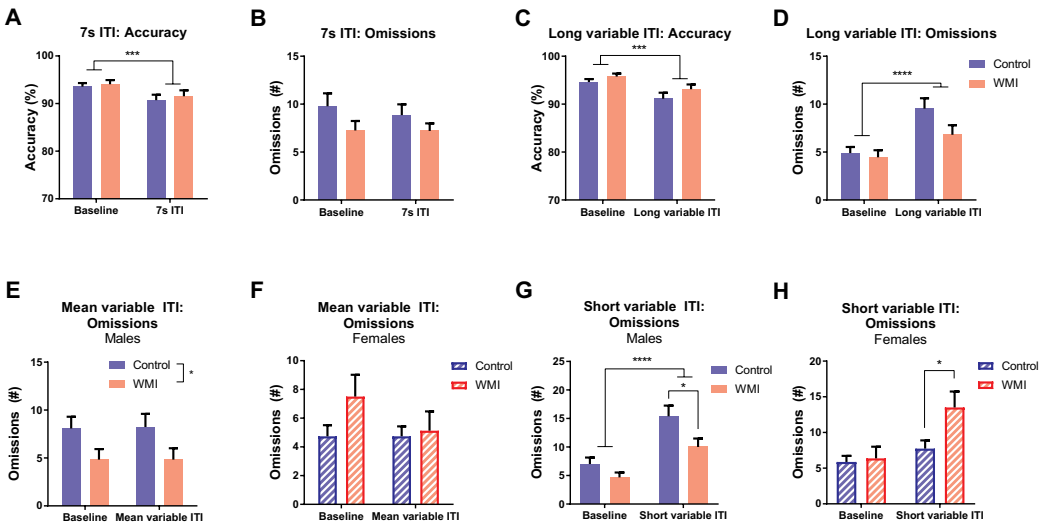


Figure S3 Accuracy (A,C) and number of omissions (B,D-H) in variants of the 5CSRTT in which the duration of the ITI was manipulated: a long 7s ITI (A,B), a long variable ITI (C,D), a mean variable ITI (E,F) or a short variable ITI (G,H). Control vs. WMI animals: *: p<0.05 (E); Multiple comparisons t-test with Bonferroni correction: *: p<0.05 (G,H); baseline vs. manipulation: ****: p<0.0001.

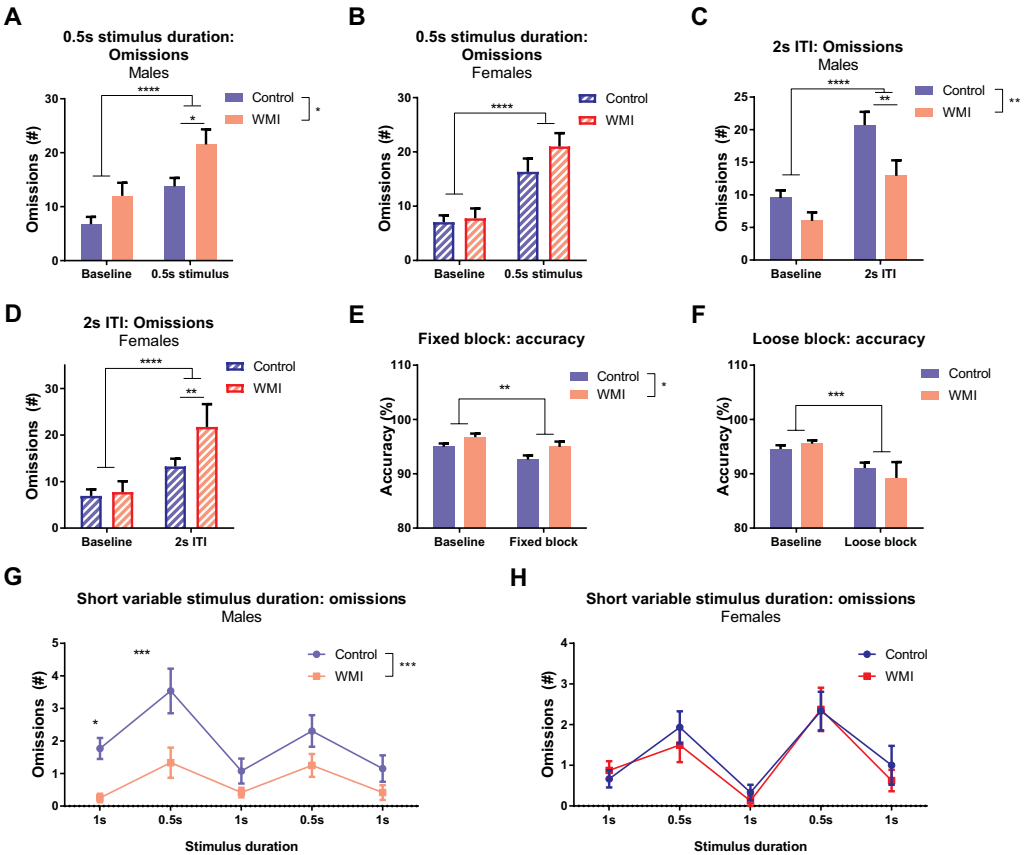


Figure S4. Additional data of the 5CSRTT in which the stimulus duration or ITI was manipulated, or in which a distractor was introduced. (A,B) number of omissions during the 0.5s stimulus duration test. (C,D) number of omissions during the 2s ITI test. (E) response accuracy after introduction of a fixed block as a distractor. (F) response accuracy after introduction of a loose block as a distractor. (G,H) number of omissions during the short variable stimulus duration test. While alternating between a stimulus duration of 1s and 0.5s every 20 trials, male WMI rats showed less omissions compared to male control rats during the first two blocks. Multiple comparisons t-test with Bonferroni correction: *: $p<0.05$; **: $p<0.01$; ***$p<0.001$ (A,C,G). Control vs. WMI animals: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (A,C,G). Baseline vs. manipulation: **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$ (A-F).

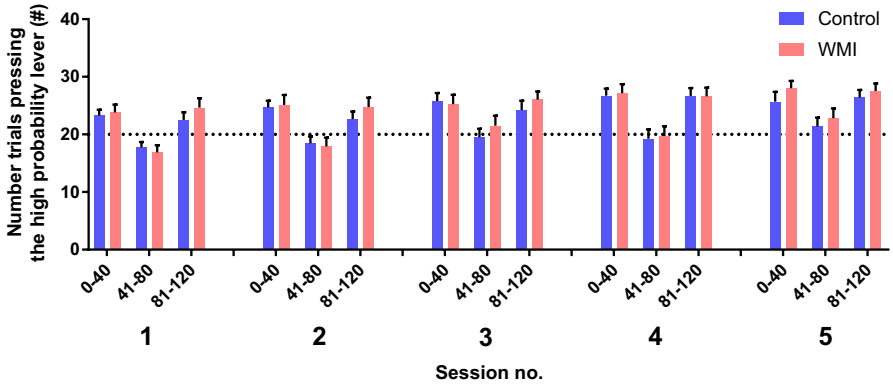
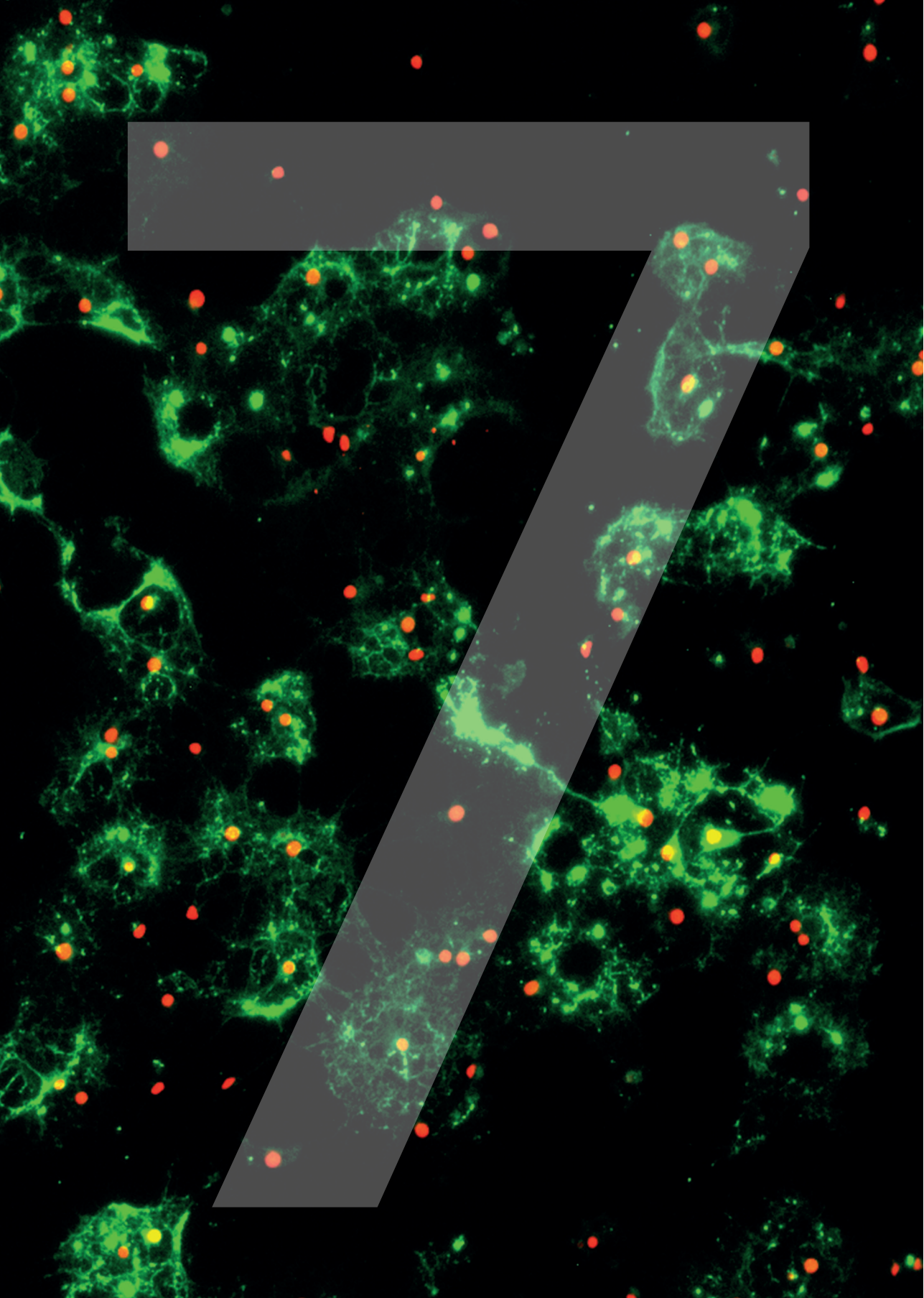


Figure S5 Additional data of the probabilistic reversal learning task. Of individual sessions (1-5) the number of trials during which animals pressed the high probability (active) lever are shown for trials 0-40, 41-80 and 81-120 separately. The expected number of lever presses in the case of no preference for a specific lever (i.e. 20; 50%) is indicated by the dotted line.



CHAPTER 7

JNK inhibitor D-JNKi promotes myelination in a rat model of diffuse white matter injury

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ABSTRACT

Diffuse white matter injury (WMI) is a major problem in infants born prematurely, causing behavioral and cognitive deficits later in life. Microglia-mediated neuroinflammation and impaired maturation of oligodendrocytes have been implicated in the etiology of diffuse WMI. Activation of the JNK MAP kinase signaling pathway has been associated with promoting neuroinflammation, negatively regulating oligodendrocyte maturation and with neonatal WMI. The small peptide JNK inhibitor D-JNKi blocks interaction of JNK with its upstream and downstream substrates, thereby inhibiting JNK pathway activation. We have previously shown that D-JNKi has neuroprotective properties in a rodent model of neonatal hypoxic-ischemic brain injury. In the present study, we investigated the potential of D-JNKi to protect the preterm brain from diffuse WMI. Using a rat model of diffuse WMI, we demonstrate that D-JNKi has the potential to rescue myelination and improve functional outcome. Furthermore, D-JNKi treatment was associated with reduced oligodendrocyte proliferation. Using primary cell cultures, we show that D-JNKi promotes myelination in oligodendrocyte precursor cells under inflammatory conditions. Whereas *in vitro* D-JNKi attenuates production of TNF–α by microglia, no changes in microglia numbers or morphology were detected following D-JNKi treatment *in vivo*. To conclude, we show that D-JNKi rescues myelination under pathological conditions *in vitro* and *in vivo*. Our results indicate that the protective actions of D-JNKi are mediated by direct interactions with oligodendrocyte lineage cells rather than attenuating microglia activation or neuroinflammation. These data highlight JNK inhibition as a promising therapeutic strategy to combat diffuse WMI and subsequently improve the outcome of preterm infants with white matter abnormalities.

INTRODUCTION

Many preterm infants suffer from diffuse white matter injury (WMI). Important risk factors include inflammatory insults and respiratory problems during the perinatal period, which negatively impact white matter development and myelination, an effect likely mediated by impaired oligodendrocyte maturation and microglia activation as observed in human postmortem brain tissue[1, 2]. Children suffering from diffuse WMI exhibit long-term sensory, behavioral and cognitive problems and to date, no treatment is available for these patients[3].

Several studies point out that c-Jun N-terminal kinase (JNK) signaling negatively regulates oligodendrocyte maturation and myelination[4, 5] and increased levels of phosphorylated (active) JNK were observed in oligodendrocytes following ischemic insults[6, 7]. Moreover, various studies revealed that JNK signaling contributes to neonatal WMI and that inhibition of JNK improves myelination after neonatal insults[7, 8].
D-JNKi (or XG-102) is a small all-D-retro-inverso peptide inhibitor of JNK with cell-permeable properties. D-JNKi consists of the JNK interacting motif of JIP1 (JNK-interacting protein 1) coupled to a HIV Tat domain to facilitate uptake, and blocks interaction between JNK and its upstream activators as well as its downstream substrates by a competitive mechanism[9-11]. We have previously shown that D-JNKi effectively attenuates neonatal hypoxic-ischemic white and gray matter injury in a rat model for term neonates. We showed that this neuroprotective effect of D-JNKi is mediated by inhibiting apoptosis, attenuating neuroinflammation and reducing reactive oxygen species (ROS) formation[12, 13].

In the present study, we aimed to investigate whether inhibition of JNK signaling with D-JNKi can promote white matter development by attenuating neuroinflammation and boosting oligodendrocyte development in a rat model of preterm WMI. Here, we report that treatment with 10 mg/kg D-JNKi partially restores OPC proliferation, myelination and motor performance in a rat model of diffuse WMI. Furthermore, using primary cell cultures we show that D-JNKi promotes myelination in oligodendrocyte cultures and attenuates pro-inflammatory activation of microglia. However, no effects of D-JNKi on neuroinflamnation were observed *in vivo*.

MATERIALS AND METHODS

Ethical statement

All procedures were carried out according to the Dutch and European international guidelines (Directive 86/609, ETS 123, Annex II) and were approved by the Experimental Animal Committee Utrecht (University Utrecht, Utrecht, Netherlands). All efforts were made to minimize animal suffering.

Reagents and culture media

DMEM20S: high glucose DMEM containing GlutaMAX and pyruvate (31966, Gibco, Thermo Fisher Scientific, Waltham, MA), supplemented with 20% fetal bovine, serum and penicillin streptomycin. **Basal medium** (BM): high glucose DMEM with pyruvate and GlutaMAX (31966, Gibco) supplemented with 0.1% BSA, 50 µg/ml Apo-transferrin, 30 nM sodium selenite, 10 nM D-biotin, 10 nM hydrocortisone and 0.5% FCS. **OPC medium**: BM supplemented with 10 ng/ml PDGF-AA and 10 ng/ml bFGF (both Peprotech, Rocky Hill, NJ). **Differentiation factors**: 10 ng/ml CNTF (Peprotech), 15 nM T3, 5 µg/ml N-acetyl-L-cysteine (both Sigma, St Louis, MO). **D-JNKi peptide** (dqsrpvqpflnlttprkpr-pp-rrrqrrkkrg; underlined amino acids represent HIV-TAT shuttle domain): synthesized at W.M. Keck facility (Yale University, New Haven, USA).

Primary cell cultures

Mixed glial cell cultures were prepared from postnatal day (P)0-2 Sprague Dawley rat (Harlan, UK) cortices, as described elsewhere[14]. Briefly, cerebral cortices were isolated and meninges were removed. Tissue was digested with 0.2 mg/ml DNase I and 0.25% trypsin in HBSS. Cells were then dissociated and passed through a 70 µm nylon cell strainer. Cells were plated on poly-D-lysine coated T75 flasks in DMEM20S and received fresh medium every 2-3 days for a minimum of 10 days before harvesting microglia and OPCs. Harvesting microglia and oligodendrocyte precursor cells (OPCs) occurred for a maximum of 2 times per flask.

For primary microglia cultures, mixed glial cell cultures were shaken for 1 hour at 200 rpm at 37°C to detach microglia from the flask. Medium with detached microglia was removed from the flask, followed by centrifugation for 10 minutes at 1200 rpm. Pellets were resuspended in a small volume of DMEM20S. Cells were counted using a trypan blue assay and plated onto poly-L-ornithine coated 24-well plates at a density of 0.5 x 10^6 cells/well for production of microglia-conditioned medium (MCM), immunocytochemistry and TNF-α measurements. Prior to starting experiments,

plated microglia were left overnight in the incubator to allow attachment to the plate. For production of microglia-conditioned medium (MCM) of unstimulated microglia (MCM-LPS) or LPS-stimulated microglia (MCM+LPS), microglia were kept in 1 ml basal medium with or without 50 ng/ml LPS for 24 hours. Subsequently, supernatant was collected, filtered through a 0.20 µm filter and stored at -80°C before adding it to OPC cultures. For TNF-α measurements, microglia were then exposed to DMEM20S containing 0 or 100 ng/ml LPS in combination with 0-2 µM D-JNKi for 24 hours.

After removal of detached microglia (as described above), culture flasks were shaken for 18-20 hours at 200 rpm to detach OPCs. Medium with detached OPCs was removed from the flasks and passed through a 20 µm-pore nylon mesh, prior to centrifugation for 10 min at 800 rpm and trypan blue cell counting. Cells were diluted in OPC medium and plated onto poly-D,L-ornithine coated 24-well plates at a density of 40.000 cells/well. OPCs were kept in OPC medium for 4-6 days, until the majority of cells had differentiated into immature oligodendrocytes as characterized by a round cell body with numerous processes. Cells were then exposed to MCM-LPS or MCM+LPS, supplemented with differentiation factors and 0-4 µM D-JNKi for 4 days. Medium, growth factors and treatment conditions were refreshed every 2 days.

ELISA

TNF-α levels in the supernatant of microglia cultures were determined using an ELISA kit for rat TNF-α (Ucytech, Utrecht, The Netherlands) according to manufacturer's instructions. Due to varying levels of TNF-α between independent experiments, TNF-α data were normalized to positive control conditions (100 ng/ml LPS; 0 µM D-JNKi).

Immunocytochemistry

Microglia cultures or oligodendrocyte cultures were fixed with 4% PFA in PBS for 10 minutes and blocked with a solution of 2% BSA and 0.1% saponin in PBS. Plates were incubated with primary antibodies (rabbit-anti-Iba1, 1:500, Wako, Osaka, Japan; rabbit-anti-Olig2, 1:1000, AB9610, Millipore, Burlington, MA; mouse-anti-myelin basic protein (MBP), 1:1000, SMI-94, Sternberger Monoclonals, Lutherville, MD) overnight at 4°C, followed by incubation with secondary antibodies (alexafluor488- or alexafluor594-conjugated goat-anti-rabbit or goat anti-mouse, both 1:1000, Invitrogen, Carlsbad, CA) for 1 hour at room temperature. Cells were counterstained with Hoechst 33342 (Sigma) and embedded with fluorsave.

In vivo model of diffuse WMI

Diffuse WMI was induced as described previously[15]. In short, time-pregnant Wistar rats (Envigo, Horst, The Netherlands) received intraperitoneal injections with 100 µg/kg LPS or vehicle on E18 and E19. Pups were exposed to hypoxia (litters from LPS mothers; 8% O_2) or normoxia (litters from vehicle mothers) for 140 minutes on P4. D-JNKi was dissolved in PBS (vehicle) and administered i.p. at 10 mg/kg immediately after hypoxia. For RT-QPCR and western blots, animals were sacrificed by overdose pentobarbital. Brains were removed, snap-frozen in liquid nitrogen and stored at -80°C until further processing.

RT-QPCR

At 3, 6 and 24 hours after hypoxia, cerebral tissue was dissected and total RNA was isolated using TRIzol (Invitrogen). cDNA was synthesized using SuperScript Reverse Transcriptase (Invitrogen) and a PCR was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with the following primers: TNF-α (forward: CCCAGACCCTCACACTCAGATCAT; reverse: GCAGCCTTGTCCCTTGAAGAGAA), IL1β (forward: CTCTGTGACTCGTGGGATGATG; reverse: CACTTGTTGGCTTATGTTCTGTCC), IL6 (forward: AACGAAAGTCAA CTCCATCTG; reverse: GGTATCCTCTGTGAAGTCTCC), MCP1 (forward: CAGAAACCAGCCAACTCTCA; reverse: GTGGGGCATTAACTGCATCT), and MIP2 (forward: TTGTCTCAACCCTGAAGCCC, reverse: TGCCCGTTGAGGTACAGGAG). Data were normalized to expression of β-actin (forward: CACTATCGGCAATG AGCGGTTCC, reverse: CAGCACTGTGTTGGCATAGAGGTC).

Western blot

Protein homogenates from OPC cultures were pooled from two wells. For protein measurements in the *in vivo* model, cerebral tissue of P5 rats was pulverized using a liquid nitrogen-cooled mortar and pestle, and stored at − 80 °C. Cytosolic, mitochondrial and nuclear protein fractions were prepared from pulverized cerebral tissue as described previously[16]. Proteins were separated by SDS-PAGE and transferred to Hybond-C membranes. Membranes were blocked with 5% BSA and stained using primary antibodies (rabbit-anti-P-JNK, #4668, 1:1000, Cell Signaling Technology, Danvers, MA; rabbit-anti-JNK, #9252, 1:1000, Cell Signaling Technology; β-actin, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with peroxidase-labeled secondary antibodies, and exposure by chemiluminescence. β-actin was used as a loading control. For P-JNK and JNK measurements, the additive chemiluminesence of both JNK isoforms (p54 and p46) was determined.

Immunohistochemistry

On P15, a subset of animals was transcardially perfused with PBS, followed by 4% PFA in PBS for immunohistochemistry as described earlier[15]. Briefly, brains were embedded in paraffin and 8 µm sections were cut at the level of bregma. Sections were blocked and stained by overnight incubation with primary antibodies (mouse-anti-MBP, 1:1000, see above; mouse-anti-Olig2, 1:500, MABN50, Millipore; rabbit-anti-Ki67, 1:300, ab15580, Abcam, Cambridge, UK; rabbit-anti-Iba1, 1:500, see above), followed by incubation with secondary alexafluor-conjugated antibodies (see above) and counterstaining with DAPI. For all stainings, one micrograph was acquired of each hemisphere using a 20X magnification objective, values of both hemispheres were averaged for each animal. For MBP measurements, pictures were obtained at a fixed distance from the striatum, and at a fixed distance into the cortex. MBP^+ area was quantified by manual setting a threshold to determine positive staining. For quantification of $Olig2^+Ki67^+$ cells and $Iba1^+$ cells, the corpus callosum was selected as region of interest and the surface area was measured. The number of positive cells was manually counted.

Rotarod

The rotarod task was performed as described elsewhere[15]. Briefly, at the age of 5 weeks, animals were subjected to two practice trials (one per day) and two test trials (one per day) on four consecutive days. During practice trials, animals were trained to stay on the rotating rod at a speed of 5 rpm for 150 seconds. During test trials, animals were placed on a rod with a rotation speed of 5 rpm increasing to 40 rpm over the course of 5 minutes. Time spent on the rotating rod was recorded and performance over the two test trials was averaged.

Statistics

When comparing 2 groups, independent samples t-tests were used. When comparing multiple groups, one-way ANOVAs were used followed by multiple comparisons with Bonferroni correction. In case of unequal variances, a nonparametric Kruskal-Wallis test with Dunn's post-hoc correction was used. For the QPCR data, two-way ANOVAs were used with time and experimental group as factors. In case of a significant effect of either factor, multiple comparisons with Bonferroni correction were performed. p-values below 0.05 were considered statistically significant. Graphs represent mean+SEM.

RESULTS

D-JNKi attenuates JNK phosphorylation in a rat model of diffuse WMI

To study the effects of D-JNKi treatment, we used a rat model of neonatal diffuse WMI induced by combined fetal inflammation plus postnatal hypoxia. Animals were treated i.p. with D-JNKi or vehicle (PBS) immediately after hypoxia. To assess activation of JNK in this rat model, we measured levels of phosphorylated JNK (P-JNK) and total JNK in control animals (no fetal inflammation, no hypoxia), vehicle-treated WMI animals and D-JNKi-treated WMI animals at 24 hours after hypoxia. We observed increased P-JNK levels in vehicle-treated WMI rats compared to control animals (Fig. 1A). Importantly, treatment with D-JNKi reduced P-JNK levels to control levels, indicating proper JNK inhibition by the D-JNKi peptide (Fig. 1A). As a control, no changes were observed in total JNK levels (Fig. 1B).

D-JNKi improves functional outcome and myelination in a rat model of diffuse WMI

To investigate the effects of D-JNKi on functional outcome, animals were subjected to the rotarod task at the age of 5 weeks to assess motor skills. Vehicle-treated WMI rats spent less time on the rotarod compared to control animals, indicating impaired motor performance (Fig. 1C). D-JNKi treatment significantly improved in motor performance compared to vehicle-treated WMI (Fig. 1C).

To study the effects of D-JNKi treatment on myelination and oligodendrocyte proliferation, stainings for myelin-marker MBP, oligodendrocyte lineage marker Olig2 and proliferation marker Ki67 were performed on brain sections of P15 animals. Importantly, vehicle-treated WMI animals showed a reduction in cortical myelination compared to control animals, and D-JNKi treatment partially rescued myelination as indicated by increased cortical MBP^+ staining (Fig. 1D,E). Additionally, vehicle-treated WMI animals showed increased numbers of $Ki67^+Olig2^+$ as a measure for proliferating (i.e. not maturing) OPCs compared to control animals. D-JNKi treatment after induction of WMI reduced the number of $Ki67^+Olig2^+$ cells back to control conditions (Fig. 1F,G). Together, these data indicate that JNK inhibition by D-JNKi has protective effects on the white matter in a rat model of neonatal WMI.

D-JNKi rescues myelination under pro-inflammatory conditions *in vitro*

Improved myelination following D-JNKi treatment *in vivo* may result from direct actions of D-JNKi on oligodendrocyte lineage cells. However, the effects may also be mediated by an attenuated neuroinflammatory response of e.g. microglia, thereby indirectly

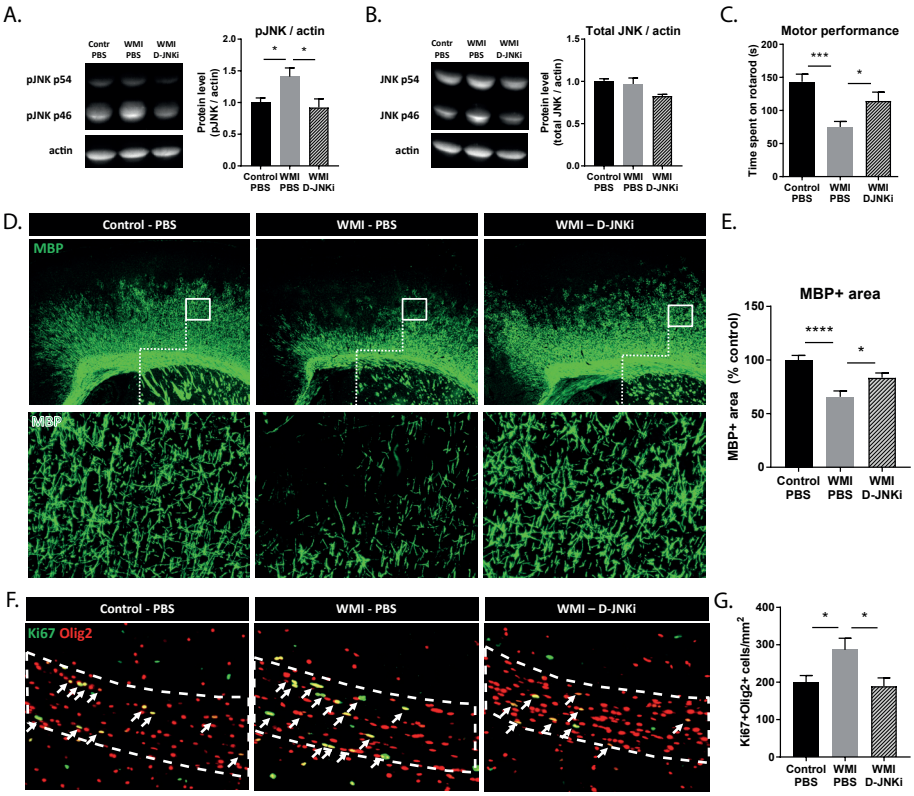


Figure 1 D-JNKi treatment inhibits JNK activation, rescues motor performance, and partially restores myelination in a neonatal rat model of diffuse WMI. **A**. Western blots at 24 hours after hypoxia confirm increased JNK phosphorylation in vehicle-treated WMI animals, which is strongly inhibited after D-JNKi treatment. **B**. Total JNK levels do not change in WMI animals, as confirmed by western blot (A,B: control-PBS: n=12; WMI-PBS: n=12; WMI-D-JNKi: n=10). **C**. Performance on the rotarod as measured by time spent on the rotating rod indicates impaired motor skills in vehicle-treated WMI animals, which improve after D-JNKi treatment (control-PBS: n=13; WMI-PBS: n=12; WMI-D-JNKi: n=15). **D**. Representative images of brain slices stained for MBP at 2.5X (upper panels) or 20X magnification (lower panels) showing cortical myelination in all experimental groups. **E**. Quantification of MBP^+ area reveals impaired cortical myelination in vehicle-treated WMI animals compared to control animals, which is partly rescued by D-JNKi treatment (control-PBS: n=13; WMI-PBS: n=11; WMI-D-JNKi: n=12). **F**. Ki67 (green) and Olig2 (red) staining on brain tissue of P15 rats. The corpus callosum as region of interest is outlined (dashed lines). The number of $Ki67^+Olig2^+$ cells (arrows) was quantified. **G**. Quantification reveals an increased number of $Ki67^+Olig2^+$ cells (i.e. proliferating oligodendrocyte lineage cells) in the white matter of vehicle-treated WMI animals compared to control animals. D-JNKi-treated WMI animals show similar numbers to that of control rats (control-PBS: n=11; WMI-PBS: n=11; WMI-D-JNKi: n=9). *: $p<0.05$; ***: $p<0.001$; ****: $p<0.0001$.

affecting oligodendrocyte maturation. To study whether D-JNKi can act on OPCs directly, we cultured primary OPCs in either MCM-LPS or MCM+LPS, in the presence of 0-4 μM D-JNKi. Compared to MCM-LPS, MCM+LPS decreased the percentage of $MBP^{+}Olig2^{+}$ cells in the OPC cultures, indicating impaired myelination. Furthermore, MCM+LPS reduced the number of $Olig2^{+}$ cells, indicating impaired OPC survival (Fig. 2A-C). Western blots confirmed that exposure to MCM+LPS increased P-JNK levels in OPC cultures (Fig. 2D). Whereas treatment with D-JNKi did not significantly affect survival of oligodendrocytes under pro-inflammatory conditions, treatment with 4 μM D-JNKi increased the proportion of MBP+ oligodendrocytes (Fig. 2A-C). These results indicate that a pro-inflammatory environment negatively affects myelination in OPC cultures and that D-JNKi has the ability to act directly on surviving OPCs to promote myelination under inflammatory conditions.

D-JNKi attenuates microglia activation *in vitro*, but not *in vivo*

Based on previous studies in which we showed that D-JNKi treatment strongly reduced neuroinflammation in a neonatal rat model of hypoxic-ischemic brain injury[12], we hypothesized that D-JNKi attenuates the inflammatory response of microglia. To study the effects of D-JNKi on microglia activation directly, we stimulated primary cultured microglia with 100 ng/ml LPS and concurrently treated them with 0-2 μM D-JNKi. LPS stimulation changed the morphology of the microglia into a more pro-inflammatory, amoeboid shape (Fig. 3A). Whereas the supernatant of unstimulated microglia did not contain detectable levels of TNFα, the supernatant of LPS-stimulated microglia contained high levels of TNF-α (>1000 pg/ml). After addition of D-JNKi to the microglia their morphology more closely resembled the unstimulated condition, and the release of TNF-α was significantly reduced (Fig. 3A,B), indicating that D-JNKi has an inhibitory effect on the pro-inflammatory response of microglia.

To assess whether D-JNKi treatment attenuates neuroinflammation and/or microglia activation *in vivo*, we measured mRNA levels of various inflammatory mediators at 3, 6 and 24 hours post-hypoxia and we stained brain sections of animals at P15 for the microglia marker Iba1. Expression of TNF-α and MIP2 (CXCL2) mRNA was significantly increased at 3h after hypoxia in vehicle-treated WMI animals, and we observed a trend towards upregulation of MCP1 (CCL2) expression at 3h post-hypoxia in vehicle-treated WMI animals compared to controls (Fig. 3C,E,F). No statistically significant changes were observed in mRNA expression of IL1β at any of the studied timepoints (Fig 3D). Surprisingly, treatment with D-JNKi did not attenuate mRNA expression of any of the cytokines/chemokines induced by WMI (Fig. 3C-F). We showed recently that combined fetal inflammation plus postnatal hypoxia causes an increase in the density of microglia

populating the white matter and that the morphology of microglia changes towards a more amoeboid phenotype[15]. In brain sections of P15 vehicle-treated WMI animals, we again observed an increase in the number of microglia in the corpus callosum compared to control animals, but no significant changes in microglia morphology were observed (Fig. 3G-I). Interestingly, D-JNKi treatment in WMI animals did not affect microglia numbers or microglia morphology compared to vehicle-treated WMI rats (Fig. 3G-I). Together, these results indicate that attenuating neuroinflammation and/or microglia activation is not the primary mechanism by which D-JNKi protects myelination in WMI animals.

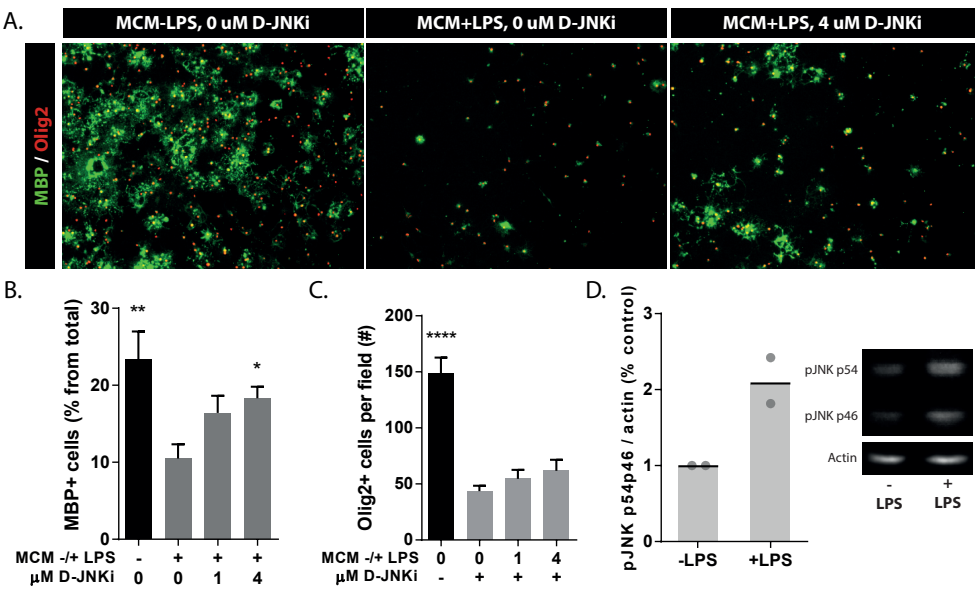
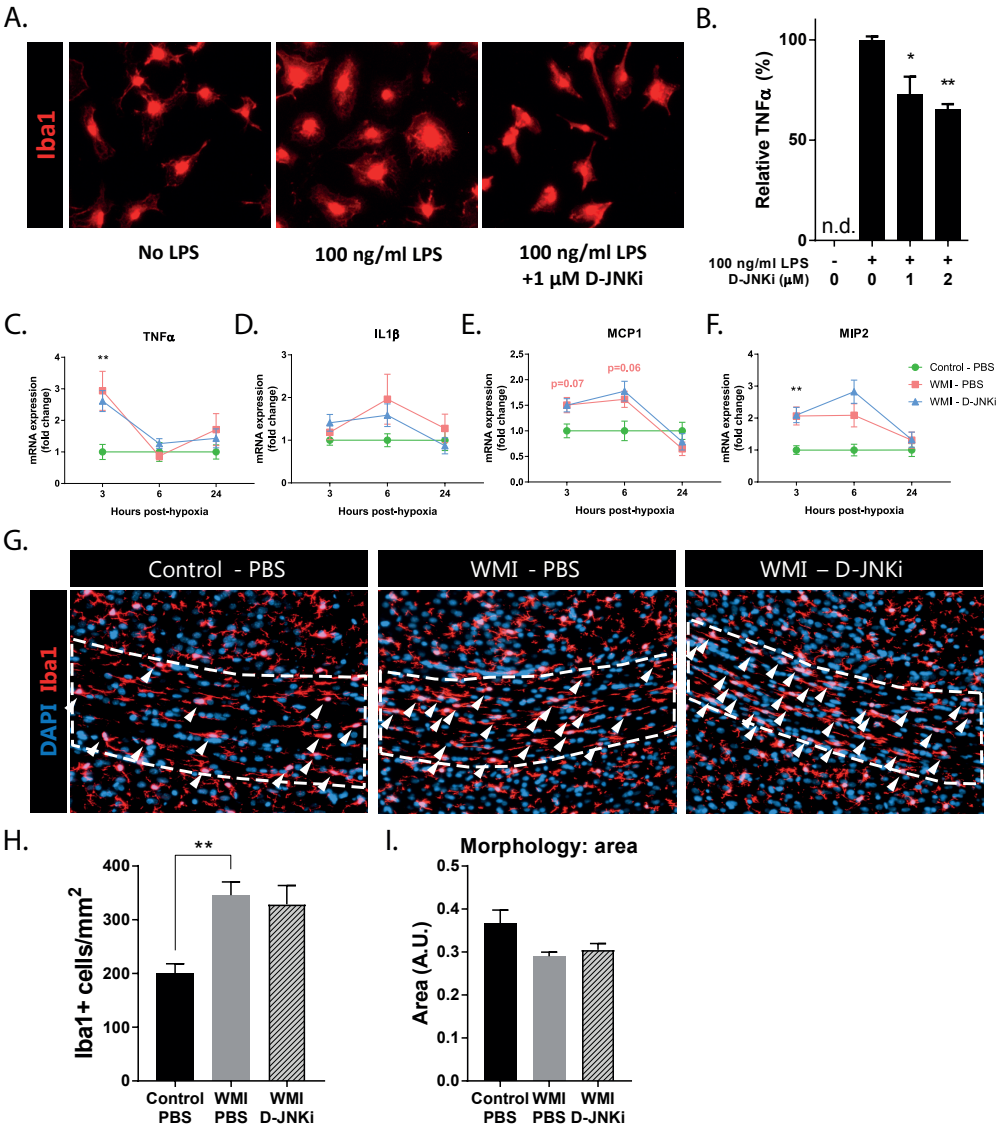


Figure 2 Treatment with D-JNKi promotes myelination under pro-inflammatory conditions in vitro. **A**. Representative fluorescent images of primary cultured oligodendrocytes stained for oligodendrocyte marker Olig2 (red) and myelin component MBP (green). Cells were exposed to unstimulated or LPS-stimulated microglia-conditioned medium (MCM-LPS or MCM+LPS, respectively) and 0, 1 or 4 µM D-JNKi. **B**. MCM+LPS impairs myelin production by oligodendrocytes and D-JNKi treatment restores the proportion of MBP$^+$ oligodendrocytes. **C**. MCM+LPS causes a reduction in the number of Olig2+ cells, indicating impaired survival. D-JNKi treatment does not significantly improve cell survival (B,C: n=5 independent experiments, 3-4 observations per experiment; nonparametric Kruskal-Wallis test with Dunn's post-hoc correction). **D**. Western blot reveals a 2-fold increase in P-JNK in OPCs after exposure to MCM+LPS compared to MCM-LPS. β-actin is used as a loading control (n=2 independent experiments). *: $p < 0.05$; **: $p<0.01$; ****: $p<0.0001$.


A.
Iba1
No LPS
100 ng/ml LPS
100 ng/ml LPS
+1 µM D-JNKi
B.
Relative TNFα (%)
100
50
0
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*
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100 ng/ml LPS - + + +
D-JNKi (µM) 0 0 1 2
C.
TNFα
D.
IL1β
E.
MCP1
p=0,07
p=0,06
F.
MIP2
mRNA expression (fold change)
Hours post-hypoxia
Control - PBS
WMI - PBS
WMI - D-JNKi
G.
Control - PBS
WMI - PBS
WMI – D-JNKi
DAPI Iba1
H.
Iba1+ cells/mm2
Control PBS
WMI PBS
WMI D-JNKi
I.
Morphology: area
Area (A.U.)

Figure 3 D-JNKi attenuates microglia activation in vitro, but not in vivo. **A**. Representative images showing primary microglia cultures stained for microglia marker Iba1 showing morphological changes following LPS stimulation, which are restored after D-JNKi treatment. **B**. Treatment with 1 or 2 μM D-JNKi attenuates microglial TNF-α production. Data are obtained from n=3 independent experiments, 1 pooled observation per experiment. One-way ANOVA followed by multiple comparisons with Bonferroni post-hoc correction. n.d.: not detectable. **C-F**. mRNA expression levels of TNF-α, IL1β, MCP1 and MIP2 measured at 3, 6 and 24 hours post-hypoxia. Vehicle-treated WMI animals show a (border-line) significant increased expression of inflammatory mediators TNF-α, MCP1 and MIP2 at 3h (and/or 6h) post-hypoxia. D-JNKi treatment did not affect mRNA expression levels (for 3,6,24 hours post-hypoxia: control-PBS: n=8,5,12; WMI-PBS: n=13,12,13; WMI-D-JNKi: n=13,12,10).Two-way ANOVA followed by multiple comparisons with Bonferroni post-hoc correction. **G**. Microglia Iba1 staining (red) and nuclear marker DAPI (blue) staining in the white matter of rats. The corpus callosum as region of interest is outlined (dashed lines). The number of $Iba1^+$ cells with a clear $DAPI^+$ nucleus (arrowheads) was quantified and morphological aspects of microglia were assessed. **H**. Quantification of the microglia density in the corpus callosum reveals an increase in microglia in vehicle-treated WMI animals compared to control animals. D-JNKi treatment does not affect microglia density. One-way ANOVA followed by multiple comparisons with Bonferroni post-hoc correction. **I**. Pro-inflammatory microglia activation is associated with a more amoeboid morphology that is paired with a smaller average surface area of individual cells. No significant changes in microglia morphology were observed in WMI animals (H,I: control-PBS: n=6; WMI-PBS: n=6; WMI-D-JNKi: n=5). One-way ANOVA followed by multiple comparisons with Bonferroni post-hoc correction. n.d.: not detected; *: $p<0.05$; **: $p<0.01$.

DISCUSSION

In the present study, we investigated the potential of the JNK-inhibiting peptide D-JNKi as a treatment option for diffuse WMI in the preterm brain. Here, we report that D-JNKi treatment rescues myelination and functional outcome in a rat model of neonatal diffuse WMI evoked by fetal inflammation and postnatal hypoxia. Furthermore, we demonstrate *in vitro* that D-JNKi attenuates microglia activation and promotes OPC maturation and subsequent myelination under pro-inflammatory conditions. However, *in vivo* microglia appear unaffected by D-JNKi treatment, yet OPC proliferation was restored following D-JNKi treatment. Collectively, these results indicate that D-JNKi has neuroprotective effects in the immature brain exposed to perinatal insults, and that the beneficial effects of D-JNKi are likely mediated by improved maturation of oligodendrocytes, rather than attenuated microglia activation.

Previously, we showed that D-JNKi treatment potently reduced hypoxic-ischemic brain damage in a rat model for term neonates, by reducing apoptotic cell death, reactive ROS production and pro-inflammatory cytokine expression[12]. In contrast to the presently used model for diffuse WMI, the hypoxia-ischemia rat model is mainly associated with neuronal injury and JNK activation was primarily observed in neurons, indicating that neurons were primarily targeted by D-JNKi. In the diffuse WMI rat model, no signs of neuronal (axonal) injury were observed as reported earlier[15]. However, other studies revealed that increased JNK signaling in oligodendrocytes and microglia may contribute to myelination impairments in the immature brain[4, 7, 8]. More specifically, JNK activation was observed specifically in microglia and oligodendrocytes in a rat model of neonatal WMI, and inhibition of JNK signaling, either genetically by antisense oligodeoxynucleotides or pharmacologically by the compound AS601245, resulted in improved myelination and attenuated microglia activation[7, 8]. These findings raised the hypothesis that D-JNKi may also prove to have beneficial effects on diffuse WMI. Indeed, we observed that D-JNKi treatment rescues myelination and improves functional outcome in a neonatal rat model of diffuse WMI in preterm infants.

Previous studies reported that JNK activation mediates the pro-inflammatory response of microglia[17] and that D-JNKi potently reduced the expression of inflammatory mediators following neonatal hypoxia-ischemia[12]. In line with these reports, we observed that the inflammatory response of primary cultured microglia was attenuated by D-JNKi *in vitro*. However, we did not observe any effects of D-JNKi on the neuroinflammatory response and/or activation of microglia *in vivo*. WMI induced an increase in microglia

numbers in the white matter, which is associated with a response to injury[18], but D-JNKi treatment did not affect microglia density. Nor did D-JNKi attenuate mRNA levels of proinflammatory mediators induced early after WMI. These surprising data may be explained by various reasons. For example, JNK signaling may not be the main regulator of neuroinflammation in our model or alternative molecular pathways (e.g. NFκB) may compensate for the loss of JNK signaling in regulating the cytokine response[19]. It should be noted that the 2-3-fold increase in mRNA levels of e.g. TNF-α and MIP2 are relatively low compared to the increase of cytokine levels following other insults, such as hypoxia-ischemia which causes a 8-10-fold increase[12]. Alternatively, in the presently used diffuse WMI model the peak in the cytokine response may occur following maternal LPS injections at E18 and E19, rather than after the second hit of postnatal hypoxia. As a consequence, D-JNKi treatment after hypoxia may be too late to efficiently reduce the acute inflammatory consequences of fetal inflammation. It could be hypothesized that instead of evoking an acute JNK-dependent peak of inflammation, in the diffuse WMI model sustained JNK-independent low-grade inflammation is the main issue postnatally. In this respect, it would be interesting to monitor the fetal cytokine response following maternal LPS injections and to study the efficacy of antenatal treatment with D-JNKi.

We observed *in vitro* that D-JNKi directly acts on OPCs, promoting maturation and myelination under inflammatory conditions. Additionally, we observed that *in vivo* D-JNKi reversed increased OPC proliferation in WMI animals. As repression of OPC proliferation is normally associated with initiation of OPC differentiation[20], these data indicate that D-JNKi may improve myelination by directly acting on OPCs, promoting their maturation into myelinating oligodendrocytes. These findings are in line with earlier reports demonstrating that JNK signaling promotes OPC proliferation[5] and represses expression of myelin-associated genes via its downstream effector c-jun[4, 21]. However, the possibility of an indirect effect of D-JNKi on myelination via other cell types (e.g. astrocytes) *in vivo* cannot be excluded at this time.

Our western blot data show that one dose of D-JNKi treatment directly after hypoxia completely blocked activation of JNK evoked by WMI. As these measurements were performed in total brain homogenates, further investigations should reveal to which specific celltype(s) the *in vivo* actions of D-JNKi can be attributed, and which downstream substrates of JNK are specifically targeted. Importantly, D-JNKi did not completely abolish JNK signaling but restored P-JNK levels to control conditions, allowing normal JNK signaling to remain possible for the regulation of normal

cellular functioning. Considering the broad range of organs and cellular processes that require JNK signaling, future safety studies should investigate whether D-JNKi affects developmental processes and whether side effects may occur. Optimistically, clinical trials revealed that subconjunctival D-JNKi administration successfully reduced inflammation following ocular surgery without causing severe adverse events[22, 23], and that intravenous infusion of D-JNKi in healthy adult males was safe and well tolerated[24]. However, the effects of D-JNKi during development remain unstudied in the human population.

To conclude, we identified the JNK signaling pathway as a therapeutic target in diffuse WMI in the preterm brain. JNK inhibition might also prove beneficial in other white matter disorders that are associated with impaired oligodendrocyte maturation and remyelination, such as multiple sclerosis[25]. The pharmacokinetic properties of D-JNKi, including its high cell-permeability, as well as the prolonged half-life compared to other small inhibitors, make D-JNKi an interesting treatment option to further explore, in order to combat diffuse WMI in preterm infants who are in desperate need of novel therapeutic options.

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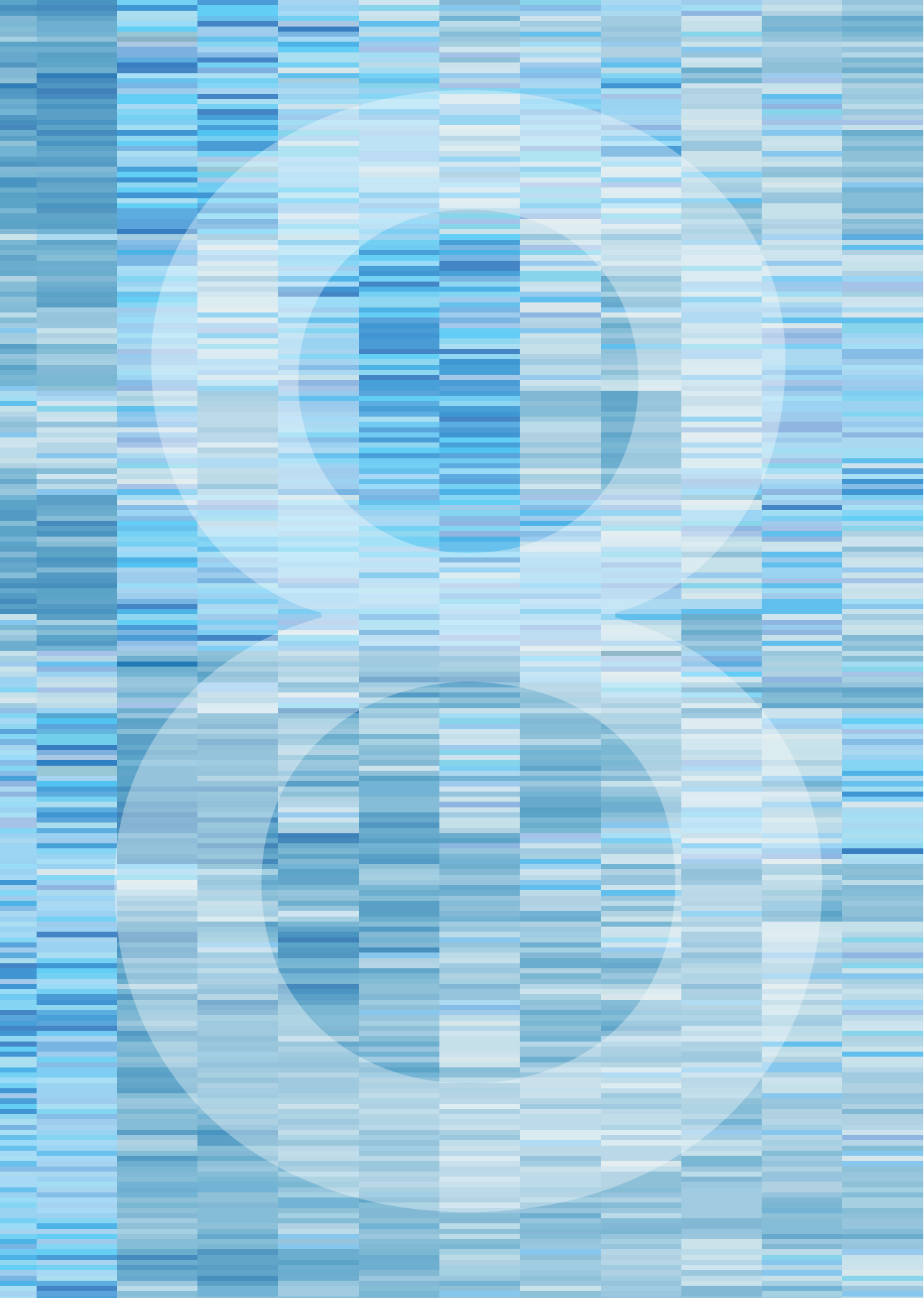

CHAPTER 8

Inflammation alters the transcriptional profile of developing oligodendrocytes

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ABSTRACT

Inflammation negatively affects oligodendrocyte precursor cell (OPC) differentiation into mature myelin-producing oligodendrocytes. Arrested oligodendrocyte maturation elicits impaired (re)myelination as observed in for example white matter injury in preterm infants and multiple sclerosis. Over the past years, the complex mechanisms underlying OPC differentiation have been well characterized. However, exactly which pathways that contribute to OPC differentiation are negatively affected by inflammatory mediators remains unknown. In this study, we used RNA sequencing on primary cultured rodent OPCs to assess how inflammation affects the transcriptional changes required for OPC differentiation. We show that the transcriptional profile of OPCs changes during maturation. Moreover, we demonstrate that an inflammatory environment affects the transcription of the genes involved in the regulation of OPC maturation. The identification of genes involved in the regulation of OPC maturation that are affected by inflammation, may reveal novel therapeutic targets to boost differentiation of OPCs and, consequently, myelination under pathological inflammatory conditions. Such treatment strategies may be beneficial for white matter disorders associated with impaired oligodendrocyte maturation, such as white matter injury in preterm infants and multiple sclerosis.

INTRODUCTION

Oligodendrocytes (OLs) are the myelinating cells of the central nervous system. Myelination is crucial for establishing neuronal connectivity, as myelination ensures efficient signal transduction and provides protection and nutritional support to neuronal axons[1, 2]. During brain development, OLs are derived from neural stem cells that commit to the OL lineage under the regulation of transcription factors such as OLIG1, OLIG2, NKX2.2 and SOX10, which enable the cells to differentiate into OL precursor cells (OPCs). Under healthy conditions, complex transcriptional machinery will be activated to promote differentiation of OPCs into immature pre-myelinating OLs. Then, under optimal circumstances immature pre-myelinating OLs differentiate into mature OLs that contact neuronal axons and start the production of myelin[3, 4].

In a number of white matter disorders, OLs play an important role. For example in diffuse white matter injury (WMI) in preterm infants, impaired maturation of OLs contributes to myelination defects, thereby causing impaired motor skills, decreased cognitive performance and increased risk of psychiatric disorders[5, 6]. Additionally, in multiple sclerosis (MS) demyelinating lesions cause axonal injury[7]. Newly formed OPCs are recruited towards the demyelinating lesions in MS, however it has been shown that these OPCs are unable to differentiate into mature myelin-producing OLs[8]. In both neonatal WMI and MS, neuroinflammation is a key factor in the underlying pathophysiology. For instance, activated proinflammatory microglia occupy the white matter in the developing brain in preterm WMI[6], or in the white matter surround demyelinating lesions in MS[7]. It has been shown previously that proinflammatory mediators like TNFα, IL1β and other cytokines negatively affect the development of OPCs (e.g. [9-11]). Therefore, microglia-mediated neuroinflammation is suggested to play an important role in the maturational arrest of OLs in both preterm WMI and MS. Over the past years, numerous complex mechanisms regulating OPC differentiation have been characterized[3, 12, 13], however exactly how these mechanisms are affected by inflammation remains unknown.

In this study, we sequenced RNA of primary cultured rodent OPCs to reveal the transcriptomic changes that occur in OPCs during differentiation and how these changes are affected by inflammation. We reveal that a pro-inflammatory environment causes changes in the transcriptomic profile of differentiating OPCs that may contribute to their maturational arrest. Furthermore, we identify a number of genes that could play an important role in impeded OPC development in pathological conditions like

preterm WMI and MS. These candidate genes may represent novel therapeutic targets to boost OPC differentiation and myelination in white matter disorders.

METHODS

Animals

All animal experiments were carried out according to Dutch ("Wet op de dierproeven", 1996) and European regulations (Guideline 86/609/EEC) and were approved by the Animal Ethics Committee of Utrecht University.

Glial cell cultures

Mixed glial cell cultures were isolated from postnatal day (P)0-2 Sprague Dawley rat pups as described earlier[14]. Briefly, cortical tissue was isolated, dissociated with DNase I (0.2 mg/ml) and trypsin (0.25%), homogenized, and filtered through a 70 µm cell strainer and plated onto T75 flasks in high glucose DMEM with pyruvate and GlutaMAX (31966, Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 20% fetal bovine serum (FCS), that was refreshed every 2-3 days. The mixed glial culture was allowed to expand for 10-12 days, so a monolayer of astrocytes, with microglia and OPCs would form. Microglia and OPCs were separated by differential adhesion; microglia were detached from the glia culture by shaking for 1 hour at 200 rpm and were plated at 400.000 cells/well onto poly-L-ornithine coated 24-well plates. After 24 hours, medium was removed and replaced by 1 ml of basal medium (BM): high glucose DMEM with pyruvate and GlutaMAX (31966, Gibco) supplemented with 0.1% BSA, 50 µg/ml Apo-transferrin, 30 nM sodium selenite, 10 nM D-biotin, 10 nM hydrocortisone and 0.5% FCS. BM contained either no LPS or 50 ng/ml LPS (from E. Coli O127:B8, Sigma, St. Louis, MO). After 24 hours incubation, the non-stimulated microglia-conditioned medium (MCM-LPS) and the LPS-stimulated microglia-conditioned medium (MCM+LPS) were collected, pooled, filtered through a 0.2 µm filter and frozen in aliquots at -80°C.

OPCs were detached from the glia culture by shaking for 18-20 hours at 200 rpm and were plated at 40.000 cells/well onto poly-D,L-ornitine coated 24-well plates in BM supplemented with mitogens PDGF-AA and bFGF. After three days, the medium was replaced by MCM-LPS or MCM+LPS supplemented with T3, CNTF and NAC to induce differentiation. OPCs were cultured for a maximum of three more days after induction of differentiation (Fig. 1A). Throughout the OPC experiments, the respective medium was refreshed every 1-2 days. OPC cultures were used for RNA harvest at 3 experimental

conditions: (1) prior to inducing differentiation (T0), (2) 24 hours after inducing differentiation in MCM-LPS (T1C), and (3) 24 hours after inducing differentiation in MCM+LPS (T1I) (Fig. 1A). Or, OPC cultures were fixed for immunostainings at 24 hours (T1C, T1I) or 72 hours (T2) after inducing differentiation.

ELISA

IGF-1 and TNFα levels in MCM-LPS and MCM+LPS were determined using commercially available ELISA kits (IGF-1, Sigma; TNFα, Ucytech, Utrecht, The Netherlands) according to manufacturer's instructions.

Immunostainings

For stainings, fixation of OPC cultures at T1 and T2 (Fig 1A) was performed by incubation in paraformaldehyde for 10 minutes. After washing, fixed cells were incubated in blocking buffer (2% BSA and 0.1% saponin in PBS) for 1 hour, followed by overnight incubation in primary antibody solutions: rabbit-anti-OLIG2 (1:1000; Millipore, Burlington, MA; AB9610), mouse-anti-MBP (1:1000; Sternberger Monoclonals, Lutherville, MD; SMI-94), rabbit-anti-IBA1 (1:1000; Wako, Osaka, Japan), mouse-anti-GFAP (1:1000; Cymbus, Southamptom, UK; CBL411) diluted in PBS. Next, wells were washed and incubated in secondary antibody solutions: alexafluor 488-conjugated goat-anti-mouse and alexafluor 594-conjugated goat-anti-rabbit (both 1:1000; Invitrogen), followed by Hoechst (1:5000, Sigma) counterstaining. The number of OLIG2+ cells was determined by automated cell counting using ImageJ and MBP+ area was assessed by setting a threshold to exclude any background signal and measure the MBP+ area. Iba-1 and GFAP antibodies were used as control stainings to assess the purity of the OPC cultures.

RNA isolation and sequencing

For each experiment, RNA of OPCs pooled from 8 wells per condition was isolated using a RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. In total, RNA was isolated and sequenced from 5 biologically independent experiments. RIN values of RNA samples were between 8.6 and 9.7.

Polyadenylated mRNA was isolated using Poly(A) Beads (NEXTflex) and sequencing libraries were made using the Rapid Directional RNA-seq kit (NEXTflex). Libraries were sequenced using the Nextseq500 platform (Illumina), producing single end reads of 75bp (Utrecht Sequencing Facility).

Data processing and analysis

Reads were aligned to the human reference genome Rnor5 using STAR version 2.4.2a. Picard's AddOrReplaceReadGroups (v1.98) was used to add read groups to the BAM files, which were sorted with Sambamba v0.4.5 and transcript abundances were quantified with HTSeq-count version 0.6.1p1 using the union mode. Subsequently, reads per kilobase million reads sequenced (RPKMs) were calculated with edgeR's RPKM function.

Differentially expressed genes were identified using the DESeq2 package while correcting for donor/animal specific baseline (desig() <- ~treatment + animal). Genes with absolute log2 fold change larger than 0.6 and padj < 0.1 were considered as differentially expressed. PCA plots were made using DESeq2::plotPCA() command with ntop = 1000.

A comparison between the presented dataset and the dataset of expression values of different types of brain cells was made by selecting the 200 most enriched genes in each condition and comparing the overlap with the 200 most enriched genes of each cell type as characterized previously[15]. Data were visualized using ClustVis[16].
For heatmaps, a selection of the 50 or 150 most significant up- and/or downregulated genes was made based on adjusted p-values, and RPKM values were plotted using ClustVis[16].

Gene ontology enrichment analysis was performed by selecting all significantly up and/or downregulated genes in a single comparison and overrepresented gene sets were calculated by PANTHER[17, 18]. Bars represent fold-change compared to expected enrichment.

RESULTS

The secretome of LPS-stimulated microglia impairs myelination in primary OPC cultures

In order to simulate the inflammatory environment that hampers OL maturation in white matter disorders such as MS and preterm WMI, we cultured primary microglia from neonatal rat brains for 24 hours with (MCM+LPS) or without (MCM-LPS) exposure to LPS. Supernatants of these microglia cultures were collected and then added to primary OPC cultures for 1 day (T1) or 3 days (T2) (Fig. 1A). To confirm microglia

polarization, IGF1 and TNFα levels in the conditioned medium were determined using ELISAs as indicators of non-polarized and proinflammatory microglia, respectively. MCM-LPS contained 12.4 pg/ml IGF1, but no detectable amount of TNFα, whereas MCM+LPS contained no detectable amount of IGF1 and >3000 pg/ml TNFα. These levels indicate that LPS skewed microglia towards a proinflammatory phenotype. One day after adding MCM to the OPC cultures (T1), cells exposed to MCM-LPS had differentiated into immature OLs as indicated by their morphology which changed from a bipolar toward more complex multipolar cells (Fig. 1A), but OPCs exposed to MCM+LPS showed much less morphological complexity (i.e. less processes) (Fig. 1B, upper panel). On T1, most cells in culture were OLIG2+, indicative for the OL lineage. However, no cells had differentiated into mature MBP+ myelinating OLs yet (Fig. 1B, middle panel). At T1 a number of GFAP+ cells (i.e. astrocytes) was observed in addition to the OLs, but no contaminating IBA1+ microglia were detected. Three days after adding MCM-LPS (T2), several cells had differentiated into mature MBP+ myelinating OLs (Fig 1B, lower panel). Cells exposed to MCM+LPS showed a slight but not statistically significant reduction in the number of Olig2+ cells and showed decreased MBP+ area, indicating impaired differentiation and myelin production by the OLs in response to a proinflammatory environment (Fig. 1B, lower panel; Fig. D,E).

RNA sequencing reveals the transcriptomic profile of OPCs

In order to assess the transcriptional changes that occur during differentiation of OPCs and how inflammation affects the differentiation process, we performed RNA sequencing on OPCs before induction of differentiation (T0), 24 hours after inducing differentiation in the presence of MCM-LPS (T1C), and 24 hours after differentiation in the presence of MCM+LPS (T1I) (Fig. 1A). Principle component analysis revealed that in particular OPCs exposed to inflammation cluster together, separately from the OPCs not exposed to inflammation (Fig. 2A). In total, 1302 genes were differentially regulated after induction of differentiation (T0 vs. T1C). After induction of differentiation MCM+LPS caused differential expression of 860 genes (T1C vs. T1I) of which 319 genes were also implicated in regulating differentiation (overlap T0 vs. T1C and T1C vs. T1I). When comparing pre-differentiation (T0) OPCs to differentiating MCM+LPS OPCs (T1I) 1541 genes were differentially regulated (T0 vs. T1I) (Fig. 2B).

The 200 most highly enriched genes in in different conditions showed most resemblance to that of OPCs, newly formed OLs and myelinating OLs as characterized by Zhang et al.[15], and less resemblance to astrocytes, microglia and endothelial cells (Fig. 2C), indicating that our OPC population was comparable to that of others. Additionally,

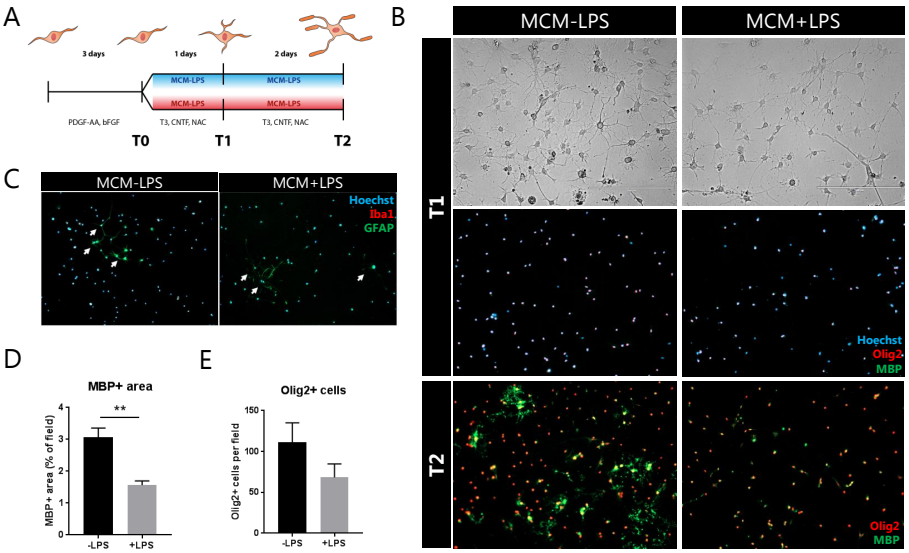

Figure 1 The secretome of LPS-stimulated microglia impairs myelination in primary OPC cultures. **A**: Schematic representation of experimental setup. OPCs were either stained or harvested for RNA isolation at T0, T1 and T2. **B**: Stainings of OPC cultures at T1 (24 hours after mitogen withdrawal) and T2 (3 days after mitogen withdrawal). Upper panels: brightfield images at T1 reveal that OPCs exposed to MCM-LPS show more arborization of their processes, indicative of maturation, compared to OPCs exposed to MCM+LPS. Middle panels: OPCs at T1 exposed to MCM-LPS or MCM+LPS stained for nuclear marker Hoechst, OL marker OLIG2 and myelin marker MBP. These stainings indicate that no myelination has occurred yet at T1. Lower panels: OPC cultures at T2 stained for OLIG2 and MBP show differentiated, myelin-producing OLs after exposure to MCM-LPS, but to a much lesser extent after exposure to MCM+LPS. **C**: OPC cultures at T1 show some presence of GFAP+ astrocytes. No IBA1+ microglia were observed. **D**: Quantification of MBP+ area in OPC cultures at T2. A significant decrease in MBP+ area was observed in OPC cultures exposed to MCM+LPS compared to MCM-LPS. **E**. Quantification of the number of OLIG2+ cells per field at T2. Data represent n=5 independent experiments; **: $p<0.01$.

when assessing the expression of cell type-specific genes, genes specific to the OL-lineage, like *Olig1* and *Sox10*, are mostly enriched (Fig. 2D). Fig. 2E shows that induction of differentiation (T0 vs T1C) caused genes to be up- and downregulated in OPCs, and that inflammation mostly caused an upregulation of genes (Fig. 2F,G). Collectively, these data confirm that the transcriptomic profile of the samples shows high resemblance to that of OLs, and that both induction of differentiation and exposure to a pro-inflammatory environment alter the gene expression in OPCs.

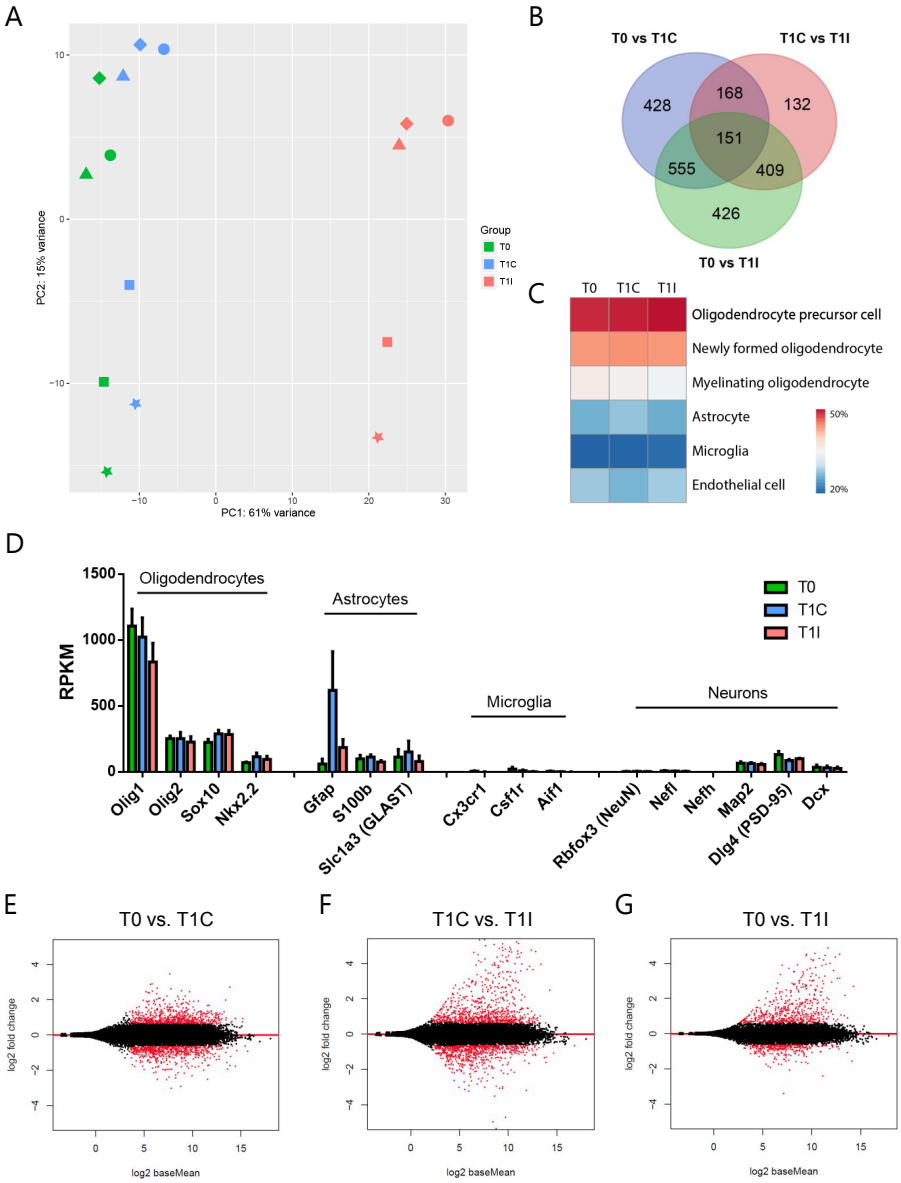

Figure 2 Changes in the transcriptomic profile of OPCs occur during differentiation, and in the presence of an inflammatory environment. **A**: Principle components analysis of sequencing data of OPCs prior to differentiation (T0), post-differentiation without inflammation (T1C) and post-differentiation with inflammation (T1I). Shapes represent biologically independent replicates. **B**: Venn-diagram of the number of differentially regulated genes when comparing the three different conditions. **C**: When comparing the most highly expressed genes to the mostly enriched genes in various brain cell types as published by Zhang et al.[15], the RNA profile of all conditions show most similarity with OPCs and newly formed OLs. **D**: High enrichment of OL-specific genes, less enrichment of astrocyte-specific genes and hardly any presence of microglia- or neuron-specific genes. **E-G**: MA plots showing differentially regulated genes when making comparisons between the three different experimental conditions, with significantly up- or downregulated genes shown in red.

Inflammation alters the transcriptional profile of developing OPCs

To investigate which genes may contribute to differentiation, the 150 genes that were most significantly upregulated and the 150 genes that were most significantly downregulated after induction of differentiation were selected and their expression levels were plotted in a heatmap (Fig. 3A T0 vs. T1C).

Gene ontology enrichment analysis of the 150 most upregulated genes revealed that many of these genes have been previously implicated in 'oligodendrocyte development' and 'myelination', and that these genes particularly exert functions in the cellular component 'myelin sheath' (Fig. 3B,C). These results highlight that after mitogen withdrawal OPCs start differentiating into mature OLs and additionally validate the specificity of the OL culture and sequencing data. In addition, the data in Fig 3D-K highlight that the regulation of genes specifically associated with OL differentiation and myelination, like *Myrf, Mbp* and *Mog*, are increased at T1C compared to T0 (Table 1).

When differentiating OPCs were exposed to inflammation (i.e. MCM+LPS), the expression of upregulated genes during OPC differentiation was diminished (T1I vs T1C in heatmap Fig. 3A). In line, myelin-associated genes show decreased expression in OPCs exposed to MCM+LPS compared to OPCs exposed to MCM-LPS, more resembling expression in T0 OPCs (Fig. 3D-K) (Table 1). These data indicate that the transcriptional changes that are triggered during differentiation are hampered in the presence of inflammation.

Exposure to MCM+LPS triggers an immune response in differentiating OPCs

In order to more closely investigate the transcriptional changes induced by inflammation, the 150 most up- and 150 most downregulated genes when comparing T1C to T1I were selected. Many genes in the T1I condition show a different pattern of expression compared to T0 and T1C (Fig. 4A). Gene ontology enrichment analysis verified that most genes differentially expressed by exposure to MCM+LPS are involved in inflammation (Fig. 4B). A closer look at specific genes increased in T1I confirms upregulation of factors associated with inflammatory pathways, including cytokine signaling pathways (Fig. 4C-L) (Table 1). In line with our observations displayed in Fig. 3, also genes regulating OPC differentiation (e.g. *Myrf* and *Sirt2*) were downregulated in T1I vs T1C (heatmap Fig 4).

Table 1

	T0 vs. T1C		T1C vs. T1I		T0 vs. T1I	
Gene	log2 fold change	adjusted p-value	log2 fold change	adjusted p-value	log2 fold change	adjusted p-value
Cnp	0.49	1.44E-07	-0.12	5.40E-02	0.37	2.25E-03
Cldn11	1.21	2.79E-32	-0.39	5.11E-08	0.80	9.40E-11
Cxcl1	2.62	7.40E-33	2.84	5.54E-60	5.13	4.99E-92
Cxcl10	1.95	1.69E-12	4.86	2.53E-198	7.00	2.89E-217
Dynll2	1.10	1.06E-23	-0.59	5.56E-30	0.52	5.90E-05
Hcn2	1.59	2.03E-37	-0.32	4.03E-04	1.25	3.11E-19
Ifi27	-1.57	1.56E-10	3.26	0	3.56	0
Ifi27l2b	-1.57	1.56E-10	4.42	1.75E-282	2.37	2.86E-20
Irf7	0.30	4.90E-02	2.04	1.16E-19	7.74	0
Kif19	2.49	1.44E-55	-0.81	4.43E-11	1.68	4.03E-24
Lhfpl2	1.21	2.10E-31	-0.38	3.19E-13	0.83	4.77E-14
Mag	0.93	2.42E-20	-0.54	5.54E-17	0.38	4.59E-03
Mbp	0.93	2.85E-18	-0.57	1.57E-27	0.35	1.54E-02
miR-675	0.79	1.41E-02	-1.12	3.89E-06	0.56	1.62E-01
Mog	0.95	6.98E-09	-0.50	7.28E-04	0.27	5.78E-01
Myrf	0.94	1.24E-14	-0.45	1.83E-16	0.49	4.29E-04
Nfkb1	0.01	9.73E-01	1.25	7.53E-66	1.26	8.9E-38
Nfkb2	0.25	2.25E-01	1.77	1.36E-77	2.05	1.1E-73
Plp1	0.75	1.07E-10	-0.51	8.68E-37	0.24	0.13
Ras-gef1b	2.04	2.54E-37	-0.51	3.56E-07	1.53	4.04E-20
Sirt2	0.68	7.50E-15	-0.40	1.90E-18	0.28	0.01
Stat1	0.51	7.86E-06	3.14	0	3.65	0
Stat2	0.74	1.28E-11	2.93	0	3.66	0
Tns3	0.97	3.28E-29	-0.37	1.28E-13	0.60	3.88E-09
Vcam1	2.00	8.73E-22	2.74	1.06E-178	4.79	4.10E-200

Log2 fold change values and adjusted p-values of genes featured in figures 3-5

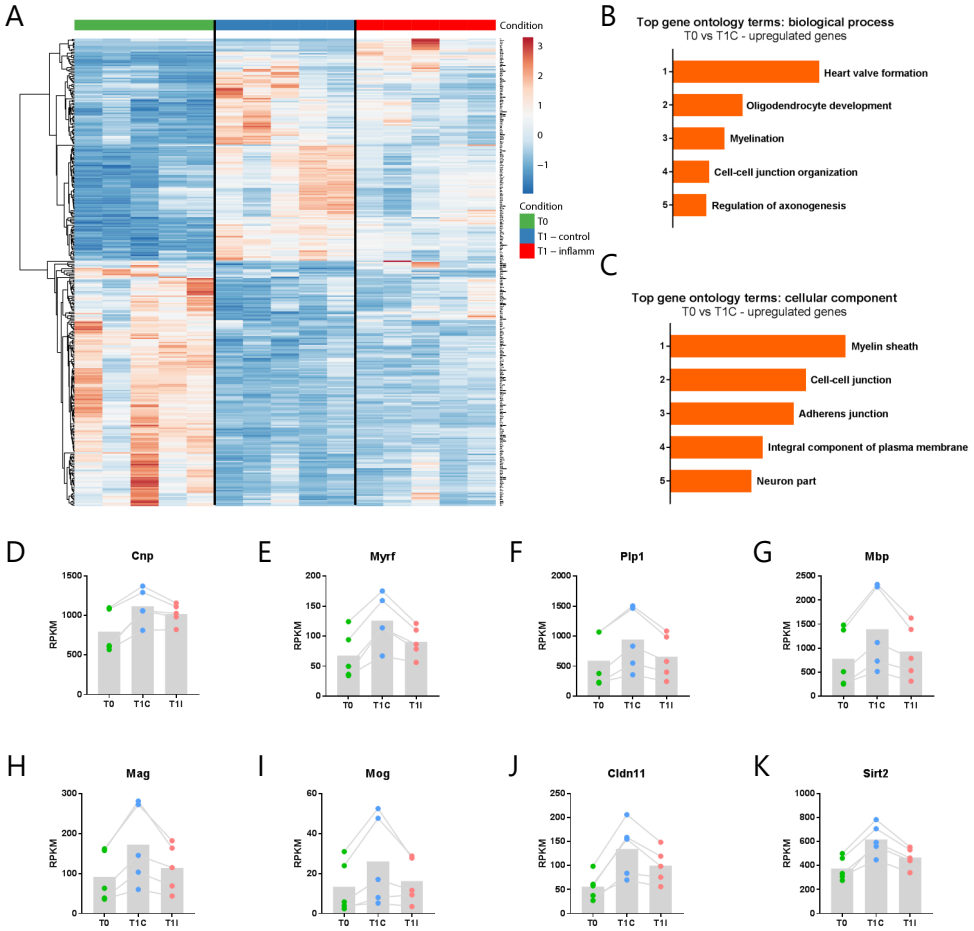

Figure 3 Inflammation attenuates the expression of genes associated with OPC differentiation. **A**: Heatmap of the 150 most upregulated and 150 most downregulated genes when comparing OPCs pre- and post-differentiation (T0 vs. T1C). Clearly, the expression of a wide range of genes is increased during differentiation. In the presence of inflammation (T1I) the upregulation of these genes is diminished. **B, C**: Gene ontology enrichment analysis confirms that genes upregulated during differentiation are involved in OPC differentiation, with "OL development" and "myelination" being among the top 5 mostly enriched biological process gene ontology terms, and "myelin sheath" being the mostly enriched cellular component gene ontology term. **D-K**: The expression of eight specific genes associated with OPC differentiation and myelination show increased expression levels during differentiation (T1C vs. T0). However, no increase or an attenuated increase is observed in the presence of MCM+LPS (T0 vs. T1I). For p-values see Table 1.

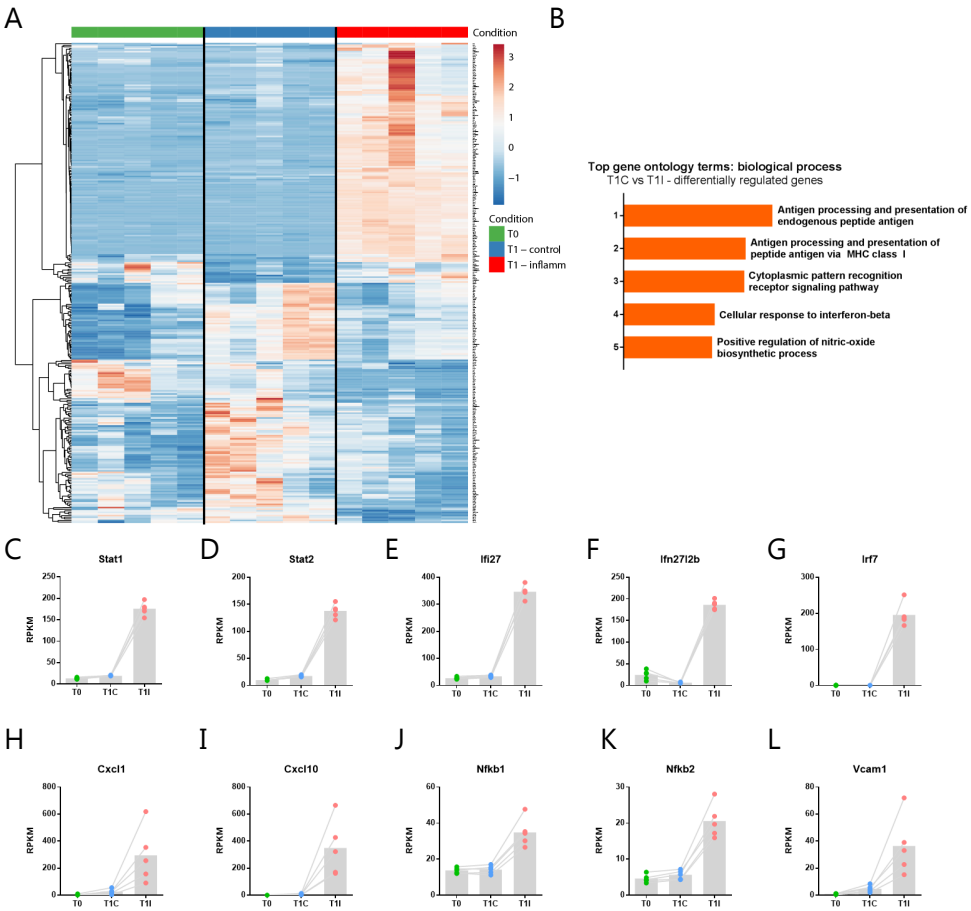

Figure 4 MCM+LPS triggers an immune response in differentiating OPCs. **A**: Heatmap of the 150 most upregulated and the 150 most downregulated genes when comparing differentiating OPCs in the absence or presence of inflammation (T1C vs. T1I). A striking increase in the expression of genes compared to either condition without inflammation is visible in the MCM+LPS condition (T1I vs T0/T1C). **B**. Gene ontology enrichment analysis of the genes upregulated during inflammation (T1I vs. T1C) reveals that many of these genes are involved in the regulation of immune processes, such as antigen presentation, pattern recognition and responses to inflammatory cytokines. **C-L.** Examples of genes that are highly increased during inflammation (T1I vs T0/T1C) that are involved in cytokine signaling or other inflammatory processes. For p-values, see Table 1.

Factors increased during differentiation, but decreased during inflammation

To identify genes that drive OPC differentiation but are affected during inflammation, the top 50 genes that are both upregulated in T0 vs. T1C, and that are downregulated at T1C vs. T1I were selected (Fig. 5A). As expected, this list contains genes that are associated with OL maturation and myelination, such as *Mbp* and *Plp1*. However, also a number of genes that have not been previously implicated in OL development show increased expression after induction of differentiation, with diminished expression under inflammatory conditions. For example, *miR-675, Kif19, Dynll2, Lhfpl2, Tns3, Rasgef1b* and *Hcn2* are all increased at T1C vs T0, but not or not as much at T1I (Fig 5B,C,E,H,J,M,O) (Table 1). Where possible, the expression of these genes was compared to previously published datasets[15, 19] of gene expression in specific stages of OL development and interestingly, most of these selected genes are highly enriched in differentiating preOLs (Fig. 5D,F,G,I,K,L,N,P,Q) (Table 1). This selection of genes, together with other genes showing similar expression profiles, represent candidates for further research to assess whether indeed these genes are novel regulators of OL maturation that contribute to inflammation-induced differentiation arrest.

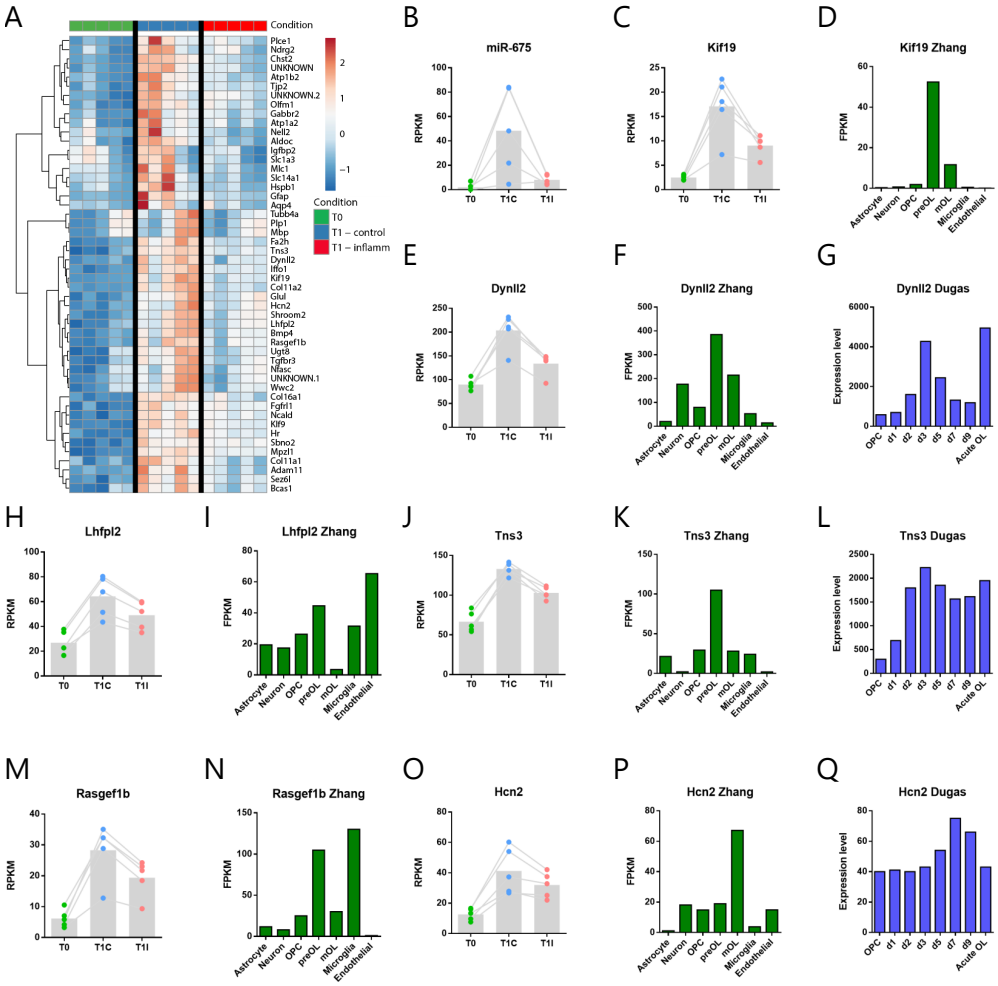

Figure 5 Identification of novel genes that may be involved in the inflammation-induced maturation arrest of OPCs. **A**: Heatmap of the top 50 genes which show increased expression during differentiation (T0 vs. T1C), but which upregulation is attenuated in the presence of inflammation (T1C vs. T1I). **B, C, E, H, J, M, O**. Examples of genes that are upregulated during differentiation, but not – or to a lesser extent – in the presence of inflammation. For p-values, see Table 1. **D, F, I, K, N, P**. Where possible, genes of interest were compared to previously published datasets by Dugas et al.[19] and Zhang et al.[15] to check the specificity of the genes for specific stages of OL maturation.

DISCUSSION

To conclude, we determined changes in the gene expression profile of OPCs that occur 24 hours after induction of differentiation, and we investigated how an inflammatory environment interacts with these genes. Clearly, inflammation attenuates the expression of genes that are positively associated with OPC differentiation and myelination, and causes an increase in expression of genes associated with an immune response. The acquired dataset may be used to identify novel genes that might 1) contribute to OPC differentiation and myelination and 2) mediate the inflammation-induced developmental arrest of OLs. We observed that exposure to inflammation prevented upregulation of genes involved in regulating OPC differentiation and myelination (Fig. 3), however genes downregulated during OPC differentiation generally remained downregulated under inflammatory conditions (Fig 3A). Moreover, inflammation was associated with increased expression of genes associated with inflammatory responses (Fig. 4). Additionally, we identified a set of genes that are upregulated in OPCs during differentiation but not in the presence of an inflammatory environment (Fig. 5). The involvement of these genes in the regulation of OPC differentiation and their potential role in inflammation-induced maturational arrest of OPCs should be further validated and studied.

We confirmed the specificity of our cells in the OL-lineage by staining OPC cultures for myelin and Olig2, by showing enriched expression of OL-specific genes, and by comparisons to published sets of OPC RNA sequencing data. However, a small set of GFAP-positive cells was also present in the OPC cultures. Contaminating astrocytes may have detached during the shaking procedure, however considering the low expression of *Gfap* in pre-differentiation (T0) samples and high expression of *Gfap* in post-differentiation (T1C) samples, it could be speculated that a proportion of OPCs differentiated into astrocytes – a fate switch that has been described extensively[20-22]. Whereas the *in vitro* approach used in this study allows the detailed analysis of the effects of differentiation and inflammation in OPCs specifically, it should be taken into account that the OPCs in culture are not residing in the complex environment that they encounter *in vivo*. Therefore, the relevance of candidate genes in the dataset should be verified *in vivo*. Furthermore, some heterogeneity exists between the biologically independent experiments as observed in e.g. the heatmaps in Fig. 3-5. Two out of five independent experiments showed already higher baseline expression of myelin-associated genes, whereas the three other experiments showed lower baseline expression of these genes, as well as differently clustered enriched expression after

induction of differentiation (Fig. 3). These data indicate that in two replicates the OPCs may have differentiated slightly faster and may have been at a somewhat further developmental stage. However, generally differentiation and inflammation affected the patterns of myelin-associated gene expression (e.g. *Plp1, Mbp, Mag, Mog*) similarly in all replicates.

The obtained dataset reveals various genes which expression changes during differentiation, or in the presence of inflammation. Genes that are significantly up- or downregulated after inducing differentiation (T0 vs. T1C) represent genes that potentially play important roles in the regulation of OPC differentiation. For instance, the specific intracellular transport genes *Kif19* and *Dynll2*, encoding for kinesin KIF19A and cytoplasmic dynein light chain 2 (DYNLL2) respectively, have not been previously implicated in OL functioning, but are increased in differentiating OPCs (Fig. 5C,E) and are enriched in pre-OLs in previously published datasets (Fig. 5D,F,G). These findings suggest a role for *Kif19* and *Dynll2* during OPC differentiation, but their exact relevance *in vivo* should be further validated.

Selecting candidate genes that are upregulated during differentiation, but which upregulation is hampered when inflammation is present, could aid in identifying novel pathways contributing to inflammation-induced maturation arrest of OPCs, eventually leading to development of novel therapeutic strategies. Examples of such genes include *Tns3* (encoding the focal adhesion protein TENSIN3 that regulates cell adhesion and migration by mediating interaction with the extracellular matrix[23]), *Lhfpl2* (encoding the relatively uncharacterized lipoma HMGIC fusion partner-like 2 protein), and *RasGEF1b* (guanine-nucleotide exchange factor) (Fig. 5H-N). Several additional interesting candidate genes that are upregulated during differentiation, but which upregulation is hampered during inflammation, are discussed below.

miR675

Whereas OPCs hardly show any expression of microRNA (*miR)675*, at 24 hours after induction of differentiation the expression of *miR675* shows a 25-fold increase. However, after exposure to inflammation the increase in *miR675* is only 4-fold (Fig. 5B). Whereas the transcript of *miR675* was not included in earlier sequencing studies, these results indicate that *miR-675* plays a role in the regulation of OPC differentiation. Other microRNAs (e.g. *miR219* and *miR338*) have been shown to play crucial roles in regulating the switch of OPCs from a proliferative state towards differentiation[24, 25]. However, *miR675* has not been previously implicated in regulating OPC differentiation,

nor has it been well characterized in general. The two isoforms of *miR675*, *miR675-3p* and *miR675-5p*, are derived from the long noncoding RNA *H19*, which is located downstream of the Igf2 gene. The IGF2-H19-miR675 locus has been highly conserved throughout evolution[26, 27]. *miR675* has been implicated in a variety of processes, including the positive regulation of osteoblast/skeletal muscle differentiation[28-30], hypoxia-induced angiogenesis[31], as well as the positive[32-34] and negatives[35, 36] regulation of tumor growth and metastasis, including that of glioma[37-39]. Interestingly, *miR675* negatively regulates proliferation[40] and negatively regulates placental growth, by downregulating expression of insulin-like growth factor 1 receptor (*Igf1r*)[40, 41]. Whether *miR675* is expressed in OPCs *in vivo* and whether it contributes to switching OPCs from a proliferative towards a differentiation stage should be further elucidated using experimental animal models.

HCN2

Another gene that was upregulated during differentiation, but to a lesser extent during inflammation is *Hcn2* (Fig. 5O). Interestingly, in the dataset by Zhang et al.[15] expression of *Hcn2* is highly enriched in mature OLs compared to earlier OL stages or to other cell types. Similarly, in the Dugas et al.[19] study *Hcn2* expression was shown to be enriched during the late stages of OL development. These data indicate that *Hcn2* is mainly expressed by mature myelinating OLs. *Hcn2* encodes the hyperpolarization-activated cyclic nucleotide-gated ion channel 2 (HCN2). Whereas the role of HCN2 in OL functioning has not been investigated, its role in neuronal functioning has been widely studied – mainly as a mediator of pain[42, 43]. Additionally, HCN2 has been implicated in memory deficits resulting from chronic cerebral hypoperfusion[44]. HCN2 is activated during hyperpolarization in the range of -60 to -90 mV and can be activated by cyclic nucleotides such as cAMP[45]. Considering the increase in expression of Hcn2 over the course of OL development, mature OLs may express HCN2 to respond to hyperpolarizing events. Thereby, it may enhance neural activity-dependent myelination. Alternatively, HCN2 may be produced by OLs to be translocated towards neuronal axons. The role of HCN2 during OPC differentiation and myelination is an interesting topic that requires further study, importantly because HCN channels may be interesting targets for therapeutic intervention[46].

Collectively, our results provide insight in the regulation of OPC differentiation and how the developmental program of OPCs is altered during inflammation. Hopefully, this dataset will contribute to the elucidation of OL-specific targets to boost myelination in the event of inflammation, such as in neonatal WMI and MS.

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CHAPTER 9

Summary & Discussion

Diffuse white matter injury (WMI) is a major complication in infants born prematurely and the need for more research to improve the outcome of these children is increasingly being recognized. In this thesis, we report various preclinical studies in which we optimized methodology to study diffuse WMI in a translational setting, and to identify and determine the relevance of novel therapeutic targets. In this final chapter, the findings presented in this thesis are summarized, and the results and potential implications of our observations are discussed.

SUMMARY

In **chapters 2 and 3**, we extensively reviewed the current state of knowledge regarding the mechanisms that contribute to healthy white matter development and how preterm birth-related insults negatively affect myelination in the brain. Over the past years, the origin of oligodendrocyte precursor cells (OPCs) has been characterized and the complex signaling pathways that regulate differentiation of OPCs into mature myelinating oligodendrocytes (OLs) have been elucidated (**chapter 3**). In humans, myelination occurs mostly postnatally and white matter volumes continue to increase until the age of 40 years. In the rodent brain, myelination follows a similar spatial and temporal pattern compared to humans (**chapter 3**). In the event of preterm birth, the developing brain is exposed to numerous perinatal insults that negatively affect the white matter, causing microglia activation, astrogliosis, and impaired maturation of OLs. Microglia and astrocytes likely play key roles in the etiology of WMI by creating an unfavorable environment for OPCs to properly differentiate into mature OLs and to start the production of myelin (**chapter 2**). In **chapter 2**, the signaling pathways through which microglia and astrocytes may contribute to impaired OL maturation in neonatal WMI are described and various treatment strategies to combat diffuse WMI are highlighted.

In **chapter 4**, we introduced a novel rat model of diffuse WMI in preterm infants. We observed that the combination of fetal inflammation and postnatal hypoxia (but not single insults) caused all pathological hallmarks of WMI, including impaired myelination, decreased OPC differentiation, microglia activation and astrogliosis. Moreover, WMI animals displayed motor deficits, anxiety-like behavior and signs of autism-like behavior. Although cortical myelination seemed restored by the age of 10 weeks, this rodent model closely resembles the clinical presentation of diffuse WMI in preterm infants until at least early adulthood. In **chapter 5** we validated methodology

to study cortical myelination patterns in great detail using our rat model of WMI. This methodology can be used to detect more subtle changes in myelination, as demonstrated in three different neonatal and one adult rodent model of brain injury. In **chapter 6**, we investigated cognitive outcome in our WMI model in 7-9 months old adult rats using the 5-choice serial reaction time task and the probabilistic reversal learning task. No changes were observed in terms of impulsivity, attention, or cognitive flexibility in adult WMI animals. These findings may be partially explained by restored myelination at later ages, as observed in **chapter 4**.

In **chapter 7** the novel rat model of diffuse WMI was used to determine the efficacy of the JNK inhibiting peptide D-JNKi as a treatment option for WMI patients. Interestingly, treatment with D-JNKi immediately after hypoxia partially restored myelination and motor performance in rats with WMI. D-JNKi treatment likely promoted myelination by acting directly on OPCs as observed *in vitro* and as indicated by reduced OPC proliferation (i.e. increased OPC maturation) in D-JNKi-treated WMI animals *in vivo*. The inflammatory response in brains of WMI animals was not affected by D-JNKi treatment, indicating that attenuation of neuroinflammation is not the major mechanism of action of D-JNKi in this model. The exact mechanisms underlying the protective actions of D-JNKi remain to be further elucidated.

In **chapter 8**, RNA sequencing was performed on primary cultured OPCs to study the transcriptomic changes that occur during OPC differentiation, and how genes involved in regulating differentiation are affected by inflammation. This experiment resulted in an extensive dataset that can be used to both identify novel genes that are implicated in the regulation of OPC differentiation, and to identify genes that may contribute to the maturation arrest of OPCs during inflammation as observed in diffuse WMI. The obtained dataset will be an important resource for further studies.

DISCUSSION

Animal models of diffuse WMI: can we mimic the human situation?

Several rodent models to study diffuse, non-cystic WMI in the immature brain have been used in the past. Maternal inflammation during late pregnancy has been shown to induce myelination impairments at P7 in the offspring[1, 2], and daily postnatal systemic injections with proinflammatory cytokine IL1β from P1-P5 causes myelination deficits that persist until at least P30[3]. Similarly, hypoxic rearing (9.5-10.5% O_2 during P3-P11)

has been shown to induce myelination deficits, together with long-term changes in white matter integrity (FA-values) until P51[4-6]. Whereas these rodent models do lead to myelination impairments, they only take one specific type of preterm birth-related insult (i.e. inflammation or hypoxia) into account whereas generally preterm infants are exposed to a combination of different insults[7, 8]. The exposure to multiple hits has been suggested to play a crucial role in the etiology of perinatal WMI with early insults sensitizing the brain to become more vulnerable to subsequent insults[9, 10]. In line, clinically, exposure to multiple hits is associated with a dramatic increase in the risk of and severity of white matter abnormalities[11-13]. In **chapters 4 and 6**, we investigated the effects of combined fetal inflammation and postnatal hypoxia, in an attempt to induce a clinically relevant pattern of brain injury that closely resembles pathology observed in the brains of preterm infants with diffuse WMI. Whereas single insults of fetal inflammation or postnatal hypoxia did not induce myelination deficits, the combination of both insults induced impaired myelination until at least P30. Similarly, Brehmer et al. demonstrated that combined early postnatal inflammation and hyperoxia caused myelination deficits at P11[14].

In **chapter 4**, we demonstrated that combined fetal inflammation and postnatal hypoxia initially induced clinically relevant behavioral impairments, including impaired motor skills, anxiety-like behavior and signs of autism-like behavior (i.e. reduced social play and more repetitive grooming). Other diffuse WMI models have also been associated with functional impairments: maternal inflammation has been shown to induce motor impairments[1, 2], postnatal systemic inflammation induces memory deficits[3], and hypoxic rearing causes increased locomotor activity and impaired spatial memory[4]. Interestingly, in animals exposed to fetal inflammation and postnatal hypoxia we observed no changes in cognitive performance during adulthood (**chapter 6**). Although the functional consequences during adulthood in other diffuse WMI models have not been extensively characterized, motor impairments following maternal inflammation were restored at P23[2] and locomotor activity following hypoxic rearing was increased at P16, but had returned to control levels by P40[4]. Collectively, these results indicate that perinatal insults initially induce functional impairments in rodents, but that behavioral performance restores during adulthood.

Improved behavioral outcome in rodent models of diffuse WMI may be explained by delayed, rather than permanently damaged myelination in the brain. In **chapter 4**, we observed that cortical myelination following fetal inflammation and postnatal hypoxia was restored to control levels at the age of 10 weeks. Similarly, in the maternal

inflammation model myelination was restored at the age of 4 months[2] and following combined postnatal inflammation and hyperoxia myelination deficits were restored at P21[14]. The recovering capacity of rodent myelination may result from compensatory mechanisms that ensure the constant availability of undifferentiated 'backup' OPCs that have the potential to mature, and thereby replace injured OLs or compensate for insufficient myelination (**chapter 3**). Oligodendrocyte lineage cells are intrinsically driven to obtain a homeostatic balance of OPC numbers, and injury to OPCs rapidly triggers proliferation and migration of neighboring OPCs to restore OPC density[15]. In our rat WMI model, we observed an initial increase in proliferating OPCs which could contribute to the availability of sufficient OPC numbers (**chapter 4**). Similarly, following hypoxic rearing the production of new OPCs is increased[6, 16, 17]. Also in human postmortem tissue of preterm WMI patients increased numbers of OPCs were observed, indicating that similar mechanisms may occur in human preterm infants[18].

The currently available animal models of neonatal diffuse WMI all initially induce a clinically relevant pattern of non-cystic injury to the white matter. However, none have been confirmed to induce myelination impairments and behavioral deficits that persist throughout adulthood. This is not in line with clinical observations, where white matter abnormalities and behavioral problems persist until adulthood[19, 20]. These findings indicate that translational mechanistic and therapeutic studies in rodents should focus on the initial time-window of approximately the first month during which myelination impairments and functional consequences are apparent. The restorative capacity of the white matter may represent an inherent difference between rodents and humans. Therefore, it is essential that novel neuroprotective treatment options are also validated in large animal models, such as in an ovine model of preterm birth-related brain injury[21], before being translated toward a clinical setting.

Glia pathology in diffuse WMI: targets for treatment?

The vulnerable oligodendrocyte precursor: death or arrest?

Pre-myelinating (pre-)OLs are particularly enriched in the brain during the crucial period in which preterm infants are exposed to inflammatory and/or hypoxic insults[22]. The highly enriched pool of pre-OLs is extremely vulnerable to preterm birth-related insults, due to their lack of antioxidant defenses and expression of highly calcium-permeable glutamate receptors (due to their subunit composition)[23]. Consequently, injury to OL lineage cells is key in the etiology of neonatal diffuse WMI (**chapters 2 and 3**). Human pathology studies on postmortem brains of preterm infants revealed impaired differentiation of pre-OLs in diffuse WMI areas[18, 24, 25], and some studies even

report apoptosis of pre-OLs[26-28]. Whether pre-OL cell death occurs may depend on the nature and severity of insults that preterm infants are exposed to and the observation of both presence and absence of pre-OL cell death in different studies likely reflects the heterogeneity of complications that preterm infants are exposed to. In our rat model of diffuse WMI introduced in **chapter 4**, we did not find evidence of OL cell death at 1 day after hypoxia. Likely, in the event of OPC/pre-OL cell death, the population of OPCs is rapidly restored by increased proliferation as described in **chapter 3**[15, 17]. However, an arrest in the maturation of OLs still results in myelination impairments. Therefore, promoting proper differentiation and maturation of OLs should be the main focus of novel treatment options to reduce the burden of diffuse WMI in preterm infants.

Microglia and astrocytes: innocent bystanders or key mediators of WMI?
As observed in postmortem brain tissue of WMI patients, microglia activation and astrogliosis are additional pathological hallmarks of diffuse WMI[18, 24-26]. As described in **chapter 2**, microglia and astrocytes responding to cerebral injury may contribute to the arrest of OL maturation in neonatal WMI by secreting pro-inflammatory cytokines or other factors that inhibit differentiation of OPCs and pre-OLs.

Pro-inflammatory activation of microglia contributes to diffuse WMI by release of pro-inflammatory cytokines and reactive oxygen/nitrogen species, as well as exacerbating excitotoxicity by release of glutamate[23]. Clearly such processes negatively affect the health and proper maturation of vulnerable OPCs and pre-OLs (**chapters 2, 7 and 8**). After an inflammatory insult, microglia may be primed to show an exaggerated response to subsequent insults, thereby aggravating injury following a second hit[29-31]. In rats exposed to fetal inflammation and postnatal hypoxia, we observed signs of microglia activation up to 2 weeks post-hypoxia, indicating that long-term changes were induced in the microglia (**chapter 4**). Since single insults did not induce WMI in this novel rat model, the synergistic effects of combined inflammation and hypoxia could be explained by the fetal inflammation priming the microglia to become more responsive to the subsequent hypoxic insult – however this remains to be demonstrated directly. Besides adopting a pro-inflammatory profile, microglia may also adopt an anti-inflammatory profile to resolve inflammation and promote regenerative processes[32]. Anti-inflammatory microglia polarization promotes differentiation of OPCs, by release of anti-inflammatory cytokines and growth factors[33, 34]. Therefore, attenuation of pro-inflammatory microglia responses or boosting anti-inflammatory polarization of microglia may be interesting therapeutic strategies to overcome the harmful effects of microglia on white matter development during perinatal insults.

Astrocyte reactivity is another hallmark of neonatal diffuse WMI (**chapter 2**). Reactive astrocytes have been implicated in hampering OL maturation via multiple mechanisms, e.g. the production of pro-inflammatory cytokines, hyaluronic acid, bone morphogenic proteins (BMPs) and prostaglandin E2[35-37]. Moreover, reactive astrocytes reduce their uptake of glutamate via glutamate transporters GLAST and GLT1, thereby contributing to excitotoxicity[38, 39]. In our rat WMI model described in **chapter 4** we indeed observed reduced expression of GLAST and GLT1 following perinatal insults, indicating that glutamate-induced excitotoxicity may be a significant factor in mediating WMI. Therefore, inhibition of excitotoxic glutamate signaling or increasing glutamate uptake by astrocytes may be an interesting therapeutic strategy.

Blood-brain barrier integrity

As described in **chapter 3**, recent studies revealed that close interactions exist between OPCs and the cerebral vasculature [40, 41]. The enormous amount of protein synthesis that occurs during myelin production by OLs requires adequate oxygen supply, and OPCs therefore promote angiogenesis in brain regions that require more oxygen[42, 43]. In addition, OPCs promote blood-brain barrier (BBB) integrity[44] and perinatal insults have previously been shown to negatively affect integrity of the BBB, resulting in infiltration of peripheral immune cells (e.g. macrophages, leukocytes, T-cells) that can aggravate WMI for instance by release of pro-inflammatory mediators[45, 46]. Proper maintenance of BBB integrity by enforcing endothelial cells and astrocytic end-feet tight junctions could restrict the extent of WMI.

Contribution of neural defects to WMI

Besides impaired myelination, other neurodevelopmental processes may also be affected by neonatal insults, and may contribute to the adverse functional outcome of preterm infants. The generation and migration of gamma-aminobutyric acid (GABA) ergic neurons during brain development coincides with the time-window during which preterm infants are exposed to various perinatal insults[28]. Both in human tissue of preterm infants with WMI and in rodent models of neonatal WMI, a reduction in GABAergic neurons has been demonstrated[28, 47]. Additionally, GABAergic signaling has been implicated in regulating proper proliferation and differentiation of OPCs, indicating that perturbed interneuron development may contribute to the white matter abnormalities associated with preterm birth[48]. Another neurodevelopmental process that occurs during early postnatal brain development is synaptic pruning by microglia[49]. During this process, microglia phagocytose excess neuronal synapses, which is crucial for normal brain development[49]. Activation of microglia during brain development may

cause excessive or insufficient synaptic pruning, which could underlie the causal link between early inflammation and neurodevelopmental disorders[50]. Collectively, these studies indicate that besides myelination deficits, neural circuitry may also be affected by preterm birth. Therefore, rescuing myelination may not be sufficient to overcome all functional consequences of preterm birth-related brain injury. This should be taken into consideration when developing novel treatment options.

Towards improved treatment options for diffuse WMI in preterm infants

Which cells should be targeted in diffuse WMI?

As microglia are significant contributors to WMI, attenuation of the inflammatory response or polarization of microglia towards an anti-inflammatory phenotype may have beneficial effects on OL maturation and white matter development. Treatment with the TNFα inhibitor etanercept has been shown to ameliorate WMI following perinatal insults in mice[51, 52]. These findings highlight the potential of etanercept as a treatment option, but it should be taken into account that protective actions of TNFα by acting on TNF receptor 2 are also inhibited by etanercept. Minocycline is a tetracycline that potently inhibits microglia activation, and treatment with minocycline has been shown to protect the white matter from perinatal insults in various rodent models of neonatal WMI[53-55]. Moreover, treatment with the anti-inflammatory cytokine interleukin-10 has been shown to be neuroprotective in the neonatal white matter[56, 57]. These studies highlight the potential of targeting neuroinflammation to improve outcome in diffuse WMI. However, the main hazard of anti-inflammatory and immunoregulatory treatment strategies, is that suppression of the immune response should be tightly regulated as this might impair the infant's defense against bacterial or viral infections[58].

The precise pathways underlying astrocyte reactivity remain poorly understood. Therefore, inhibiting the onset of astrogliosis remains difficult. However, the downstream effects of astrocyte reactivity may be targeted by novel treatment options. Activation of the JAK/STAT pathway may prevent the downregulation of glutamate transporters[39], or inhibition of BMP signaling with the antagonist Noggin may protect OLs from injury[59]. Alternatively, reactive oxygen species may be counteracted by treatment with antioxidants as demonstrated earlier[60].

Whether attenuation of the pro-inflammatory cerebral response, promoting anti-inflammatory microglia polarization, or attenuation of astrocyte reactivity are sufficient strategies to reduce the effects of perinatal insults on OL maturation is an important issue. Alternatively, developing OLs could be targeted directly. As discussed in **chapters 2 and 3**, the wide range of pathways that have been implicated in regulating OPC

differentiation and myelination have been uncovered over the past decades. Activation of pathways that promote OPC differentiation could promote myelination during stressful conditions, or conversely blocking pathways that inhibit OPC differentiation and myelination may also be beneficial (for examples see table 1, **chapter 3**). Although many pathways that regulate OL development and myelination have been revealed over the past years, it is essential that we obtain an even better understanding of OL biology during healthy development and during injurious events. In **chapter 8**, we reveal that the expression of a wide range of genes involved in the regulation of OPC differentiation is altered in the presence of inflammation. Future studies should elucidate which of these genes actually regulate OPC differentiation *in vivo* and which genes may represent targets for novel treatment options to counteract the negative effects of inflammation on OL maturation. The valuable dataset acquired in **chapter 8** therefore provides an important basis for further research into the mechanisms underlying impaired OL maturation under inflammatory conditions.

When defining novel treatment options for the developing brain, it should be taken into consideration that potential treatment options do not interfere with normal neurodevelopmental processes or healthy neuronal functioning. To illustrate, antagonizing glutamate receptors may protect developing OLs from injury, but may also hamper normal glutamatergic communication between neurons. Ideally, a treatment option or combination therapy that attenuates microglia activation and astrogliosis, promotes blood-brain barrier integrity, but also directly promotes differentiation of OLs should be developed in order to protect the preterm brain from injury. An interesting target in this respect would be the Sonic hedgehog (Shh)-Smoothened (Smo) pathway, which has been implicated in regulating OPC generation, OPC differentiation, angiogenesis, BBB integrity and reducing inflammation[61-65]. Interestingly, activation of the Shh-Smo pathway indeed had protective effects in a mouse model of glucocorticoid-induced neonatal brain injury[66, 67]. However, its potential as a treatment against specifically diffuse WMI requires further study.

JNK signaling: a key modulator of maturation?

Although some studies indicate that activation of JNK signaling occurs in OLs following injury, and thereby negatively regulates OL differentiation and myelination, the precise role of JNK signaling in OLs has not been thoroughly investigated. As in other cell types, in OLs stressful conditions (including aberrant AMPA-receptor activation) induce JNK-dependent apoptosis[68, 69]. Moreover, JNK activation specifically in OLs has been demonstrated in an adult rat model of ischemic brain injury and in human postmortem

brain tissue of multiple sclerosis patients[70, 71]. Moreover, Chew et al. demonstrated that platelet-derived growth factor (a potent inhibitor of OPC differentiation) acutely activates JNK signaling and that inhibition of JNK promotes OPC differentiation and expression of myelin-associated genes[72]. The negative regulation of OPC differentiation by JNK was shown to be dependent on the downstream target c-jun, which forms the AP1 transcription factor that represses the expression of the myelin-basic protein gene[72, 73]. Additionally, JNK signaling was shown to promote OPC proliferation via activation of c-jun, c-fos and c-myc, further reinforcing the notion that JNK signaling maintains OPCs in an immature state[74]. These observations point out a role of JNK signaling in negatively regulating OPC differentiation and myelination.

Collectively, these studies indicate that JNK activation following perinatal insults might play a crucial role in WMI in preterm infants. Indeed, Wang et al. demonstrated that JNK signaling was increased in pre-OLs and microglia in a rat model of neonatal WMI induced by LPS-sensitized hypoxia-ischemia[45]. Moreover, pharmacological and genetic inhibition of JNK attenuated neuroinflammation and rescued myelination, further supporting the role of JNK in neonatal WMI[45, 52]. In **chapter 7** we studied the potential of the JNK inhibitor D-JNKi as a treatment to improve neonatal diffuse WMI. D-JNKi has previously been shown to partially inhibit OPC cell death following UV irradiation *in vitro*[75]. Additionally, previous studies in our lab have shown that D-JNKi has neuroprotective effects in a rat model of neonatal hypoxic-ischemic brain injury[76, 77]. In these earlier studies the neuroprotective actions of D-JNKi were mainly mediated by preventing neuronal apoptosis, however the pathophysiology underlying diffuse WMI is very different with impaired OL maturation and neuroinflammation being crucial factors. In **chapter 7**, we show that D-JNKi treatment promotes myelination under pathological conditions *in vitro* and *in vivo*. Our studies using primary OPC cultures indicate that D-JNKi is able to directly act on OPCs, thereby promoting differentiation and myelination under inflammatory conditions. Our *in vivo* data also indicate that D-JNKi may have acted on OPCs and pre-OLs directly, reducing JNK-dependent repression of differentiation, and thereby improving myelination and functional outcome. Interestingly, we did not observe any effects of D-JNKi treatment on microglia activation as indicated by cytokine expression levels, morphology and cell numbers. These results indicate that microglia activation in our rat model is not fully dependent on JNK signaling or that the treatment regime of D-JNKi used in this study cannot effectively target microglia. Therefore our main conclusion was that D-JNKi promotes myelination by directly acting on OPCs, although it cannot be excluded that neuroprotective effects are partially mediated by effects on astrocytes

or by acting on peripheral immune cells. Thus, our results indicate that increased JNK signaling contributes to diffuse WMI and that inhibition of JNK signaling by D-JNKi peptide promotes myelination under pathological conditions. However, it should be taken into account that JNK signaling is not specific to glial cells and regulates numerous developmental and cellular processes[78]. Therefore, caution should be taken when inhibiting JNK signaling in a clinical setting. D-JNKi should probably not be considered as a treatment for all preterm infants, regardless of their situation. Likely, JNK inhibition using D-JNKi will be most effective in the event of acute perinatal events during which a peak in JNK activation may occur, which can be specifically blocked by D-JNKi without affecting basal JNK levels required for development. It would be a major step forward if we could develop a validation of increased JNK phosphorylation using laboratory tests on e.g. blood samples that would reflect JNK phosphorylation in the brain. In this way, D-JNKi may not only prevent WMI following acute perinatal insults when applied systemically, but may also benefit other vulnerable developing organs in preterm infants such as the lungs or the intestines.

All in all, D-JNKi is an interesting treatment option to further explore for clinical application. Further rodent studies should focus on elucidating the precise mechanisms of action, and should reveal whether systemic D-JNKi treatment is safe without causing long-term adverse effects on any organs. Although phase I and phase II clinical trials using D-JNKi show promising prospects for treatment in adults[79-81], efficacy and toxicity studies of D-JNKi treatment during development should be validated using larger animal models, before translation towards the clinic.

Growth factor treatment

As described in **chapter 2**, growth factors are essential in the regulation of OL maturation. Two growth factors are of particular interest for therapeutic purposes. Intranasal epidermal growth factor treatment has been shown to improve outcome in a mouse model of neonatal diffuse WMI, by downregulating signaling elements of the differentiation inhibiting Notch pathway[6]. Additionally, insulin-like growth factor 1 (IGF-1) potently promotes differentiation of OPCs[82]. Compared to full-term infants, preterm neonates show lower circulatory IGF-1 levels and lower IGF-1 levels have been associated with adverse neurodevelopmental outcome[83, 84]. Increasing IGF-1 levels in preterm neonates may promote OL maturation and improve myelination, as demonstrated in rodent models[85, 86]. Recently, a phase II clinical trial aimed at reducing retinopathy of prematurity was performed in which IGF-1 (together with its binding protein IGFBP-3 to increase the half-life) was infused into preterm infants, without

causing any serious adverse effects[87]. It will be important to further study IGF-1 treatment for preterm infants in the context of brain development and WMI.

MSC treatment

Treatment with mesenchymal stem cells (MSCs) has proven to be a highly successful strategy to protect the neonatal brain from hypoxic-ischemic brain injury. More specifically, MSCs can be administered intranasally and have been shown to migrate towards brain lesions to initiate regenerative responses, causing improvements in grey and white matter volumes[88-90]. Whereas MSCs do not integrate into the brain tissue, they produce numerous trophic and anti-inflammatory factors that promote recovery[88-90]. Therefore, intranasal MSC treatment may also be beneficial in diffuse WMI following preterm birth-related insults. One obvious difference, however, is that in case of hypoxic-ischemic lesions MSCs have a clear destination to migrate towards. It will be uncertain to know where MSCs will exactly migrate to in the case of diffuse WMI – if they migrate towards the brain at all. Future studies will have to determine the efficacy of MSC treatment for neonatal diffuse WMI. An interesting future application for MSCs is their genetic modification to overexpress therapeutic factors (e.g. anti-inflammatory cytokines, growth factors), thereby acting as vehicles that locally release bioactive drugs in injured brain regions.

Optimal care for preterm infants: much to learn

Over the past decades, major progress has been made in providing optimal care for infants born prematurely. Yet, there is still room for improvement as many preterm infants show high rates of morbidity and adverse functional outcome. From a clinical perspective, there is a high need to better understand which infants are at high risk to develop diffuse WMI and which time-windows are suitable for potential clinical interventions. Epidemiological studies into how different types of insults and the timing of the insults affect the pattern of brain injury and functional outcome of preterm infants may help to identify critical periods in brain development during which interventions should be carried out to improve outcome.

Whereas the link between preterm birth and an increased risk of neurodevelopmental disorders, including autism-spectrum disorders, schizophrenia and ADHD, has been well established[91-97] the exact mechanisms underlying preterm birth-induced neurodevelopmental disorders remains unknown. Yet, numerous studies have indicated that white matter integrity is impaired in autism-spectrum disorder, schizophrenia and ADHD[98-103]. Further studies should aim at investigating whether neurological disorders

in children born prematurely are mediated by changes in white matter structure. This will provide information regarding the pathophysiological processes that should be targeted by novel treatments to reduce the high risk of preterm infants to develop neurological disorders.

Another important question remains whether genetic influences can predispose preterm infants for developing diffuse WMI or related neurological impairments. Only few studies have investigated the role of genetic factors in preterm birth-related brain injury (**chapter 2**). Large-scale multicenter studies in which large amounts of data are integrated regarding the clinical presentation of preterm infants, blood values, MRI measures, short- and long-term functional outcome, together with their genetic makeup may reveal associations between certain genotypic variances and pathology or neurological outcome. Screening for genetic vulnerability may aid in selecting a subpopulation of preterm infants that may particularly benefit from certain treatment options.

Brain development, and in particular myelin production, requires enormous amounts of energy, fatty acids and amino acids. It has been demonstrated that insufficient food intake is related to adverse outcome[104], indicating that sufficient energy levels are required for proper brain maturation. However, the optimal nutritional profile remains to be further developed, as the optimal nutritional composition of different types of fatty acids, amino acids, probiotics, and other supplements remains unknown[105]. Improving general nutrition for preterm infants may benefit their development. Moreover, determining the optimal amount of nutritional supplements based on blood levels of nutritional parameters may allow tailored nutrition for each individual case in the heterogeneous preterm population.

As discussed in **chapter 3**, neural activity promotes myelination. This raises the possibility of promoting myelination by providing preterm infants with an enriched environment that provokes sensory stimulation and/or motor activity. Although the efficacy of sensory stimulation in improving white matter integrity in infants remains controversial[106, 107], sensory stimulation therapy may be used as a supplement to bioactive treatment options.

Concluding remarks

To conclude, in this thesis we describe and validate new methodology to study diffuse WMI in rodents. The novel rat model of WMI and the advanced analysis of cortical

myelination enable detailed investigation of mechanisms underlying inflammation and hypoxia-induced WMI, and of new treatment options to protect the brain from preterm-birth related injury. Moreover, we show that JNK inhibition by D-JNKi peptide promotes OL differentiation and myelination under pathological circumstances. These data indicate that D-JNKi is an interesting therapeutic option, which should be further developed as a treatment option for clinical application. Furthermore, detailed analysis of OL gene expression patterns that are affected by inflammation may reveal additional pathways that could be targeted to ameliorate the negative effects of preterm birth on white matter development. Together, these studies contribute to the development of novel treatment options, which are desperately needed to improve outcome of preterm infants with diffuse WMI.

9

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NEDERLANDSE SAMENVATTING (SUMMARY IN DUTCH)

Witte stof hersenschade in te vroeg geboren baby's

Vroeggeboorte is een groot probleem in neonatale intensive care units. In Nederland vindt 8% van alle geboortes te vroeg plaats, dat wil zeggen na een zwangerschapsduur van 37 weken of korter – in plaats van de normale zwangerschapsduur van ca. 40 weken. Een deel van deze kinderen wordt zelfs al geboren na een zwangerschapsduur van slechts 24-27 weken. Vanwege de extreem vroegtijdige geboorte wordt deze kwetsbare groep aangeduid als 'extreem premature' kinderen. Wanneer extreem prematuren geboren worden, zijn veel organen nog niet ver genoeg ontwikkeld om hun functies buiten de baarmoeder correct te kunnen uitvoeren. Zo ontwikkelen veel extreem prematuren long- en darmproblemen. Tevens is het immuunsysteem in deze kinderen vaak nog niet ver genoeg ontwikkeld om bescherming te bieden tegen schadelijke invloeden van buitenaf, wat een verhoogde gevoeligheid voor infecties met zich mee brengt. Daarnaast zorgt een combinatie van ademhalingsproblemen en onderontwikkelde vaatstelsels vaak voor een verminderde zuurstoftoevoer naar organen. Schade aan organen, blootstelling aan infecties en zuurstoftekort kunnen een ontstekingsreactie veroorzaken in het lichaam van prematuur geboren baby's, wat de schadelijke processen kan verergeren.

Ook de hersenen kunnen door blootstelling aan ontstekingsreacties en zuurstoftekort beschadigd worden. Tijdens het derde trimester van de zwangerschap ondergaat het brein een belangrijke groeispurt. Met name de witte stof in de hersenen (die belangrijke verbindingen tussen verschillende hersengebieden bevat) ontwikkelt zich tijdens de laatste weken van de zwangerschap - precies de periode waarin prematuur geboren kinderen aan schadelijke invloeden blootgesteld worden. Daardoor is vooral de witte stof in de hersenen van te vroeg geboren baby's vaak beschadigd. In het ergste geval veroorzaakt dit grote cystes in de witte stof (*cystische periventriculaire leukomalacie*), wat leidt tot ernstige motorische handicaps zoals cerebrale parese (hersenverlamming). Gelukkig komt deze vorm van witte stof hersenschade vanwege de vele verbeteringen in neonatale zorg steeds minder voor in te vroeg geboren baby's. Daarentegen komen mildere vormen van witte stof hersenschade (aangeduid als *diffuse* witte stof hersenschade) nog steeds erg vaak voor in premature kinderen. Diffuse witte stof schade wordt gekenmerkt door verkleinde volumes en verminderde structurele integriteit van witte stof banen, de aanwezigheid van microscopische laesies in de witte stof en glia activatie/gliosis, welke kunnen worden waargenomen middels MRI scans.

De afgelopen jaren is duidelijk geworden dat diffuse witte stof hersenschade later in het leven kan leiden tot verminderde cognitieve prestaties, sociale problematiek en moeite met complexe motorische taken. Daarnaast is witte stof schade geassocieerd met een verhoogd risico op het ontwikkelen van psychiatrische aandoeningen, zoals autisme, ADHD, schizofrenie en depressie. Momenteel zijn er geen behandelingen beschikbaar om witte stof schade in premature baby's te voorkomen of te genezen. Het is daarom van groot belang om onderzoek te doen naar zowel de mechanismen die ten grondslag liggen aan het ontstaan van diffuse witte stof schade in te vroeg geboren baby's, als naar nieuwe geneesmiddelen die een uitkomst zouden kunnen bieden voor deze extreem kwetsbare doelgroep.

Glia & witte stof schade

Naast zenuwcellen (neuronen) bevat het brein ook glia cellen (vernoemd naar het Griekse woord voor 'lijm') die het mogelijk maken dat zenuwcellen hun functies correct kunnen uitvoeren. Er zijn verschillende soorten glia cellen, waaronder *oligodendrocyten, astrocyten* en *microglia*, welke een rol spelen bij het ontstaan van witte stof schade in te vroeg geboren baby's.

Oligodendrocyten zijn de cellen die in het centrale zenuwstelsel verantwoordelijk zijn voor de productie van myeline. Myeline vormt een isolerend laagje om de uitlopers van zenuwcellen, wat ervoor zorgt dat de zenuwcellen efficiënt en snel signalen kunnen doorgeven naar verder gelegen hersengebieden. Daarmee levert myeline een essentiële bijdrage aan de mogelijkheid van verschillende hersengebieden om met elkaar te kunnen communiceren. Schade aan de myeline laagjes leidt dan ook tot minder snelle en efficiënte communicatie tussen hersengebieden, wat de oorzaak kan zijn van cognitieve achterstand en gedragsproblemen. Myeline is een witte, vettige substantie die vooral belangrijk is in de witte stof van de hersenen, waar zich verbindingen tussen hersengebieden bevinden – het geeft ook de witte kleur aan de witte stof. Het is aangetoond dat in de hersenen van prematuur geboren kinderen met witte stof schade, de oligodendrocyten niet goed uitrijpen tot volgroeide cellen die myeline kunnen produceren. Dit wordt gezien als een belangrijke oorzaak voor het ontstaan van diffuse witte stof schade in prematuren. Het stimuleren van de ontwikkeling/rijping en de myelineproductie van oligodendrocyten zijn belangrijke doelen van potentiële behandelmethoden om witte stof schade tegen te gaan of te herstellen.

De hersenen bezitten een bijzonder immuunsysteem; de normale immuuncellen die in de rest van ons lichaam een rol spelen bij afweer (zoals B-cellen, T-cellen, macrofagen, etc.) worden normaliter weerhouden om het hersenweefsel binnen te dringen door de bloed-hersenbarrière. Het afweersysteem in het brein wordt gereguleerd door microglia. Deze cellen hebben veel weg van macrofagen en kunnen schadelijke stoffen en afval afkomstig van beschadigde cellen opruimen. Na blootstelling aan schade of pathogene moleculen kunnen microglia geactiveerd worden om een ontstekingsreactie te reguleren – dit wordt *pro-inflammatoire* activatie genoemd, en is belangrijk om de hersenen te beschermen tegen externe invloeden zoals bijvoorbeeld infecties. Anderzijds kunnen microglia ook *anti-inflammatoir* geactiveerd worden. Dit is belangrijk voor de negatieve regulatie van ontstekingsreacties en voor het stimuleren van weefselherstel. Het is aangetoond in hersenweefsel van preterme geboren kinderen met witte stof schade dat microglia proinflammatoir geactiveerd zijn. De oorzaak hiervan ligt waarschijnlijk in de vele ontstekingsreacties die plaatsvinden elders in het lichaam, die zorgen voor de aanwezigheid van ontstekingsstoffen in de bloedbaan. Daarnaast zou ook een schommelende zuurstoftoevoer een rol kunnen spelen. Microglia produceren vele moleculen, met name cytokinen en chemokinen, die ze uitscheiden om hun omgeving te beinvloeden. Experimenten hebben aangetoond dat over het algemeen pro-inflammatoire cytokinen een negatief effect hebben op de ontwikkeling van oligodendrocyten, en daarmee ook op myelinisatie in de witte stof. Pro-inflammatoire activatie van microglia zou daarom een belangrijke rol kunnen spelen bij het ontstaan van witte stof hersenschade in prematuur geboren kinderen.

In **hoofdstukken 2 en 3** wordt gedetailleerd de huidige kennis over de rol van glia cellen in witte stof schade besproken. In **hoofdstuk 2** wordt beschreven hoe interacties tussen verschillende celtypes onder invloed van ontstekingsreacties en zuurstofschommelingen een negatief effect kunnen hebben op de ontwikkeling van de witte stof. Daarnaast wordt een aantal eiwitten en moleculen aangehaald als mogelijke doelwitten van eventuele nieuwe medicijnen en worden veelbelovende nieuwe behandelmethoden voor witte stof schade uitgelicht. In **hoofdstuk 3** wordt in detail besproken hoe de ontwikkeling van oligodendrocyten eruit ziet, en hoe de ontwikkeling van de witte stof in zowel mensen als knaagdieren verloopt.

Diermodellen

Om meer te weten te komen over de mechanismen die een rol spelen bij het ontstaan van witte stof hersenschade én om de werking van potentiële behandelmethoden te onderzoeken is het essentieel om translationeel onderzoek te doen. Door de

klinische situatie van witte stof hersenschade in dieren na te bootsen, kunnen onder gecontroleerde omstandigheden de cellulaire en moleculaire gebeurtenissen in kaart worden gebracht.

In **hoofdstuk 4** beschrijven we een nieuw ratten model om diffuse witte stof schade te bestuderen. In dit model wordt diffuse witte stof hersenschade geïnduceerd door blootstelling aan prenatale ontsteking en postnataal zuurstoftekort. Het patroon van hersenschade dat hierdoor ontstaat vertoont hoge mate van gelijkenis met de klinische situatie van diffuse witte stof schade, namelijk verminderde myelinisatie en activatie van zowel microglia als astrocyten. Daarnaast vertonen ratten met witte stof schade verminderd motorisch functioneren en autisme-achtig gedrag. Dit diermodel kan gebruikt worden om onderliggende mechanismen en nieuwe behandelmethoden te onderzoeken.

In **hoofdstuk 5** beschrijven we hoe in knaagdiermodellen van o.a. neonatale hersenschade corticale myelinisatie gedetailleerd geanalyseerd kan worden door de myeline in gesneden hersencoupes aan te kleuren en vervolgens middels beeldanalyse software te analyseren. In **hoofdstuk 6** hebben we onderzocht of er, net als in de humane situatie, ook in het nieuwe ratmodel lange-termijn afwijkingen zijn op het gebied van cognitieve flexibiliteit, aandacht en impulsiviteit. In tegenstelling tot onze verwachtingen vonden we dat in verschillende gedragstaken ratten met witte stof hersenschade op volwassen leeftijd even goed presteerden als controle ratten. Dit representeert niet de humane situatie, waar wel is aangetoond dat witte stof schade na vroeggeboorte kan leiden tot cognitieve problemen op volwassen leeftijd. Deze bevindingen belichten een belangrijke limitatie van het diermodel beschreven in **hoofdstuk 5**. Echter, tot op heden is er geen enkel knaagdiermodel beschreven dat leidt tot diffuse witte stof schade waarbij gedragsproblemen aanhouden tot op volwassen leeftijd. Het is dus belangrijk om te realiseren dat deze knaagdiermodellen vooral kunnen worden gebruikt om de vroege stadia van witte stof schade na vroeggeboorte te bestuderen.

Het remmen van JNK als behandelmethode

Verschillende veelbelovende behandelstrategieën zouden mogelijk gunstige effecten kunnen hebben op de witte stof in de hersenen van prematuur geboren kinderen. Bijvoorbeeld behandelingen met stamcellen, ontstekingsremmers of antioxidanten zouden theoretisch schade aan de witte stof moeten kunnen voorkomen of herstellen. Een ander veelbelovend medicijn is *D-JNKi*, een remmer van het eiwit JNK. JNK is

een kinase-eiwit dat een rol speelt bij het initiëren van een ontstekingsreactie, het initiëren van celdood, maar ook bij het reguleren van celdeling en -differentiatie. Daarnaast is eerder aangetoond dat JNK specifiek in oligodendrocyten geactiveerd wordt na ischemische gebeurtenissen in de hersenen. Ook is aangetoond dat *c-jun*, een eiwit dat door JNK geactiveerd wordt, een rol speelt bij de negatieve regulatie van myeline-productie. Eerder is door onze onderzoeksgroep al aangetoond dat behandeling met D-JNKi celdood kan voorkomen in de hersenen van neonatale ratten na hypoxie-ischemie. In **hoofdstuk 7** van dit proefschrift hebben we onderzocht of behandeling met D-JNKi ook gunstige effecten heeft in het ratmodel voor diffuse witte stof hersenschade. Onze hypothese was dat D-JNKi zowel de ontstekingsreactie van microglia zou dempen, als dat het de ontwikkeling en myeline-productie van oligodendrocyten zou bevorderen. Deze hypothese is gedeeltelijk bevestigd door onze studies. Ratten met witte stof hersenschade die behandeld werden met D-JNKi lieten een significante verbetering zien in motorisch functioneren. Tevens vonden we in de hersenen van de ratten behandeld met D-JNKi verbeteringen in oligodendrocyte ontwikkeling en myelinisatie. Echter, na D-JNKi behandeling bleef de ontstekingsreactie onveranderd. Onze bevindingen laten zien dat D-JNKi een veelbelovend geneesmiddel is dat gebruikt zou kunnen worden om de ontwikkeling van de witte stof in premature kinderen te verbeteren. Het lijkt erop dat D-JNKi direct effect heeft op oligodendrocyten en daarmee de productie van myeline bevordert, maar dat het minder effect heeft op microglia en de ontstekingsreactie in de hersenen. Echter, toekomstig onderzoek zal de exacte werkingsmechanismen moeten verklaren. Tevens zullen de veiligheidsaspecten van D-JNKi behandeling bestudeerd moeten worden, alvorens deze behandeling klinisch gebruikt kan worden.

Toekomstperspectief

Ook al is er de afgelopen jaren veel wetenschappelijk onderzoek gedaan op het gebied van witte stof schade in prematuren, veel vragen moeten nog beantwoord worden. Met name de moleculaire en cellulaire mechanismen die een rol spelen bij het ontstaan van witte stof schade moeten verder opgehelderd worden, zodat er nieuwe behandelstrategieën ontwikkeld kunnen worden. Om hiermee een begin te maken hebben we middels next-generation RNA sequencing in kaart gebracht van welke genen de expressie verandert tijdens normale rijping van oligodendrocyten, zoals beschreven in **hoofdstuk 8**. Daarnaast hebben we van deze selectie genen onderzocht wat er met hun expressie gebeurt onder inflammatoire condities, om zo kandidaat-genen te identificeren die mogelijk een belangrijke rol spelen bij het afremmen van de rijping van oligodendrocyten. Door dit soort kandidaat-genen verder te onderzoeken

zouden in de toekomst nieuwe behandelmethoden ontwikkeld kunnen worden.
Vooralsnog blijft witte stof hersenschade een zeer groot probleem in de prematuur geboren baby. Optimistisch gezien wordt er steeds meer onderzoek gedaan naar mogelijke behandelmethoden en zijn er verschillende experimentele behandelingen die op zijn minst veelbelovend te noemen zijn. Hopelijk worden de veiligheid en effectiviteit van deze behandelingen in de komende jaren bewezen zodat ze snel vertaald kunnen worden naar de kliniek om de ernstige consequenties van witte stof schade in te vroeg geboren kinderen te minimaliseren.

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Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.

- Louis Pasteur

CURRICULUM VITAE

Erik van Tilborg was born on August 6, 1990 in Venlo, The Netherlands. After graduating from high school at College Den Hulster (Venlo) in 2008, he started studying Psychology at the faculty of Psychology and Neurosciences at Maastricht University. During the Bachelor programme, Erik entered the Maastricht Research Based Learning (MaRBLe) Programme by joining the group of dr. Jos Prickaerts (School for Mental Health and Neuroscience, Maastricht University) for three months during which he studied the effects of housing conditions on novel object recognition task performance in rats. Furthermore, he studied the time-dependent effects of the PDE4 inhibitor rolipram on object recognition memory in rats. The findings of these studies were presented in Erik's Bachelor thesis and Erik obtained his Bachelor's degree in 2011. Erik continued his education at Maastricht University with a Research Master in Fundamental Neuroscience, during which he joined the laboratory of Prof. Ted Abel, MD, PhD (University of Pennsylvania, Philadelphia, US) for eight months to study the molecular mechanisms underlying memory deficits following sleep deprivation in the mouse brain, under supervision of Jennifer Choi Tudor, PhD. In 2013, Erik received his Master's degree and started as a PhD candidate in the group of dr. Cora Nijboer at the Laboratory for Neuroimmunology and Developmental Origins of Disease (NIDOD), University Medical Center Utrecht in close collaboration with the Department of Neonatology, under (co)promotors Prof. dr. Manon Benders, Prof. dr. Cobi Heijnen, and dr. Floris Groenendaal. During his PhD, Erik studied the cellular mechanisms underlying white matter injury in preterm infants. Furthermore, using a newly developed rat model he studied the efficacy of a novel therapeutic compound (D-JNKi) to improve white matter development in preterm infants. The findings of these studies are presented in this thesis. Erik hopes to continue his academic career by making significant contributions to the field of developmental neuroscience.

Brain, *n*. An apparatus with which we think we think.

- Ambrose Bierce

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