RECENT ADVANCES IN MYELIN PLASTICITY

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RECENT ADVANCES IN MYELIN PLASTICITY

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Table of Contents

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EDITORIAL
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Editorial: Recent Advances in Myelin Plasticity

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Keywords: oligodendrocytes, microglia, astrocytes, plasticity, myelin

Editorial on the Research Topic

Recent Advances in Myelin Plasticity

The Research Topic on Myelin Plasticity in the Non Neuronal Cells Section of the Frontiers in Cellular Neuroscience aims to provide an overview and new perspectives on the myelin formation and repair mechanisms. New insights into the cellular process and advances in technological tools in this field could reveal novel therapeutic strategies that may lead to the development of new treatments for central (CNS) and peripheral (PNS) nervous system de-/dys-myelinating pathologies. This is timely work given the high impact of CNS and PNS demyelinating disorders and is expected to contribute to the prevention of severe disabilities that have a serious impact on a patient's everyday life as well as on the society. Myelin plasticity is also a key mechanism that is involved in repair processes, a major challenge in human disorders.

The majority of myelin is formed postnatally in the rodents and by adulthood in humans. Although myelin plasticity in response to neuronal activity is an old observation, its extent has been appreciated relatively recently. It is now accepted that myelin can be shaped by environmental stimuli and undergo significant structural changes throughout life. This fine-tuning mechanism enhances neuronal function by orchestrating adjustments in myelin structure and axo-glial interactions. The potential link between this adaptive myelination and neuropsychiatric conditions is an active area of research.

In the special Research Topic, the review by Ronzano et al. summarizes how neuronal activity shapes the myelination profile during life emphasizing key parameters of the myelination process such as oligodendrocyte progenitor (OPC) proliferation, maintenance and differentiation, axon selection and myelination pattern. The authors examine myelination in adulthood as an adaptive mechanism and lastly but importantly, discuss how myelination and repair are also modulated by other glial cells such as astrocytes and microglia.

The review presented by Bonetto et al. focuses on myelin plasticity during adult life and described new insights into the link between this plasticity, learning and behavior, as well as mechanistic aspects of myelin formation that may underlie myelin plasticity, highlighting OPC diversity in the CNS.

Murphy et al. analyzes experience-dependent changes in myelin content in the adult visual and somatosensory cortex. Using models and conditions that drive adult plasticity and myelin basic protein (MBP) expression modulation, the authors demonstrated that induced plasticity can directly control visual activity in visual cortex. Using a deprivation visual paradigm, they showed that MBP content increased in the non-deprived hemisphere, while it decreased in the deprived hemisphere suggesting that modulation of myelin expression in adult visual cortex may reflect the levels of visually driven activity.

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The review by Traiffort et al. focuses on the important contribution of astrocytes and microglia in the myelination process in normal and pathological conditions. The authors discuss the mechanisms by which astrocytes and microglia influence developmental myelination and their interplay with oligodendrocytes. They then proceed in discussing both beneficial and detrimental roles of these two glial cell types in de- and remyelination.

Petratos et al. analyze one of the key mechanisms at play during neuronal injury, namely the Nogo receptor A (NgR1)-mediated signaling cascade during myelination. Nogo is recognized as one of the major myelin-associated inhibitors but its role in maintaining axo-myelin stability and receptor binding is not completely elucidated. The authors provide recent insights as to how neuronal NgR1 regulates myelin thickness under normal and pathological conditions with particular emphasis on the regulation of perinodal domains. They further discuss how NgR1 signaling can be targeted in animal models as a future potential therapeutic strategy.

Deboux et al. have investigated the role of Slit1, a secreted axon guidance molecule also involved in adult neurogenesis, during myelin formation and in pathological condition. Using Slit1 deficient mouse, the authors showed that while Slit1 does not affect the normal developmental process of oligodendrogenesis and myelination, it regulates adult progenitor mobilization during remyelination by controlling cell migration and renewal within lesions.

Finally, El Waly et al. describe an innovative method to characterize the cascade of cellular events involved in lysolecithin (LPC)-induced demyelination by combining intravital coherent antistoke Raman scattering microscopy with intravital two-photon fluorescence microscopy in multicolor transgenic reporter mice. Taking advantage of spinal glass window implantation, a longitudinal description of cell dynamics during focal and reversible demyelination in the same volume of interest over weeks could be obtained. The authors detected early oligodendrocyte injury followed by axon degeneration within 2 days after LPC incubation, amplified by the recruitment of peripheral proinflammatory cells at day 4. Recovery took weeks and involved a new wave of anti-inflammatory innate immune cells at day 14. Overall, the use of recurrent imaging provided new insights into the role of peripheral immune cells in regulating both the axonal and oligodendroglial fates during de- and remyelination.

In conclusion, this special issue hosted manuscripts that highlighted several aspects of myelin plasticity both as reviews of current literature but also as original research contributions. They all provide insights into how myelin can be shaped throughout life under physiological or pathological conditions. We hope this collection of articles would be a useful contribution to the myelin field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Myelin Plasticity and Repair: Neuro-Glial Choir Sets the Tuning

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The plasticity of the central nervous system (CNS) in response to neuronal activity has been suggested as early as 1894 by Cajal (1894). CNS plasticity has first been studied with a focus on neuronal structures. However, in the last decade, myelin plasticity has been unraveled as an adaptive mechanism of importance, in addition to the previously described processes of myelin repair. Indeed, it is now clear that myelin remodeling occurs along with life and adapts to the activity of neuronal networks. Until now, it has been considered as a two-part dialog between the neuron and the oligodendroglial lineage. However, other glial cell types might be at play in myelin plasticity. In the present review, we first summarize the key structural parameters for myelination, we then describe how neuronal activity modulates myelination and finally discuss how other glial cells could participate in myelinic adaptivity.

Keywords: myelin, oligodendrocytes, glia, microglia, astrocyte, myelination, plasticity, remyelination

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INTRODUCTION

Myelin is a feature of jawed vertebrates (Zalc et al., 2008), though it has also been acquired independently along with evolution by few invertebrate taxa (Hartline and Colman, 2007). Myelin is formed by lipid-rich membrane layers wrapped around axons, providing electrical insulation and metabolic support. This process ensures fast saltatory conduction (Waxman and Foster, 1980), reaching velocities that would otherwise require giant axons (Hartline and Colman, 2007). Despite its energy cost (Harris and Attwell, 2012), myelin correlates with increased population fitness, more efficient behaviors and increased body size.

In vitro and *in vivo* models showed that the axonal diameter is a key determinant for myelination (Lee S. et al., 2012; Goebbels et al., 2017; Mayoral et al., 2018). The usual threshold for myelinated axon in the peripheral nervous system (PNS) is 1 micron (Matthews, 1968). However, theoretical predictions suggest that myelination can increase axonal conduction with a diameter as low as 0.2 μm (Waxman and Bennett, 1972), which fits with central nervous system (CNS) myelination, where axons with diameters from 0.4 μm can be myelinated (Hildebrand et al., 1993). At a given axonal diameter, the conduction velocity of an action potential depends on the structural characteristics of myelin. The major parameters are the g-ratio (the axonal diameter divided by the total outer diameter of the fiber; Smith and Koles, 1970), and the internodal length (Huxley and Stampfli, 1948). Mean measured value and predicted optimum for the g-ratio are between 0.6 and 0.7 in the PNS and slightly above in the CNS white matter (Rushton, 1951; Smith and Koles, 1970; Waxman and Swadlow, 1976; Michailov et al., 2004; Chomiak and Hu, 2009). The conduction velocity also increases with the internodal length until it reaches a plateau at 1,000 μm (Brill et al., 1977; Moore et al., 1978). In the PNS, the majority of internodes exceed 500 μm

(Hildebrand et al., 1994), and variations in internodal length have little effect on conduction velocity (Wu et al., 2012; Simpson et al., 2013). In the CNS, internodes are much shorter, on average 50 μm in gray matter and 150 μm in white matter (Tomassy et al., 2014; Arancibia-Cárcamo et al., 2017; Stedehouder et al., 2017, 2019), and changes in their length have a higher impact on conduction velocity (Etxeberria et al., 2016). Thus, in the CNS, structural characteristics allow for modulation of conduction velocity.

In the CNS, *in vitro* (Watkins et al., 2008) as well as *in vivo* experiments (Czopka et al., 2013) have demonstrated that myelinating oligodendrocytes (OLs) establish myelin sheaths in only a few hours. Following this step, between 20 and 60 myelin sheaths per OL are stabilized in rodents (Matthews and Duncan, 1971; Chong et al., 2012), and about 15 per OL in zebrafish. The deposition of the successive myelin layers is led by the inner tongue which wraps around the axon and extends laterally (Snaidero et al., 2014). The dynamics of the actin cytoskeleton appears finely regulated to trigger myelin wrapping, with an actin polymerization at the leading edge of the inner tongue and subsequent depolymerization (Nawaz et al., 2015; Zuchero et al., 2015). Moreover, defects in adhesion molecules expressed at myelin membranes and axolemma affect the number, the length and the folding of myelin sheaths, disrupting target recognition and myelin extension around and along axons (Djannatian et al., 2019; Hughes and Appel, 2019; Klingseisen et al., 2019).

Myelination has long been viewed as a process ending in young adults. However, in the CNS, though some structures like the optic nerve are fully myelinated (Honjin et al., 1977; Bartsch et al., 1997; Dangata and Kaufman, 1997), most of the areas exhibit partial myelination. The corpus callosum contains 20–40% of unmyelinated fibers in adult rodents (Seggie and Berry, 1972; Gravel et al., 1990; Olivares et al., 2001), and the myelination profile of excitatory as well as inhibitory neurons show discontinuous patterns in the cortical and hippocampal areas (Tomassy et al., 2014; Micheva et al., 2016; Stedehouder et al., 2017, 2019). These myelination patterns have been suggested to regulate action potentials (APs) arrival at the presynaptic compartment (Salami et al., 2003) and provide metabolic support to fast-spiking neurons that have a high energy demand (Micheva et al., 2016). Incomplete myelination should allow for myelin plasticity, which could potentiate specific connections or provide additional metabolic support in the CNS by the addition of myelin on specifically activated networks.

NEURONAL ACTIVITY SHAPES MYELINATION PROFILE ALONG WITH LIFE

The role of neuronal activity in modulating myelination was first suggested more than 50 years ago by the effect of light deprivation on mouse optic nerves (Gyllensten and Malmfors, 1963). Later on, modulation of the oligodendroglial lineage through neuronal activity was shown *in vitro* using neurotoxins and electrical stimulations (Barres and Raff, 1993; Demerens et al., 1996; Fields and Stevens, 2000; Stevens et al., 2002). More recently, the relationship between these processes has been extensively studied with growing evidence that neuronal activity plays a key role in the modulation of every step of myelination both during development and in adulthood.

The Oligodendroglial Lineage Can Perceive the Neuronal Activity

Neuronal activity can modulate oligodendrocyte progenitor cells (OPCs) proliferation, maintenance and differentiation in zebrafish and mammals (Hill et al., 2014; Zonouzi et al., 2015; Hamilton et al., 2017; Hoche et al., 2019). Glutamatergic and GABAergic neurons have been shown to form bona fide synapses on OPCs in rodents (Bergles et al., 2000; Lin and Bergles, 2004) and humans (Gallo et al., 2008), with neuronal inputs on OPCs being consistent between brain regions (Mount et al., 2019). The activity of afferent neurons through the activation of either AMPA or GABA receptors is widely involved in the control of OPCs fate and self-maintenance along CNS development (Mangin et al., 2012; Zonouzi et al., 2015; Balia et al., 2017; Kougioumtzidou et al., 2017; Chen et al., 2018; **Figure 1**). Furthermore, OPCs are not only sensitive to the presence of neuronal activity, but also to the pattern of activity, which modulates differently their proliferation and differentiation (Nagy et al., 2017). Although the involvement of neuron-OPCs synapses has been largely documented, non-synaptic junctions between neurons and OPCs have also been involved in the facilitation of OPCs differentiation *in vitro* (Wake et al., 2015; **Figure 1**). The control of OPCs proliferation and differentiation has been showed to depend on Ca^{2+} signals triggered by neuronal activity *in vitro* in rodents (Wake et al., 2011) and *in vivo* in zebrafish (Hoche et al., 2019). However, depending on the developmental stage and the anatomical area studied, OPCs respond differently to neuronal activity, possibly related to their heterogeneous expression of voltage-gated channels and receptors to neurotransmitters (Káradóttir et al., 2008; Hoche et al., 2019; Spitzer et al., 2019).

Neuronal Activity Modulates Axon Selection as Well as Myelination Pattern

Highly specific selection of the axonal segments to be myelinated is necessary to lead to adequate myelination patterns. It has been shown *in vitro* and *in vivo* in mice and zebrafish that the choice of the target axons is promoted by neuronal activity (Hines et al., 2015; Wake et al., 2015; Mitew et al., 2018; **Figure 1**). In zebrafish, the maintenance of nascent myelin sheaths is increased on electrically active axons (Hines et al., 2015). Neuronal activity can also regulate the number of myelin sheaths per OL in zebrafish (Mensch et al., 2015) and their length in mouse optic nerves (Etxeberria et al., 2016). Activity-dependent myelination acts through the release of axonal vesicles (Hines et al., 2015; Mensch et al., 2015; Wake et al., 2015; Etxeberria et al., 2016) triggering Ca^{2+} signals in OLs. In zebrafish, Ca^{2+} signals along myelin sheaths regulate their stabilization and growth in an axonal activity-dependent manner

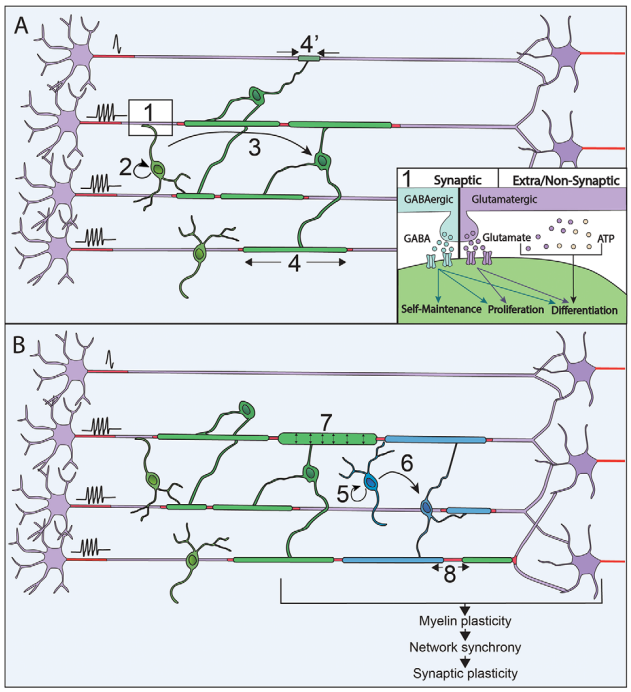

FIGURE 1 | Neuronal activity modulates myelination processes along with life. **(A)** Neuronal activity is sensed through synapses and extra/non-synaptic junctions between neurons and oligodendrocyte progenitor cells (OPCs; 1). Neurons release GABA (blue) or Glutamate (purple) that activate respectively $GABA_A$ and AMPA receptors at neuron-OPC synapses. Vesicles of ATP (orange) can also be released by neurons and modulate OPCs physiology at extra-synaptic and non-synaptic junction together with glutamate. Neuronal activity modulates every step of myelination during development: OPCs maintenance and proliferation (2), OPCs differentiation in OLs (3) and myelin sheaths stabilization and extension (4 and 4'). **(B)** In the adult, OPCs are maintained and their proliferation (5), as well as differentiation (6), can be promoted by an increase of neural activity when performing a new task. This increase in neuronal activity can also modulate the characteristics of myelin sheaths that are already formed by increasing their thickness (7) and modifying the nodal gaps (8).

(Baraban et al., 2018; Krasnow et al., 2018). The frequency, the duration and the amplitude of Ca^{2+} signals appears to be crucial for myelination and correlates with axonal activity (Krasnow et al., 2018). Based on what has been done on NG2 cells (Nagy et al., 2017), deciphering the effects of various neuronal firing patterns on OLs myelination may result in a better understanding of these complex modulations. However, the prominence of neuronal activity in the control of myelination needs to be weighted, as myelin increase could also reflect concurrent growth of axonal arborization (Stedehouder et al., 2018). Moreover, non-neuronal activity related mechanisms concomitantly participate to axon selection during myelination (Rosenberg et al., 2008; Bechler et al., 2018; Mayoral et al., 2018) and, for some neuronal populations, myelination occurs independently of neuronal activity (Koudelka et al., 2016). It can, therefore, be considered that neuronal activity is rather acting as a modulator allowing to adapt myelination pattern to the activity of the neuronal networks.

Myelination in Adulthood as an Adaptive Mechanism

In mice, OPCs keep proliferating and differentiating in adult CNS, with 5–20% of OLs generated during adulthood (Rivers et al., 2008; Kang et al., 2010; Simon et al., 2011; Young et al., 2013). The OLs generated in adulthood could contribute to cellular turnover or adaptive myelination. However, in mice, except in the optic nerves, OLs survival rate is over 90% at 8 months suggesting that the new OLs generated may rather participate in adaptive processes (Tripathi et al., 2017). Remodeling of existing myelin has first been observed, in social isolation of adult mice, where induction of behavioral changes correlate with myelin sheath thinning and transcriptional changes in OLs in the medial prefrontal cortex (Liu et al., 2012). Myelin plasticity could further be associated with changes in internodal or nodal gap length, both of which have been described to tune conduction velocity (Ford et al., 2015; Arancibia-Cárcamo et al., 2017; **Figure 1**). Indeed, myelin sheath length can be remodeled once it is established; however, these changes are relatively rare in adulthood and sensory enrichment failed to induce any measurable changes in sheath length in rodents (Hill et al., 2018; Hughes et al., 2018). Alternatively, conduction velocity could be tuned by changes in nodal gap length, which can be modulated in adult mice (Dutta et al., 2018), upon neuronal activity changes (Cullen et al., 2019; Korrell et al., 2019).

So far, adaptive myelination has mainly been associated with the generation of new OLs and the addition of new myelin sheaths (**Figure 1**). First, the learning of complex motor tasks has been shown to trigger OPCs proliferation, OLs maturation and myelin deposition (Sampaio-Baptista et al., 2013; McKenzie et al., 2014). Furthermore, in the same paradigm of complex wheel running, OPCs differentiation occurred within the range of a few hours (Xiao et al., 2016). Relatively short optogenetic stimulations of the premotor areas at a physio mimetic frequency triggered OPCs proliferation, oligodendrogenesis and myelin thickening, coupled to behavioral improvement (Gibson et al., 2014), corroborating the involvement of adaptive myelination in motor learning. Lastly, spatial learning was shown to trigger adaptive myelination, and impairment in adaptive myelination leads to defect in memory consolidation (Steadman et al., 2020) and short term memory (Geraghty et al., 2019). In humans, a link between neuronal activity and the addition of new myelin sheaths in adult CNS has been shown by studies on healthy subjects achieving motor and memorization tasks. White matter microstructural changes were demonstrated (Scholz et al., 2009; Takeuchi et al., 2010), and the amplitude of the effect correlated with the training duration (Taubert et al., 2010). These changes could be due to myelin deposition *per se* or reflect axonal remodeling (Zatorre et al., 2012). The origin of the newly added myelin has been investigated by immunohistochemical studies, which provided evidence of proliferating OPCs in the adult brain (Geha et al., 2010). This was further supported by studies on non-human primates showing an increase in the number of OLs during adulthood (Peters and Sethares, 2004; Peters et al., 2008). Alternatively, myelin could also arise from pre-existing

OLs persisting into adulthood, as identified in humans (Yeung et al., 2014; Fard et al., 2017; Jäkel et al., 2019). Thus, although adaptive myelination also occurs in the human brain, to which extent mechanisms are shared between rodents and humans is still under debate.

Myelin adaptation could be involved in the fine-tuning of neural network synchrony, and action potential arrival at the presynaptic compartment (Pajevic et al., 2014; Ford et al., 2015), that are thought to govern learning and memory (Feldman, 2012; Kandel et al., 2014; Korte and Schmitz, 2016). The effect of adaptive myelination on short term memory and memory consolidation supports this hypothesis (Geraghty et al., 2019; Steadman et al., 2020), but future studies will be needed to determine how adaptive myelination modulates the electrophysiological parameters of specific parts of neuronal circuits, and further creates a synchronization at specific connections. Moreover, feedback signals from the myelinated axon/neuron allowing for the fine control of myelin addition and removal should be required to tune finely AP arrival at the synapses and further synchronize the circuits. Until now, they remain unknown, with previous works on synaptic plasticity being a potential source of inspiration to investigate them (Fields et al., 2014).

Newly added myelin sheaths could further provide metabolic support to axons (Fünfschilling et al., 2012; Lee Y. et al., 2012; Meyer et al., 2018), the metabolic supply being regulated by neuronal activity (Saab et al., 2016). This myelin addition probably would not result in a global energetic advantage (Harris and Attwell, 2012), but might be needed to generate fast-spiking firing discharges and thus allow for precise axonal firing (Micheva et al., 2016; Moore et al., 2019).

Although the molecular mechanisms inducing adaptive myelination in the adult are still unclear, recent studies showed the involvement of two factors, endothelin (Swire et al., 2019) and BDNF (Geraghty et al., 2019). Neuronal activity triggers an increase in blood flow that in turn increases endothelin expression by endothelial cells (Walshe et al., 2005; Pandit et al., 2015). This has been shown to increase myelination *ex vivo* (Yuen et al., 2013). In adult mice, endothelin rescues myelination defects triggered by social isolation, thus confirming its involvement in adaptive myelination (Swire et al., 2019). BDNF had first been suggested to modulate activity-dependent myelination (Lundgaard et al., 2013) and later showed to be a regulator of adaptive myelination (Geraghty et al., 2019). It is produced by neurons in an activity-dependent manner (Balkowiec and Katz, 2000; Hartmann et al., 2001; Dieni et al., 2012) and can be released by synaptic vesicles (Park et al., 2014). Thus, BDNF secretion could specifically trigger adaptive myelination along activated axons. However, BDNF is not only released by neurons, but also by astrocytes (Fulmer et al., 2014; Zhang et al., 2014) and microglial cells (Parkhurst et al., 2013). These complex BDNF signals might have to be integrated by the oligodendroglial lineage when it comes to adaptive myelination, as well as in injury (McTigue et al., 1998; Ikeda et al., 2002; Ramos-Cejudo et al., 2015). Lastly, OPCs themselves could modulate myelination and myelin plasticity directly or indirectly, in particular through the secretion of BDNF or retinoic acid (Tanaka et al., 2009; Parolisi and Boda, 2018; Goncalves et al., 2019). Adaptive myelination and repair should thus not be considered only as direct neuronal crosstalk with the oligodendroglial lineage, but also in regard to their direct cellular environment.

MYELINATION AND REPAIR ARE ALSO MODULATED BY OTHER NEURO-GLIAL INTERACTIONS

The crosstalk between neuron and glia is complex and probably critical when it comes to myelination regulation, in adaptive processes and repair. Astrocytes and microglial cells are known to participate in (re)myelination modulation and have been described to detect neuronal activity (for review, Domingues et al., 2016; Adaikkan and Tsai, 2019; Bar and Barak, 2019; Molina-Gonzalez and Miron, 2019). Although astrocytes and microglia may be involved in molecular mechanisms modulating adaptive myelination, the understanding of their impact on adult myelination processes is still limited.

Control of Myelination and Myelin Plasticity by Astrocytes

Astrocytes are the most abundant CNS glial cell type, with a major role in metabolic support, homeostatic functions, assembly and modulation of synapses, Blood-Brain Barrier (BBB) integrity and nervous tissue scaring. They further participate in neuronal activity and myelination regulation, in plasticity and learning (for review, Barres, 2008; Fields et al., 2014). Astrocytes are heterogeneous, with protoplasmic astrocytes, in the gray matter, interacting with synapses and BBB, and fibrous astrocytes, in the white matter, contacting nodes of Ranvier and blood vessels (for review, Sofroniew and Vinters, 2010).

Astrocytes have been described to regulate all oligodendroglial lineage steps, from OPCs proliferation to differentiation and myelination (for review, Domingues et al., 2016; **Figure 2**), in particular by secretion of various factors such as IGF1, CNTF, CXCL1, TIMP-1 and LIF (Gard et al., 1995; Stankoff et al., 2002; Ye et al., 2004; Padovani-Claudio et al., 2006; Modi et al., 2013; Jiang et al., 2016). Astrocytic role in myelination is partly dependent on neuronal activity, with the activity-dependent neuronal release of ATP triggering the secretion of astrocytic LIF factor, which further promotes OL survival and myelination (Ishibashi et al., 2006). Astrocytes also provide some lipids necessary to support the metabolic costs of myelination (Camargo et al., 2017) and promote OLs survival and maturation through direct physical contacts (Sakurai et al., 1998; Corley et al., 2001). They further connect with oligodendrocytes through connexins necessary for myelin maintenance and support of OLs K^+ buffering during neuronal activity (Menichella et al., 2006; Orthmann-Murphy et al., 2008; Tress et al., 2012). Once myelin is formed, astrocytes further play a role in myelin plasticity by regulating myelin thickness and nodal gap length (Dutta et al., 2018). Lastly, astrocytes control local blood flow depending on neuronal activity (for review, Nortley and Attwell, 2017) and could thus further be involved

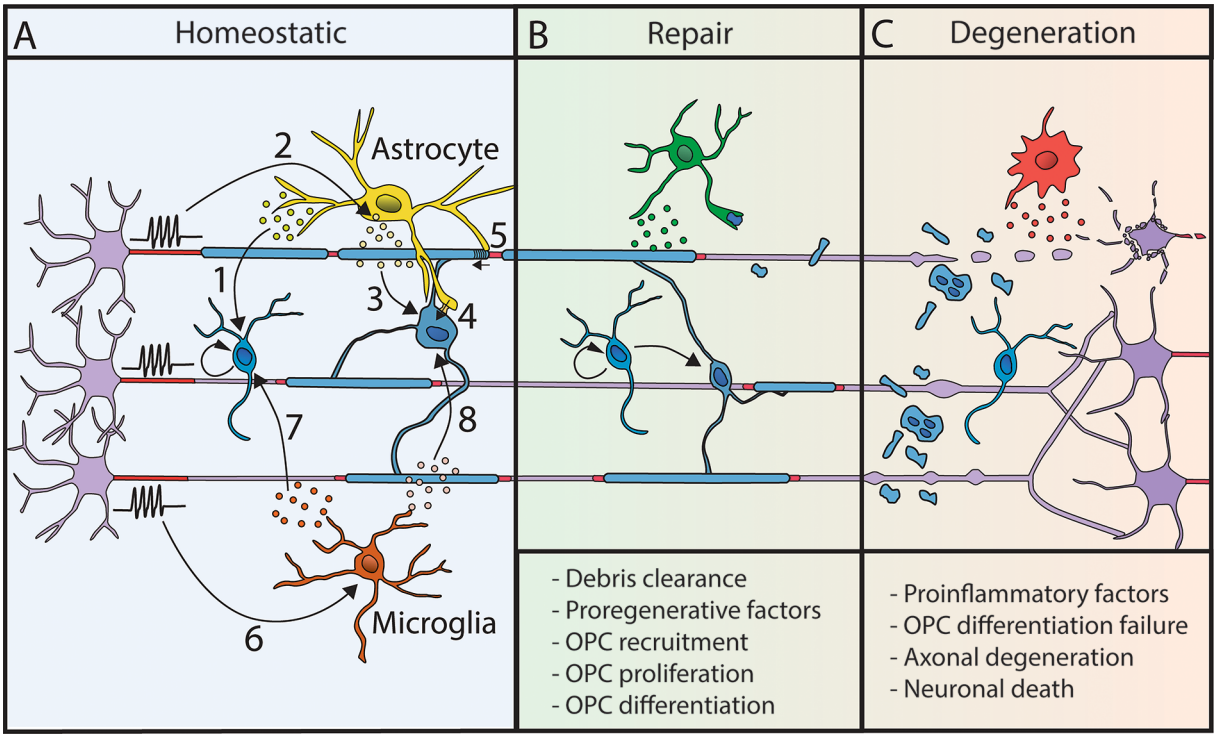

FIGURE 2 | Myelination processes are modulated by other glial cells. **(A)** In homeostatic conditions, astrocytes and microglial cells modulate myelin deposition. Astrocytes release factors regulating OPC proliferation (1). Neuronal activity triggers LIF release by astrocytes (2), which promotes myelination (3). Moreover, astrocytes are metabolically coupled to OLs (4) and modulate conduction velocity by acting on myelin thickness and nodal length (5). Microglial cell behavior is modulated by neuronal activity (6). They release factors that promote OPC proliferation and differentiation (7) and activate myelination (8). **(B)** Following demyelination, glial cells can promote repair by the clearance of myelin debris and the release of pro-regenerative factors. **(C)** However, their sustained proinflammatory activity can lead to repair failure and neurodegeneration.

in the indirect control of adaptive myelination by vasculature (Swire et al., 2019).

Astrocytes also play a complex role in demyelination and repair (**Figure 2**). They have been described to be rather beneficial *in vitro*, as well as *in vivo*, in chemically-induced demyelinating mouse models (Franklin et al., 1991; Selvaraju et al., 2004; Kramann et al., 2019). Following demyelination, they attract OPCs, promote their proliferation and differentiation (Omari et al., 2005; Patel et al., 2012). In contrast, astrocytes might play an inhibitory role in remyelination, in particular by inhibiting OLs maturation (Blakemore et al., 2003; Back et al., 2005; Sloane et al., 2010). They can further promote proinflammatory responses, circulating immune cell recruitment through BBB and modulate the number of activated microglial cells (Brambilla et al., 2014; Kim et al., 2014; Eilam et al., 2018). The complex role played by astrocytes, related to their phenotype, further depends on environmental cues and interaction with surrounding cells (Liddelow et al., 2017).

Control of Myelination and Myelin Plasticity by Microglia

Microglial cells are the resident immune cells of the CNS, where they represent 5–10% of the cells (Lawson et al., 1990). They continually monitor their environment (Nimmerjahn et al., 2005), and play complex roles in neuroplasticity, homeostasis, host defense, healing, debris clearance and peripheral cell recruitment (for review, Colonna and Butovsky, 2017; Prinz et al., 2019). They can adopt different phenotypes, with environment-dependent transcriptional profiles (Gosselin et al., 2014, 2017), and proinflammatory to pro-regenerative polarization (Miron and Franklin, 2014), though a strict dichotomy is an inadequate vision (Ransohoff, 2016). Microglial cells are further sensitive to neuronal activity (Li et al., 2012; Liu et al., 2019; Stowell et al., 2019; Cserép et al., 2020). Altered microglia activity at different stages of life is associated with developmental and acquired neurological pathologies and can impair the plasticity-related process and cognitive function (Morris et al., 2013).

In homeostatic condition, microglia can support survival, differentiation, myelinogenesis, and homeostasis of the oligodendroglial lineage (Hamilton and Rome, 1994; Butovsky et al., 2006; Pasquini et al., 2011; Shigemoto-Mogami et al., 2014; Hagemeyer et al., 2017; Wlodarczyk et al., 2017; **Figure 2**). Activated microglia associated with myelin deficits has further been described in neurodevelopmental disorders and mental conditions (Garey, 2010; Morgan et al., 2010; Janova et al., 2018; Bar and Barak, 2019; Barak et al., 2019). These defects might be partly related to a lack of adaptive myelination. Indeed, microglia activation state is modulated by neuronal activity

(Iaccarino et al., 2016; Adaikkan et al., 2019; Giorgetti et al., 2019; Martorell et al., 2019; Garza et al., 2020), and has been shown to modulate adaptive myelination in adult (Geraghty et al., 2019).

In demyelinating diseases, microglial activation is an early hallmark in multiple sclerosis (MS) together with axonal damage even prior to demyelination (Howell et al., 2010; Nikić et al., 2011). Microglia can have a dual role in repair, either impairing or promoting myelination in MS and its models in rodents (for review, Miron, 2017) depending on its phenotype (proinflammatory or pro-regenerative; Miron et al., 2013; Locatelli et al., 2018). It is considered that the pro-regenerative/pro-remyelinating effect of microglia might be related both to the secretion of pro-myelinating factors and the capacity of myelin debris clearance (Lampron et al., 2015; Cantuti-Castelvetri et al., 2018; **Figure 2**). Astrocytes can further participate in microglial recruitment at the lesion to promote debris clearance (Skripuletz et al., 2013), taking part in a global crosstalk. Reciprocally, the effect of extracellular vesicles produced by microglia on OPCs is modulated by astroglia (Lombardi et al., 2019). Finally, it has been recently described that microglial activation following cancer therapy can lead to astroglial activation and alter adaptive myelination highlighting the importance of inter-glial communication in these mechanisms (Gibson and Monje, 2019; Gibson et al., 2019).

The complex contribution of activated astrocytes and microglia in inflammatory conditions thus makes them key players in repair, able to either compromise or promote the efficacy of myelin redeposition (Franklin and Goldman, 2015). The activation states of these cells were further modulated by neuronal activity, the characterization of the complex crosstalk between glial and neuronal partners should pave the way to a better understanding of myelinic regulation and to more integrative therapeutical strategies.

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BRIEF RESEARCH REPORT
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Experience-Dependent Changes in Myelin Basic Protein Expression in Adult Visual and Somatosensory Cortex

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An experience-driven increase in oligodendrocytes and myelin in the somatosensory cortex (S1) has emerged as a new marker of adult cortical plasticity. That finding contrasts with the view that myelin is a structural brake on plasticity, and that contributes to ending the critical period (CP) in the visual cortex (V1). Despite the evidence that myelin-derived signaling acts to end CP in V1, there is no information about myelin changes during adult plasticity in V1. To address this, we quantified the effect of three manipulations that drive adult plasticity (monocular deprivation (MD), fluoxetine treatment or the combination of MD and fluoxetine) on the expression of myelin basic protein (MBP) in adult rat V1. In tandem, we validated that environmental enrichment (EE) increased cortical myelin by measuring MBP in adult S1. For comparison with the MBP measurements, three plasticity markers were also quantified, the spine markers drebrin E and drebrin A, and a plasticity maintenance marker Ube3A. First, we confirmed that EE increased MBP in S1. Next, that expression of the plasticity markers was affected in S1 by EE and in V1 by the visual manipulations. Finally, we found that after adult MD, MBP increased in the non-deprived V1 hemisphere, but it decreased in the deprived hemisphere, and those changes were not influenced by fluoxetine. Together, the findings suggest that modulation of myelin expression in adult V1 may reflect the levels of visually driven activity rather than synaptic plasticity caused by adult plasticity.

Keywords: visual cortex (V1), somatosensory cortex, monocular deprivation, environmental enrichment (EE), myelin basic protein (MBP), myelin, adult plasticity

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INTRODUCTION

In the visual cortex (V1), the developmental increase and signaling of intra-cortical myelin are described as a structural brake on critical period (CP) plasticity (Bavelier et al., 2010). Recent studies of adult somatosensory cortex (S1), however, have shown that enhancing plasticity with environmental enrichment (EE) increases cortical oligodendrocytes and myelination (Hill et al., 2018; Hughes et al., 2018). Those increases suggest that more myelin may be a marker of adult plasticity in S1. There is no similar information about plasticity-related myelin changes in adult V1, and that gap leaves unanswered if myelin plasticity in the adult cortex might differ between S1 and V1. Here, we addressed if manipulations that are known to affect plasticity in adult rodent V1 (e.g., monocular deprivation (MD) and fluoxetine administration) cause changes to myelin expression.

The idea that myelin is a brake on CP plasticity in V1 comes from two lines of evidence. First, V1 has little expression of myelin genes (Lyckman et al., 2008) or proteins (Bjelke and Seiger, 1989; Siu et al., 2015) during the CP when abnormal visual experience (e.g., MD) quickly changes cortical function. Second, myelin signaling in V1 inhibits experience-dependent neurite growth (Schoop et al., 1997) by various myelin-associated inhibitors, including Nogo, MAG, and OMgp (Wang et al., 2002; McGee et al., 2005; Akbik et al., 2012). Knocking out the receptor for Nogo (Nogo-66, NgR) prolongs ocular dominance plasticity in V1 (McGee et al., 2005) while in the somatosensory cortex (S1) it prolongs juvenile-like dendritic spine turnover (Akbik et al., 2013).

In vivo imaging of oligodendrocytes in mouse S1, however, has found that myelination develops slowly past the end of the CP when myelinating cells continue to proliferate and form discontinuous patches of myelin along cortical axons in young adult animals (Hill et al., 2018; Hughes et al., 2018). Also, sensory enrichment is known to enhance plasticity in rodents (Rosenzweig and Bennett, 1996) causes a 5-fold increase in the number of oligodendrocytes and hundreds of new myelin sheaths that remain stable for at least 3 months (Hughes et al., 2018). This rapid experience-dependent increase in myelin suggests that myelination supports plasticity in the adult cortex. Furthermore, several features of myelin sheaths on cortical inhibitory neurons suggest distinct functions related to plasticity (Micheva et al., 2018). Thus, cortical myelin has a broad spectrum of functions linked to plasticity in adult S1. Less is known, however, about myelin plasticity in adult V1.

V1 of adult rodents maintains some ocular dominance plasticity, but it is less than during the CP and requires longer MD to promote a shift (Sawtell et al., 2003; Sato and Stryker, 2008). Combining MD with treatment using fluoxetine, however, reinstates juvenile-like plasticity in adult V1 (Maya Vetencourt et al., 2008) and changes the expression of many plasticity-related proteins (Beshara et al., 2015). Despite this understanding of plasticity in adult V1, there is little information about whether MD or fluoxetine in adult animals affects cortical myelin. In particular, does fluoxetine-enhanced plasticity increase myelin in V1 similar to EE driven changes in S1. To address this, we studied the expression of myelin basic protein (MBP) in adult V1 after manipulating visual experience (MD) or enhancing plasticity (fluoxetine) and compared it with MBP changes in S1 after exposure to an enriched environment (EE). Because both MD and EE affect dendritic spines (Oray et al., 2004; Jung and Herms, 2014) we also measured a pair of markers that regulate spine plasticity and synapse formation (embryonic-type drebrin E and adult-type drebrin A; Koganezawa et al., 2017; Hanamura et al., 2018). Also, an E3 ubiquitin-protein, Ube3A, was measured because it is necessary for MD driven plasticity (Yashiro et al., 2009; Sato and Stryker, 2010), long-term potentiation (Jiang et al., 1998) and there is reduced cortical expression of MBP and other myelin proteins in Ube3A deficient mice (Grier et al., 2015). We found that EE increased MBP in S1 while MD caused hemisphere-specific changes in V1, increasing MBP in the non-deprived hemisphere and decreasing it in the deprived hemisphere. Fluoxetine did not affect the experience-driven changes in MBP even though it did affect the expression of the plasticity markers (drebrin-E, drebrin-A, and Ube3A). These results suggest that the direction of myelin plasticity in the adult cortex depends on whether the neural activity is enhanced or reduced.

MATERIALS AND METHODS

Rearing Conditions and Surgical Procedures

All procedures were approved by the McMaster University Animal Research Ethics Board. We quantified the expression of MBP and markers for dendritic spines (drebrin E and drebrin A) and plasticity maintenance (Ube3A) in S1 and V1 of adult Long-Evans rats. S1 was studied from animals reared individually (n = 12) or group-housed in an enriched environment (EE) consisting of multiple forms of physical stimulation including a large, spacious cage, with four levels connected by three ramps, and a running wheel (Short-term EE (S-EE) 2 weeks n = 6; Long-term EE (L-EE) 66 weeks n = 5; Critter Nation Double Unit Model:162, MidWest Homes For Pets). The toys and location of food and water were changed weekly. The lengths of EE and rearing conditions were selected to be similar to previous studies (Baroncelli et al., 2012). V1 was studied from animals reared individually with normal binocular vision (BV n = 6), or one of three manipulations: 1 month of fluoxetine (P70-P98, n = 8), 1 week of MD (P91–98; n = 6), 1-month fluoxetine (P70–98) plus 1 week MD (P91–98; n = 8). Fluoxetine was dissolved in the drinking water (0.2 mg/ml of drinking water), and animals were permitted to self-regulate their intake of food and water. These rearing conditions, including the use of male rats, were selected to be similar to previous studies (Maya Vetencourt et al., 2008).

MD was done by trimming the eyelid margins and suturing them together with 5–0 vicryl. The surgery was performed in an aseptic environment; anesthesia was induced and maintained with gaseous isoflurane (1.5–5%) in oxygen. Eyelids were monitored daily to check for and repair any openings. In the rat, 90% of the visual pathway is crossed so the hemisphere contralateral to the MDed eye was designated the deprived hemisphere and the hemisphere ipsilateral to the MDed eye the non-deprived hemisphere because it still received strong visual stimulation from the open eye (**Figure 1A**).

Tissue Collection

Animals were euthanized with Euthanyl (sodium pentobarbital, 150 mg/kg) and perfused with cold 0.1 M phosphate-buffered saline (PBS; 4°C; 4–5 ml/min). The brain was removed from the skull, placed in cold PBS, and tissue samples were collected from S1 for the animals reared with EE and V1 for the other animals (**Figure 1B**). The samples were immediately frozen on dry ice and stored in a −80°C freezer.

Sample Preparation and Immunoblotting

The samples were prepared from the cortical tissue pieces, protein concentrations were carefully equated, and immunoblotting was done using procedures that have been described previously (Beston et al., 2010; Murphy et al., 2014;

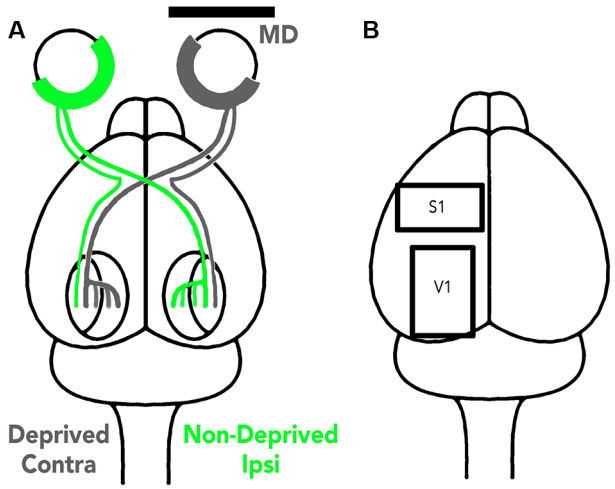

FIGURE 1 | Illustration of the visual pathway to the deprived and non-deprived V1 hemispheres **(A)** and the tissue sampling regions **(B)**. **(A)** The visual pathway is illustrated using gray lines to represent the deprived eye pathway and green lines the non-deprived eye pathway. About 90% of the rat visual pathway projection projects to the contralateral hemisphere so V1 opposite the MDed eye is the deprived hemisphere while the other V1 (ipsilateral) is non-deprived because it still receives strong input from the open eye (green). **(B)** The cortical regions sampled (S1 and V1) are illustrated in one hemisphere and in this study the samples were taken from different animals.

Beshara et al., 2015; Balsor and Murphy, 2018). Importantly, since expression levels of housekeeping proteins such as GAPDH and β-actin can be inconsistent (Lee et al., 2016; Butler et al., 2019), we followed current best practices for Western blotting (Pillai-Kastoori et al., 2020) with a rigorous multi-step protocol using stringent quality control checks at each stage of the sample preparation and immunoblotting (Balsor and Murphy, 2018). Protein concentrations were equated using three replicates of each sample and bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The colorimetric change was quantified (iMark Microplate Absorbance Reader, Bio-Rad Laboratories, Hercules, CA, USA) for the samples and standards and analyzed to ensure a correlation of >0.99 was achieved. The samples were diluted to 1 μg/μl with sample (M260 Next Gel Sample loading buffer 4×, Amresco) and Laemmli buffer (Cayman Chemical, Ann Arbor, MI, USA). A control sample was made from a small amount of each sample, run on every gel and used to normalize the bands for each sample run on a gel. Importantly, to maintain quality control at each step of the experiment, a high-quality pipette (Picus, Sartorious) was used and the calibration was checked daily. Each sample was run three or four times, and a total protein stain was used to normalize each lane.

The samples (25 μg) were separated on 4–20% Tris-Glycine gels (Novex, WedgeWell Gels, Thermo Fisher Scientific, Waltham, MA, USA), transferred to polyvinylidene difluoride (PVDF-FL) membranes (Millipore, Burlington, MA, USA), and membranes were blocked with blocking buffer (Odyssey Blocking Buffer 1:1 with PBS, 1 h; LI-COR Biosciences, Lincoln, NE, USA). Membranes were incubated in primary antibody overnight at 4°C (MBP, 1:4,000 Abcam; Ube3A, 1:1,000 Bethyl Laboratories, Montgomery, TX, USA; drebrin, 1:500 Fitzgerald) and PBS-T (Sigma-Adrich, St. Louis, MO, USA; 3 × 10 min). The membranes were incubated in secondary antibody (anti-mouse, 1:8,000; anti-rabbit, 1:10,000; LI-COR Biosciences, Lincoln, NE, USA) for 1 h at RT and washed in PBS. Blots were scanned (Odyssey scanner, LI-COR Biosciences, Lincoln, NE, USA) to visualize the bands, then stripped (Blot Restore Membrane Rejuvenation Kit, Millipore, Burlington, MA, USA) and reprobed with the next antibody.

Analyses

Densitometry was used to analyze the bands (Licor Odyssey Software version 3.0; LI-COR Biosciences, Lincoln, NE, USA; Beshara et al., 2015). The control sample run on every gel was used to normalize each band on the blot and full blots are available in the **Supplementary Datasheet S1**.

To examine expression levels of protein expression, we plotted histograms of the mean and SEM, for each condition normalized to the control group. To compare between-the-groups we used the bootstrapping method described previously (Beshara et al., 2015). Briefly, the programming language R was used to simulate a dataset of 1,000,000 points with the same mean and SEM as the group being compared. A Monte Carlo simulation was run to compare the groups by randomly sampling N times from the simulated dataset where N was the number of animals in the comparison group (e.g., $N = 6$) and repeating this step 100,000 times to generate the expected distribution for N animals. Confidence intervals (CI) were calculated from the expected distribution and compared with the observed mean of the group. Groups were considered to be significantly different (i.e., $p < 0.05$) when the observed mean was outside the 95% CI of the simulated distribution. For each comparison between groups, we ran the bootstrap analysis in both directions, and the more conservative result of the significance test was reported.

Finally, we analyzed the patterns of protein expression changes in V1 for the deprived (contralateral) and non-deprived (ipsilateral) hemispheres by combining the measurements of MBP and Ube3A from this study with data for GluA2, PSD95, Gephyrin, Synapsin, and Synaptophysin from our previous study (Beshara et al., 2015). Hierarchical cluster analysis using the Ward D2 method (Murtagh and Legendre, 2014) was run in R for each condition using all seven proteins and the dendextend function in the Hmisc package. All pairwise Pearson's R correlations were calculated using rcorr and visualized using heatmap2 in gplots. The proteins were ordered in the matrices using the dendrogram from the hierarchical clustering so proteins with similar patterns were nearby in the matrix. Each correlation in the matrix was color-coded to facilitate visualizing clusters. A table of the Pearson's R values is in **Supplementary Datasheet S2**.

Image Manipulation

The bands in each figure are representative of the group and full blots are in the **Supplementary Datasheet S1**. The size of the bands was adjusted using a uniform horizontal and vertical transformation, and a single gray-level linear adjustment was

applied to all of the bands to preserve the relative intensity of the bands in the different groups.

RESULTS

Effects of EE, Fluoxetine, MD and Fluoxetine Plus MD on Spine Markers

First, we examined whether the treatments had an effect on plasticity in the cortical areas using drebrin E and drebrin A as markers of dendritic spines. We chose these markers because both EE and MD are known to change spines in S1 and V1, respectively (Oray et al., 2004; Jung and Herms, 2014). Furthermore, there is a developmental shift from more of the immature protein drebrin E to more of the mature protein drebrin A, and it is that increase in drebrin A that facilitates spine maturation (Koganezawa et al., 2017).

In S1, the long-term EE group (−41%, SEM 6.2%, $p < 0.001$) had less drebrin E expression than normal suggesting that this treatment led to fewer nascent spines (**Figure 2A**). Short-term EE also appeared to have less drebrin E but that difference was not significant. In contrast, drebrin A expression was increased after long-term EE (+76%, SEM 36%, $p < 0.01$; **Figure 2B**), which is consistent with previous studies showing greater stability and larger mature spines after EE (Jung and Herms, 2014).

In V1, we focused on the deprived hemisphere because it is where previous studies found that MD drives spine plasticity (Oray et al., 2004). One week of MD did not significantly change the expression of either drebrin isoform compared with normals and there was only a trend toward increased expression of drebrin E (MD contra, +30%, SEM 18%, n.s.; **Figures 2C,D**). Fluoxetine treatment, however, whether alone or combined with MD reduced the expression of both drebrin isoforms in V1 (drebrin E: −32%, SEM 6.0%, $p < 0.001$; drebrin A: −32%, SEM 3.2%, $p < 0.001$; **Figures 2C,D**). This is one of the few plasticity-related synaptic markers in V1 that is affected by fluoxetine (Beshara et al., 2015).

Since the drebrin isoforms regulate different aspects of spine maturation and there is a developmental shift in the balance between the markers (immature drebrin E; mature drebrin A) we calculated an index to determine if the treatments changed the balance between the drebrin isoforms (**Figures 2E,F**). The control animals in both V1 and S1 had a balanced expression of drebrin A and E resulting in index values that overlapped zero. In V1, the drebrin isoforms balance was not changed by fluoxetine, MD or the combination of treatments (**Figure 2E**). In contrast, both short-term ($p < 0.05$) and long-term EE ($p < 0.01$) caused a shift to more drebrin A in S1 than found in the normal young adult (**Figure 2F**). Taken together, we found that fluoxetine and EE drove different patterns of drebrin plasticity.

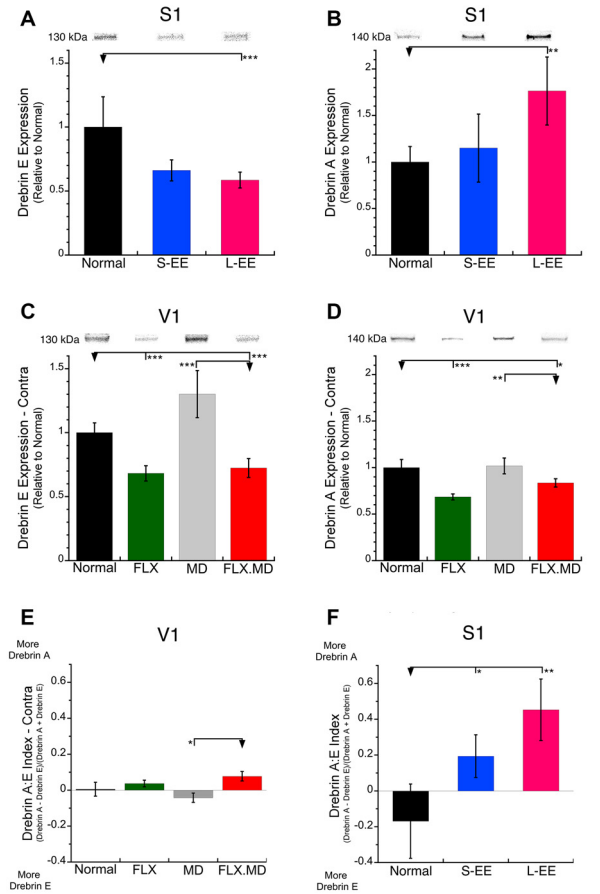

FIGURE 2 | Expression of drebrin-E and drebrin-A in S1 and the deprived V1. **(A,B)** Drebrin E and drebrin A expression in S1 of control animals ($n = 12$), short-term environmental enrichment (S-EE; $n = 6$) and long-term EE (L-EE; $n = 5$). **(A)** In S1, expression of the immature drebrin E was reduced after L-EE (−41%, SEM 6.2%, $p < 0.001$). **(B)** In contrast, the mature drebrin A was increased after L-EE (+76%, SEM 36%, $p < 0.01$). **(C,D)** Drebrin E and drebrin A expression in V1 of animals reared with normal binocular vision ($n = 6$), 1-month fluoxetine (P70–98, $n = 6$), 1 week monocular deprivation (MD; P91–98, $n = 8$) or 1-month fluoxetine (P70–98) plus 1 week MD (P91–98, $n = 8$). In V1, both drebrin E and drebrin A were reduced after fluoxetine alone (FLX: drebrin-E: −32%, SEM 6.0%, $p < 0.001$; drebrin-A: −32%, SEM 3.2%, $p < 0.001$) or when fluoxetine was combined with MD (FLX MD: drebrin-E: −28%, SEM 7.4%, $p < 0.001$; drebrin-A: −17%, SEM 4.4%, $p < 0.05$). **(E,F)** An index of the relative expression of drebrin A and drebrin E was calculated where values <0 indicate more drebrin E and values >0 more drebrin A. **(E)** In V1, none of the treatment groups shifted the drebrin balance compared with normal. **(F)** In S1, both S-EE and L-EE shifted the drebrin balance to more drebrin A (S-EE $p < 0.05$, L-EE $p < 0.01$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Effects of EE on MBP and Ube3A in Somatosensory Cortex

We examined if short-term or long-term EE changed MBP expression in S1. MBP makes up about 30% of all myelin proteins and is comprised of two families: classic- (18.5–21.5 kDa) and Golli-MBP (33–35 kDa). In this study, we quantified the Classic-MBP isoform because it is found in mature oligodendrocytes and myelin sheaths, and is necessary for activity-driven compaction of myelin around axons (Wake et al., 2011).

MBP was increased after short-term EE (+56%, SEM 30%, $p < 0.05$) and appeared to be increased after long-term EE but it was not significant (+27%, SEM 9%, $p < 0.06$; **Figure 3A**). Those

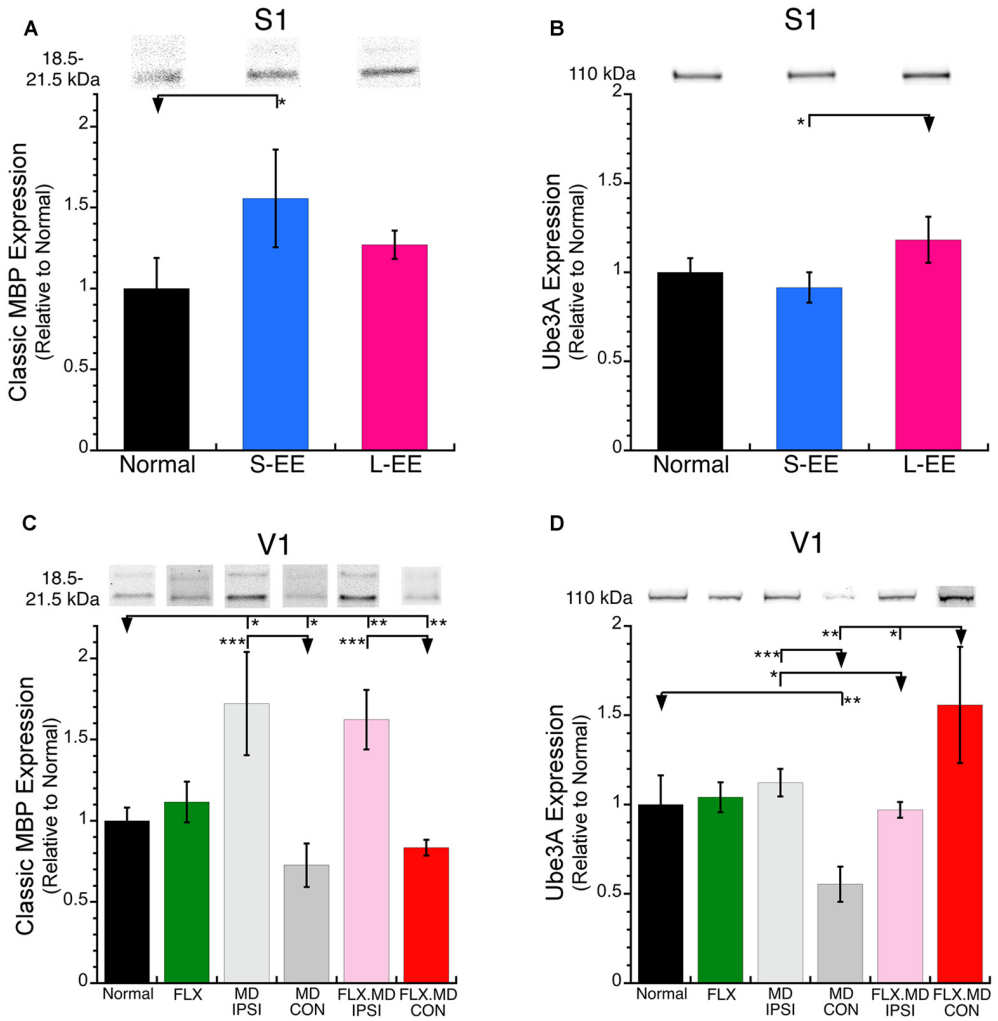

FIGURE 3 | Expression of myelin basic protein (MBP) and Ube3A in S1 and V1. **(A)** S-EE increased MBP expression (+56%, SEM 30%, $p < 0.05$) and **(B)** L-EE increased Ube3A (+18%, SEM 13%, $p < 0.05$). **(C)** MBP expression was increased in the non-deprived (ipsilateral) hemisphere after both MD (light gray; MD ipsi, +72%, SEM 32%, $p < 0.05$) and fluoxetine plus MD (pink; flx MD ipsi, +62%, SEM 18%, $p < 0.01$). There was a loss of MBP expression in the deprived (contralateral) hemisphere after both MD (dark gray; MD con, −27%, SEM 13%, $p < 0.05$) and fluoxetine plus MD (red; flx MD con, −17%, SEM 4.8%, $p < 0.01$). **(D)** There was a loss of Ube3A expression after MD (MD con, −45%, SEM 9.9%, $p < 0.001$) but an increase after MD plus fluoxetine (flx MD con, +56%, SEM 33%, $p < 0.01$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

MBP results show a similar pattern to the EE driven increase reported for oligodendrocytes and myelination in S1 (Hughes et al., 2018). Next, we quantified Ube3A which is necessary for the maintenance of CP experience-dependent plasticity (Yashiro et al., 2009). Ube3A after both the short- and long-term EE was not different from normal, however, the long-term EE group had greater Ube3A expression than the short-term EE group (**Figure 3B**).

Effects of Fluoxetine and MD on MBP and Ube3A in the Visual Cortex

We examined how fluoxetine, MD and the combination of treatments changed the expression of MBP and Ube3A in V1 (**Figure 1B**). Fluoxetine alone did not change the expression of MBP (**Figure 3C**) or Ube3A in V1 (**Figure 3D**) which contrasts with the reduced expression of both drebrin isoforms (**Figures 2C,D**). MD, however, caused hemisphere specific changes to MBP expression (**Figure 3C**). In the non-deprived (ipsi) hemisphere, there was an increase in MBP (MD ipsi: +72%, SEM 32%, $p < 0.05$) while in the deprived (contra) hemisphere there was a loss of MBP (MD con: −27%, SEM 13%, $p < 0.05$). When fluoxetine was combined with MD it did not affect the pattern of MBP expression found after MD alone and there continued to be increased expression in the non-deprived hemisphere and loss of expression in the deprived hemisphere (flx MD ipsi: +62%, SEM 18%, $p < 0.01$; flx MD con: −17%, SEM 4.8%, $p < 0.01$; **Figure 3C**).

The pattern of Ube3A changes in V1 did not follow MBP changes. MD caused no change in Ube3A in the non-deprived (ipsi) hemisphere but a loss in the deprived hemisphere

(MD con: −45%, SEM 9.9%, $p < 0.001$; **Figure 3D**). The combination of fluoxetine and MD caused a recovery of Ube3A in the deprived hemisphere (flx MD con: +56%, SEM 33%, $p < 0.01$; **Figure 3D**). While MBP was bidirectionally changed driven by visual experience, increasing in the non-deprived and decreasing in the deprived hemisphere, Ube3A only decreased in the deprived hemisphere. Furthermore, fluoxetine treatment did not affect MBP, but it did affect the other plasticity markers (drebrin and Ube3A), especially when it was combined with MD.

We used unsupervised hierarchical cluster analysis of the data from the deprived (contralateral) and non-deprived (ipsilateral) V1 hemispheres to identify high dimensional patterns in the data. Along with the expression of MBP and Ube3A, protein expression was included for five markers of synaptic plasticity (GluA2, PSD95, Gephyrin, Synapsin, and Synaptophysin) measured previously using tissue samples from the same animals (Beshara et al., 2015). MBP and Ube3A were in separate clusters for both hemispheres of all conditions (**Figure 4**). In normal animals, MBP clustered with GluA2 while Ube3A clustered with Synapsin, Gephyrin, and PSD95 (**Figure 4A**). Fluoxetine changed the relationship between MBP and GluA2 to a negative correlation and partitioned them into different clusters, but the Ube3A cluster remained similar to the normal pattern (**Figure 4B**). After MD, MBP clustered with the postsynaptic markers Gephyrin and PSD95 in the deprived hemisphere while Ube3A clustered with the presynaptic marker Synapsin (**Figure 4C**). The combination of fluoxetine with MD led to a different pattern in the deprived hemisphere. There MBP clustered with Synapsin while Ube3A clustered with the postsynaptic markers and Synaptophysin (**Figure 4D**).

In the non-deprived hemisphere, the pattern for MBP and GluA2 was different from normal and those proteins were partitioned into separate clusters (**Figure 4E**). Ube3A, however, clustered with the same synaptic markers as in normal animals (**Figure 4E**). Finally, the combination of fluoxetine and MD led to a unique pattern of clusters with MBP and GluA2 in a cluster and Ube3A and Synaptophysin in another cluster (**Figure 4F**). Interestingly, the increase in MBP in the non-deprived hemisphere led to a negative relationship with GluA2 (**Figure 4E**), but adding fluoxetine changed it to a positive relationship (**Figure 4F**) that was similar to the normal pattern between MBP and GluA2 (**Figure 4A**). Together, these cluster analyses showed that the combination of fluoxetine with MD changed the relationships of MBP with this collection of plasticity markers even though the expression levels of MBP were similarly affected by MD alone or with fluoxetine (**Figure 3C**).

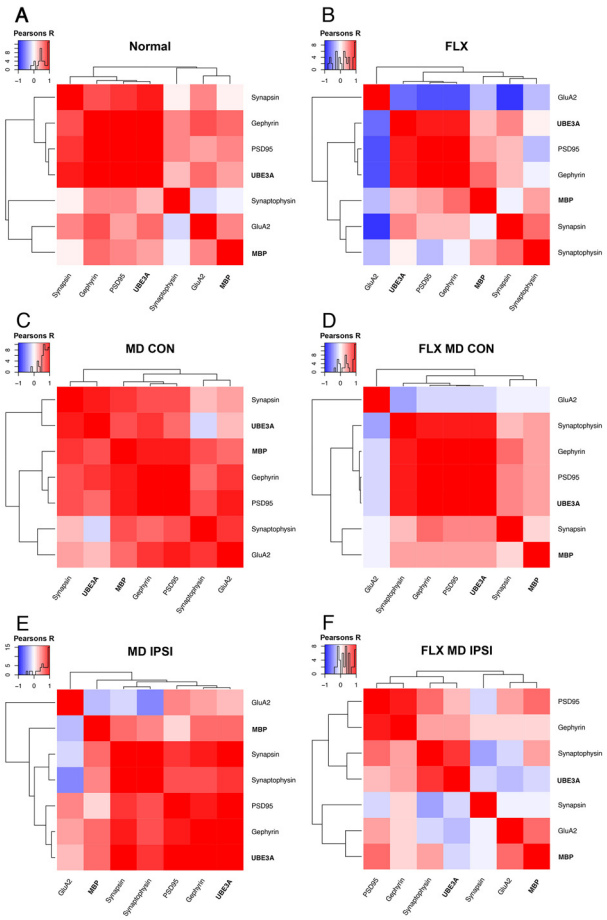

FIGURE 4 | Hierarchical cluster analysis of adult V1. The high dimensional pattern of protein expression changes in V1 for the deprived (contralateral) and non-deprived (ipsilateral) hemispheres were analyzed by combining the measurements of MBP and Ube3A from this study with data for GluA2, PSD95, Gephyrin, Synapsin, and Synaptophysin from our previous study (Beshara et al., 2015). Correlation matrices are plotted to show the strength and direction (blue: negative; red: positive) of the pairwise Pearson's R correlations between proteins for each condition and hemisphere. The inset with each panel shows the color-code and distribution of *R* values. The order of proteins was determined using unsupervised hierarchical clustering such that proteins with stronger correlations were nearby in the matrix: **(A)** normal, **(B)** fluoxetine, **(C)** MD contralateral (deprived) hemisphere, **(D)** fluoxetine and MD contralateral (deprived) hemisphere, **(E)** MD ipsilateral (non-deprived) hemisphere, and **(F)** fluoxetine and MD ipsilateral (non-deprived) hemisphere.

DISCUSSION

In this study, we quantified MBP expression in S1 and V1 after treatments known to affect adult plasticity in those cortical areas. Sensory enrichment caused an increase in MBP expression in S1 that was similar to a previous finding of an increase in the number of oligodendroctyes (Hughes et al., 2018). Furthermore, the S1 changes to markers of dendritic spines (drebrin A) and plasticity maintenance (Ube3A) were consistent with previous studies showing that enrichment causes an increase in spines (Jung and Herms, 2014) and functional plasticity (Polley et al., 2004). The changes in V1 after MD and fluoxetine treatments were more complicated than the EE driven changes in S1. The increase in the nascent spine marker drebrin E in the deprived hemisphere was consistent with an MD-driven increase in spine motility (Oray et al., 2004; Hofer et al., 2008). The MBP levels in V1, however, appeared to reflect activity levels with increased MBP in the non-deprived hemisphere and decreased MBP in the deprived hemisphere. The high dimensional cluster analyses uncovered more subtle changes including showing that fluoxetine did affect the overall

pattern of relationships between MBP and the other proteins. Together, these findings suggest that multiple mechanisms, including activity levels and specific plasticity mechanisms, may be involved in regulating experience-dependent changes in MBP expression in adult V1.

Much of existing research on the role of myelin in V1 plasticity has focused on the CP when myelin is typically viewed as a structural brake on visual plasticity (Bavelier et al., 2010). Thus, it was unexpected to find that MD in adults changed MBP. In the deprived hemisphere, the loss of MBP led to clustering with PSD95 and Gephyrin, which are also reduced by MD in adults (Beshara et al., 2015). In the non-deprived hemisphere, MBP increased, but the other markers are not changed (Beshara et al., 2015). That increase in MBP partitioned it into an individual cluster, suggesting that it had weak or no relationships with the other plasticity markers. Glutamatergic receptor proteins, however, are known to go through transient changes during MD and can return to normal levels within a week (Williams et al., 2015). Thus, the MBP changes measured here may reflect the longer-term impact of fluctuations in neural activity that can support more efficient neural transmission (Fields, 2015). It is well-known that neuronal activity can increase myelination (Demerens et al., 1996; Gibson et al., 2014) and perhaps the increased MBP contributes to increased metabolic support (Saab et al., 2013; Philips and Rothstein, 2017) for more active neurons in the non-deprived V1. In that framework, myelin in adult V1 could serve an adaptive role supporting responses to heightened demands of the increased visually driven activity. New studies will be needed to tease apart the mechanisms that contribute to regulating these activity-dependent MBP changes in adult V1 to determine the role of indirect signaling vs. direct glutamatergic synaptic input to oligodendrocyte precursors (OP). For example, OP cells express AMPA receptors and signaling through those receptors can stimulate the production of white matter myelin during development (Kougioumtzidou et al., 2017).

In young adult rats, 1 month of fluoxetine treatment reinstates juvenile-like ocular dominance plasticity (Maya Vetencourt et al., 2008) and increases spine density and size (Ampuero et al., 2010). Here, we did not find an effect of fluoxetine on the amount of MBP expression in V1. There was no change in MBP levels after 1 month of fluoxetine and no recovery of the MD-induced loss of MBP when fluoxetine was combined with MD. In contrast, there was evidence from drebrin and Ube3A that fluoxetine alone or combined with MD affected V1 because the drebrin isoforms were reduced, and Ube3A was increased in the deprived hemisphere. Furthermore, the cluster analysis showed that the pattern of relationships between MBP expression and plasticity markers measure in a previous study (Beshara et al., 2015) was changed by fluoxetine. Fluoxetine is known to regulate NMDA and AMPA receptors (Szasz et al., 2007; Kiss et al., 2012; Vizi et al., 2013; Barygin et al., 2017) and both receptors are found on oligodendroglia where they participate in myelination (Káradóttir and Attwell, 2007; Kougioumtzidou et al., 2017). Thus, fluoxetine could have a direct effect on the regulation of MBP in adult V1 separate from the effects on synaptic plasticity mechanisms.

Ube3A deficient mice have an experience-dependent loss of dendritic spines in V1 (Kim et al., 2016), increased excitability, weaker orientation tuning (Wallace et al., 2017) and a 25% reduction in cortical MBP (Grier et al., 2015). Here, the Ube3A and MBP changes caused by MD and fluoxetine were not the same. MD reduced both Ube3A and MBP by similar amounts in the deprived hemisphere, but only MBP was increased in the non-deprived hemisphere. Although adding fluoxetine rescued the MD-induced loss of Ube3A, it did not rescue MBP expression. It is interesting to note that all of the high dimensional analyses partitioned Ube3A and MBP into different clusters suggesting that MBP and Ube3A reflect different aspects of adult V1 function and plasticity.

In S1, the relationships between Ube3A, drebrin, and MBP after EE found here were more consistent with previous results about the effects of EE on the somatosensory cortex. For example, long-term EE increased both Ube3A and drebrin A with a trend to increased MBP, providing additional support for the idea that EE maintains enhanced adult plasticity by increasing spines (Jung and Herms, 2014) and myelin (Hughes et al., 2018). The V1 findings suggest a more complicated relationship and raise new questions about how MBP and the various components of myelin interact with other mechanisms to enhance or reduce plasticity in the adult cortex.

The experience-dependent changes in MBP expression in the adult cortex probably involve many cortical circuits, including a subset of GABAergic neurons: the fast-spiking parvalbumin-positive (PV+) cells (Kawaguchi and Kubota, 1997) that play an essential role in stimulus-selective response potentiation in adult V1 (Kaplan et al., 2016). In both mice and the human cortex, the axons of many PV+ neurons have patches of myelination that are mainly confined to the proximal axonal segment (Micheva et al., 2016; Stedehouder et al., 2017). Furthermore, the myelin sheaths around PV+ axons have more MBP than non-GABAergic axons (Micheva et al., 2018). Neuronal activity in S1 elongates the myelin patches on PV+ interneurons (Stedehouder et al., 2018), raising the possibility that increased MBP in the non-deprived V1 may reflect changes in PV+ cells myelination. In contrast, PV+ cells do not contribute to MD-driven ocular dominance in the deprived adult V1 (Kaplan et al., 2016), so the loss of MBP found there may involve other cell types. It will be necessary to follow-up these findings with high-resolution anatomical studies to determine the cells and circuits in V1 where MD changes MBP.

CONCLUSIONS

The current findings and those of other recent studies highlight that increases in cortical myelin can be more than a structural brake on experience-dependent plasticity. Instead, cortical myelin may have a role in adaptive plasticity in the adult cortex. Future studies are needed to address the functional contributions that experience-dependent changes in cortical myelin have on adult plasticity. Those studies will be timely since interventions, such as the use of the antihistamine clemastine, are being tested to increase cortical myelin in both neurodegeneration and

neurodevelopmental disorders (Liu et al., 2016; Green et al., 2017; Barak et al., 2019).

DATA AVAILABILITY STATEMENT

The Western Blot data used to support the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

The animal study was reviewed and approved by McMaster University Animal Research Ethics Board.

AUTHOR CONTRIBUTIONS

KM designed the research, analyzed the data, and wrote/revised the article. SM designed the research, performed research, analyzed the data, and wrote/revised the article. KC performed research and analyzed the data. KA analyzed the data and revised the article. SB designed the research, performed research, analyzed the data, and wrote/revised the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00056/full#supplementary-material.

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REVIEW
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Astrocytes and Microglia as Major Players of Myelin Production in Normal and Pathological Conditions

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Myelination is an essential process that consists of the ensheathment of axons by myelin. In the central nervous system (CNS), myelin is synthesized by oligodendrocytes. The proliferation, migration, and differentiation of oligodendrocyte precursor cells constitute a prerequisite before mature oligodendrocytes extend their processes around the axons and progressively generate a multilamellar lipidic sheath. Although myelination is predominately driven by oligodendrocytes, the other glial cells including astrocytes and microglia, also contribute to this process. The present review is an update of the most recent emerging mechanisms involving astrocyte and microglia in myelin production. The contribution of these cells will be first described during developmental myelination that occurs in the early postnatal period and is critical for the proper development of cognition and behavior. Then, we will report the novel findings regarding the beneficial or deleterious effects of astroglia and microglia, which respectively promote or impair the endogenous capacity of oligodendrocyte progenitor cells (OPCs) to induce spontaneous remyelination after myelin loss. Acute delineation of astrocyte and microglia activities and cross-talk should uncover the way towards novel therapeutic perspectives aimed at recovering proper myelination during development or at breaking down the barriers impeding the regeneration of the damaged myelin that occurs in CNS demyelinating diseases.

Keywords: oligodendrocyte, myelination, remyelination, astrocyte, microglia

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During the last decades, the production of myelin in the central nervous system (CNS) has been the focus of a multitude of studies in the context of development or regeneration. Oligodendrocytes, the cells synthesizing myelin in the CNS, were initially found in hind-jawed fishes (Zalc and Colman, 2000; Hartline and Colman, 2007; Zalc, 2016). These cells derive from oligodendrocyte progenitor cells (OPCs) occurring in several waves. In mice, the first wave arises at embryonic day (E) 12.5 in the ventral domain of the ventricular wall in the developing brain and spinal cord. The second one emerges more dorsally at E15.5 in both regions and the last one arises specifically in the perinatal cortex around birth (Pringle and Richardson, 1993; Timsit et al., 1995; Trousse et al., 1995; Spassky et al., 1998; Olivier et al., 2001). Unexpectedly, the destruction of one of these waves in the telencephalon induces the remaining cells to compensate for the lost oligodendrocytes thus suggesting functional similarity. In agreement with this observation, ventrally derived oligodendroglial cells strongly decline early after birth in the postnatal dorsal

forebrain and are replaced by dorsally derived cells, which thus constitute more than 80% of oligodendroglial cells present in the adult corpus callosum and cerebral cortex (Kessaris et al., 2006; Tripathi et al., 2011). Myelination itself progresses according to typical sequences, which are spatially and temporally determined. In humans, after 16 weeks of gestation, myelin can be detected in the fasciculus cuneatus, and shortly after occurs in the cerebellar and pyramidal tracts. Then, myelination proceeds rapidly during the first year from the occipital to the fronto-temporal lobes. Regions devoted to basic homeostasis are proposed to be myelinated before areas involved in more intricate tasks like the frontal cortex. Late myelinated areas are typically myelinated at a lower level than early myelinating regions (Brody et al., 1987; Kinney et al., 1988). More recent data derived from serial reconstructions of the adult mouse cortex showed that the degree of myelination can even vary along a single axon. Thus, neurons located in superficial cortical layers exhibit both myelinated- and large unmyelinated segments, contrasting with the pattern observed for instance in the spinal cord or the optic nerve where myelinated axons display regular internodes (Tomassy et al., 2014).

The finding that the CNS can regenerate myelin lost upon various types of insults was observed much earlier than the characterization of oligodendrocyte and myelin generation. Indeed, almost 60 years ago, occasional myelin sheaths were visualized a short time after demyelination was induced into the subpial cord by repeated withdrawal and reinjection of cerebrospinal fluid (Bunge et al., 1961). However, the demonstration that central remyelination was able to restore secure conduction in the demyelinated spinal cord and to lead to functional recovery was provided only two decades later (Smith et al., 1979). Since then, thousands of publications were dedicated to this process notably in the aim to investigate how new OPCs are recruited to the demyelinating area and differentiate into myelinating oligodendrocytes (Gallo and Armstrong, 2008; Clemente et al., 2013; Domingues et al., 2016; Lloyd et al., 2017).

Although in both development and repair, the oligodendrocyte is the pivotal cell type for myelin production, its function is widely influenced by the other glial cells, namely astrocytes and microglia. This review is aimed at revisiting the present knowledge regarding communication between oligodendroglial, astroglial and microglial cells in the context of myelin development and regeneration with a specific focus on most recent advances.

ASTROCYTE-OLIGODENDROCYTE COMMUNICATION IN DEVELOPMENTAL MYELIN PRODUCTION

In the mouse spinal cord and cerebral cortex, the first mature astrocytes expressing the glial fibrillary acidic protein (GFAP) can be detected at the embryonic day (E) 12.5 and 16.5 respectively, thus before the start of OPC maturation and axon myelination taking place during the first three postnatal weeks (Miller et al., 1985; Qian et al., 2000). Like neurons and oligodendrocytes, astrocytes are derived from the neuroepithelium from which they migrate throughout the CNS along radial glia processes (Molofsky and Deneen, 2015).

Astrocyte-Derived Trophic Factors and Cytokines Control Oligodendrogenesis

The crucial role of astrocytes was initially related to the finding that these cells are the main producers of the platelet-derived growth factor (PDGF). Acting through its receptor PDGFRa, PDGF promotes OPC proliferation and mobility and, also inhibits OPC differentiation towards mature oligodendrocytes, by generating a graded response requiring activation of the phosphoinositide 3-kinase and/or phospholipase C γ pathways. Astrocyte-derived PDGF was thus proposed to drive the clock that times oligodendrocyte development (Raff et al., 1988; Richardson et al., 1988; McKinnon et al., 2005). Besides PDGF, astrocytes secrete other growth factors. Brain-derived neurotrophic factor (BDNF) is namely required to potentiate myelination during the early postnatal development as indicated by BDNF knockout heterozygous mice, which exhibit delayed CNS myelination (Cellerino et al., 1997). In the same line, under stress conditions such as those occurring during prolonged cerebral hypoperfusion at birth, astrocyte-derived BDNF also supports OPC maturation in a TrkB-dependent manner as shown *in vitro* in sublethal CoCl2 exposition of OPC / astrocyte primary co-cultures and, *in vivo*, in a transgenic mouse line in which BDNF expression was specifically downregulated in astrocytes (Miyamoto et al., 2015). The observation that ciliary neurotrophic factor (CNTF) synthesis by astrocytes starts at the onset of myelination in the rodent optic nerve (Stöckli et al., 1991; Dobrea et al., 1992) led to suggest its physiological role on myelination, as well. The strong pro-myelinating effects of CNTF and other members of the family such as the leukemia inhibitory factor (LIF) in myelinating co-cultures further supported this hypothesis. CNTF acts on oligodendrocytes by favoring their final maturation *via* a mechanism involving the 130 kDa glycoprotein receptor common to the CNTF family and transduced through the Janus kinase (JAK) pathway (Stankoff et al., 2002). In contrast, other astrocyte-derived secreted proteins negatively regulate oligodendrocyte biology. For instance, the chemokine CXCL1 transiently expressed at a high level during spinal cord development signals through the chemokine receptor CXCR2 expressed by immature OPCs. Migrating OPCs were proposed to enter the presumptive white matter where they may encounter an environment in which astrocytes transiently and locally express high CXCL1 levels. Through CXCR2, CXCL1 may inhibit PDGF-stimulated OPC migration before subsequently increasing OPC proliferation in concert with PDGF (Tsai et al., 2002).

Astrocytes Control the Availability of Cues Regulating Oligodendrocyte Production

Besides the secretion of trophic factors, astrocytes are also able to control the bioavailability of oligodendrocyte-regulating cues (**Figure 1A**). The best-documented example was provided by the capacity of astrocytes to control the concentration of the morphogen Sonic Hedgehog (Shh) during OPC production in the optic nerve. Shh participates in the proliferation and

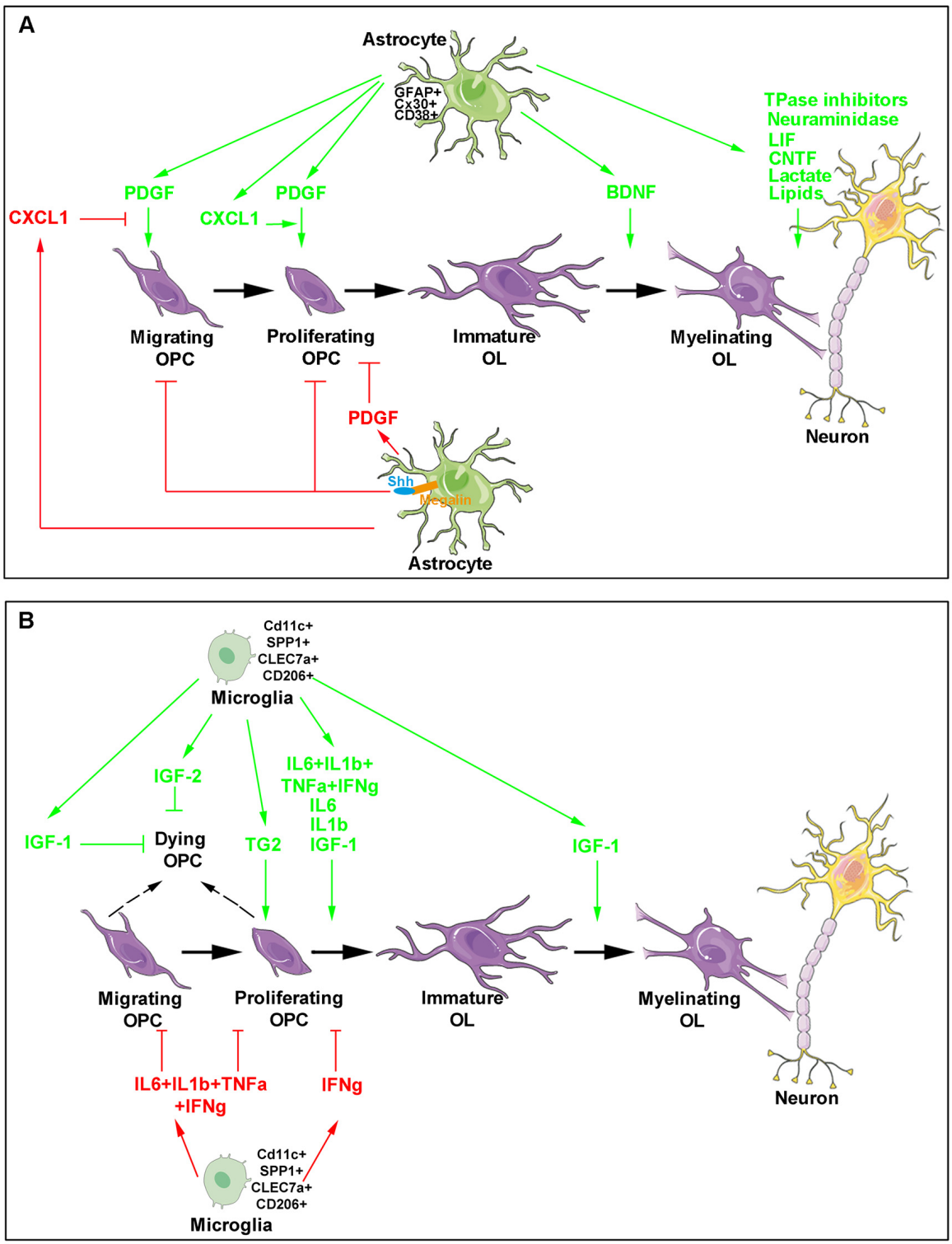

FIGURE 1 | Major roles of astrocytes and microglia during developmental oligodendrogenesis and myelination. The different steps of oligodendroglial cell production (purple) are shown and include oligodendrocyte progenitor cell (OPC) migration, proliferation, differentiation as well as the maturation of immature oligodendrocytes into cells able to myelinate neuronal axons. Astrocyte- **(A)** and microglia- **(B)** derived molecules are indicated. The green arrows indicate molecules exhibiting a positive activity on oligodendrocyte lineage and myelin production (top in each panel) whereas the red arrows and blocking symbols indicate molecules displaying an inhibiting activity on oligodendroglial lineage production (bottom in each panel).

migration of OPCs during the colonization of this nerve (Merchán et al., 2007). The multiligand receptor megalin, a member of the low-density lipoprotein receptor family able to bind Shh, was found to be exclusively expressed by astrocytes according to a dynamic pattern paralleling optic nerve colonization by OPCs arising from the optic chiasm and migrating to the retina. Indeed, when OPCs start their migration throughout the nerve at E14.5 in the mouse, megalin is more widely distributed in the region close to the optic chiasm whereas the distribution is reversed at E16.5 when the first OPCs reach the retina. Since, thereafter, megalin was found to be weakly and uniformly expressed all along the nerve, this receptor was proposed to control Shh internalization and its subsequent release at the suitable concentration during the various steps leading to oligodendrogenesis (Ortega et al., 2012).

Astrocyte Integrity Is Required for Proper Myelination

Consistent with the above findings, the requirement of astrocyte integrity for the normal development of oligodendrocytes was shown by the analysis of transgenic mouse strains devoid of GFAP expression. Although viable, GFAP knockout mice exhibit abnormal myelination including the presence of actively myelinating oligodendrocytes in adults, nonmyelinated axons in the optic nerve, reduced myelin thickness in spinal cord and ultrastructural defects such as loosening of myelin sheaths (Liedtke et al., 1996). More recently, the invalidation of the transmembrane protein CD38, which possess ADP-ribosyl cyclase activity and is highly expressed in astrocytes, was similarly reported to result in altered astrocyte maturation and delayed oligodendrocyte differentiation during the postnatal development (Hattori et al., 2017). Furthermore, physical interactions between oligodendrocytes and astrocytes appear to be also required for myelination as shown by analysis of the role of connexins (Cx) 30 and Cx43 present on astrocytes and forming gap junctions with Cx32 and Cx47 present on oligodendrocytes. First, Cx47 mutants that cause the Pelizaeus–Merzbacher-like disease do not efficiently form functional channels with Cx43 (Orthmann-Murphy et al., 2007). Second, the deletion of both Cx47 and Cx30 in mice induces severe myelination defects including vacuole formation and thin myelin sheaths as well as a decreased number of oligodendrocytes (Tress et al., 2012). A mechanism possibly involved is proposed to be disruption of the metabolic support provided by astrocytes to oligodendrocytes in agreement with the gap junction-mediated unidirectional flux that transports the molecules preferentially from astrocytes to oligodendrocytes (Robinson et al., 1993).

Astrocytes Are Indispensable for Axon Myelination

Accurate delineation of the molecular mechanisms supporting the role of astrocytes in myelination itself has never ceased for the last decades. Among the most relevant findings, the requirement of astrocytes for alignment and adherence of oligodendrocyte processes to axons was shown by using cocultures of retinal ganglion cells and optic nerve oligodendrocytes that led to propose a mechanism implicating neuraminidase-mediated removal of polysialic acid (PSA) from both cell types (Meyer-Franke et al., 1999). In agreement with this result, the abolition of the postnatal downregulation of PSA in oligodendrocytes from transgenic mice expressing the polysialyltransferase ST8SiaIV under the control of the proteolipid protein promoter indicated that PSA downregulation is required for efficient differentiation of oligodendrocytes, as well as for myelin formation and maintenance. However, *in vivo* implication of astrocytes is still lacking (Fewou et al., 2007). Another link between astrocytes and myelination was established by the demonstration that electrical activity in pre-myelinated axons increases myelination after OPCs mature to a promyelinating stage *via* the activity-dependent release of ATP from axons. ATP acts in a paracrine manner on astrocytes *via* the purinergic P2 receptor and induces the release of the LIF cytokine that ultimately stimulates myelination (Ishibashi et al., 2006). A specific myelinating CNS coculture system provided evidence that astrocytes are implicated rather in the promotion of rapid myelin growth than in initiation of myelination (Watkins et al., 2008).

Astrocytes Provide Lipids for Myelin Sheath Production

Still consistent with their implication in myelination, astrocytes were also proposed to supply lactate to oligodendrocytes. Lactate constitutes a source of energy and a precursor of lipid synthesis including cholesterol altogether necessary for myelin production. Although astrocytes and oligodendrocytes are both in direct contact with blood vessels and may thus take up lactate from blood, the hypothesis is that lactate released from astrocytes through the monocarboxylate transporter (MCT) 4 may be supplied to oligodendrocytes possibly importing the molecule through MCT1 (Sánchez-Abarca et al., 2001; Rinholm et al., 2011). The requirement for local synthesis of cholesterol is consistent with the fact that myelin comprises about 80% of the brain cholesterol content and that peripheral cholesterol entry into the brain is largely precluded by the blood-brain barrier. During postnatal myelination, *de novo* synthesis of cholesterol is carried out by both astrocytes and oligodendrocytes. Astrocyte-derived cholesterol is then distributed to oligodendrocytes *via* lipoproteins contained in apolipoproteins, mostly the apolipoprotein E (ApoE) in the CNS, and secreted *via* the ATP-binding cassette transporter ABCA1 (Saher and Stumpf, 2015). Astrocytes provide a substantial fraction of the lipids incorporated into CNS myelin and in the absence of astrocyte lipid synthesis, oligodendrocytes are unable to finalize CNS myelination, leading to hypomyelinated and slower-conducting fibers in adulthood. Indeed, the specific inactivation of an essential co-activator of the transcription factor SREBP, the SREB cleavage activating protein (SCAP) in oligodendrocytes, resulted in lipid biosynthesis and myelination delay nevertheless recovering in adulthood. In contrast, when the enzyme was deleted in astrocytes or both astrocytes and oligodendrocytes, a persistent hypomyelination was observed. Thus, extracellular lipids seem to be supplied by astrocytes under

conditions of compromised oligodendrocyte lipid synthesis. Moreover, full myelin synthesis requires an astrocyte lipid supply in addition to endogenous oligodendrocyte lipid synthesis (Camargo et al., 2017).

Besides providing lipids for the synthesis of myelin sheaths during development, astrocytes, namely those contacting the nodes of Ranvier, were also reported to reversibly modify myelin thickness and nodal gap length thus appropriately regulating conduction velocity in individual axons. In support of this observation, the reduction of exocytosis induced in transgenic mice expressing a dominant-negative fragment of the vesicle-associated membrane protein 2 (VAMP2) in astrocytes, exhibited detachment of adjacent paranodal loops of myelin from the axon, increased nodal gap length, thinning of myelin sheath in the optic nerve and finally decrease in visual acuity. These data led to propose that thrombin-dependent proteolysis of the cell adhesion molecule neurofascin 155 that attaches myelin to the axon, is inhibited by the vesicular release of thrombin protease inhibitors from perinodal astrocytes, which likely involves these cells in myelin remodeling necessary for optimal electrical conduction (Dutta et al., 2018).

The last evidence of the remarkable and specific relationship between astrocytes and myelination is finally provided in a model of neuromyelitis optica, a chronic inflammatory demyelinating disease characterized by the destruction of astrocytes and their foot processes in early lesions. Indeed, in a model of this disease, the death of oligodendrocytes was found to occur before granulocyte or macrophage/microglia infiltration, but only a few hours after the death of astrocytes induced in a complement-dependent manner (Wrzos et al., 2014).

MICROGLIA-OLIGODENDROCYTE COMMUNICATION IN DEVELOPMENTAL MYELIN PRODUCTION

Considered as resident myeloid cells of the CNS, microglia have been only recently characterized as the progeny of yolk sac-derived macrophages that start to enter the CNS at E9 through the blood vasculature before closure of the blood-brain barrier that occurs by E13 in mice (Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013). Thereafter, at least under non-pathological conditions, microglia cells are autonomously maintained through proliferation (Askew et al., 2017). Microglia is crucial during neurodevelopment namely *via* its interaction with neuronal cells for wiring and neural circuit regulation (Thion et al., 2018).

Distinct Patterns of Secreted Molecules for Microglia and Astrocyte-Mediated Control of OPC Generation

The first arguments supporting a role for microglia in developmental myelination were mainly derived from microglia-oligodendrocyte cocultures. They showed that microglia was able to stimulate the synthesis of sulfatide, a myelin-specific galactolipid, and to increase myelin-specific proteins, MBP and PLP in oligodendrocytes (Hamilton and Rome, 1994). Microglia activity was mimicked by conditioned medium derived from microglia cultures suggesting that the cells acted *via* the secretion of appropriate molecules. Together with promoting cell maturation, microglia were found to prevent OPC apoptosis by upregulating the nuclear factor-kappa B (NF-κB) p65 subunit *via* the recruitment of PI-3 kinase to the PDGFRa thus leading to a synergism with PDGF (Nicholas et al., 2001).

Although both microglia- and astrocyte-conditioned media were able to prevent OPC degeneration induced by growth factor deprivation in the culture medium during a short time, microglia-derived medium was unable to support OPC survival in the long-term compared to astrocytes. These differential effects were supported by distinct patterns of cytokine/growth factors correlated with differentially activated intracellular signaling pathways in OPCs (Pang et al., 2013). More specifically, mechanisms underlying enhanced microglia-induced oligodendrocyte differentiation were proposed to be related to IGF-1. IGF-1 levels were more than six-fold higher in microglia- than in astrocyte-derived medium and its expression was consistently detected in amoeboid microglial cells located in the corpus callosum until the seventh postnatal day, which corresponds to the time of morphological change of microglia towards the ramified phenotype present in adulthood unless microglia become activated. Consistently, exposure of microglia cultures to lipopolysaccharide leading to microglial activation, increased IGF-1 secretion (Kaur et al., 2006) and IGF-1 knockout mice display reduced oligodendrocyte survival, differentiation, and maturation (Ye et al., 2002). Moreover, IGF-2 also derived from microglia was found to prevent the TNFα-induced death of mature oligodendrocytes *in vitro* (Nicholas et al., 2002).

Unique Phenotype of Neonatal Microglia Implicated in Developmental Myelination

As soon as the end of the 1970s, the existence of a specific subpopulation of microglial cells had been proposed in the early postnatal corpus callosum (Imamoto and Leblond, 1978). Unexpectedly, the pharmacological suppression of microglia activation by using the anti-inflammatory drug minocycline was reported to significantly inhibit oligodendrogenesis both *in vitro* in neurosphere culture and *in vivo* in the subventricular zone (SVZ) of the dorsal forebrain. This inhibition was mediated by the blockade of several proinflammatory cytokines including IL-1β, IL-6, TNF-α, and IFN-γ. Also, the observation that activated microglia significantly increased $O4^+$ cells but decreased $PDGFRa^+$ OPCs (**Figure 1B**) again confirmed microglia activity on oligodendrocyte maturation (Shigemoto-Mogami et al., 2014).

More recent data provided by two independent groups led to a major advance in the understanding of the identity and role of this microglial subpopulation that appeared to highly proliferate and display a rhomboid shape in contrast to microglia detected at the same time in the neighboring cortex and exhibiting small processes comparable to adult microglia (Hagemeyer et al., 2017; Wlodarczyk et al., 2017). Presently, neonatal microglia implicated in developmental oligodendrogenesis appear as a cell population with a dramatically different gene expression profile compared to the one from adult healthy mice. Based

on the observation that during neuroinflammation, microglia expressing the integrin complement receptor CD11c are a major source of IGF-1, Wlodarczyk and collaborators detected a substantial increase of $CD11c^+$ microglia during the first days after birth before a sharp decrease mainly in highly myelinating areas such as the developing corpus callosum and cerebellum. IGF-1 depletion in this subset led to a reduction in brain weight, a decrease in the expression of myelin proteins including PLP, MBP, MAG and MOG, and was finally associated with higher frequency of less myelinated fibers in the corpus callosum. Thus, as the major source of IGF-1, the $CD11c^+$ microglia subset was proposed to play a critical role in primary myelination and neuronal support in the neonatal CNS (Wlodarczyk et al., 2017). Interestingly, the comparison of transcriptomes from sorted $CD11c^+$ and $CD11c^-$ microglia revealed that neonatal microglia, naive adult microglia and microglia derived from the experimental autoimmune encephalomyelitis (EAE) model of demyelination formed distinct global expression clusters. Unexpectedly, $CD11c^+$ and $CD11c^-$ subpopulations were relatively close together indicating that the major difference was related to developmental age rather than subpopulation phenotype. The identification of gene expression profiles associated with microglia from the different conditions showed that under demyelinating conditions, microglia cells were enriched for immune system genes consistent with their activated state. On the contrary, in the neonatal brain, microglia gene profile included genes involved in nervous system development displaying a neurogenic phenotype. Remarkably, upon a quite total ablation of microglia in the adult brain by using an inducible mouse strain, clusters of highly proliferating microglia were detectable throughout the CNS. Although expressing CD11c and nestin, the proliferating cells did not display a neurogenic gene expression profile confirming that the myelinogenic $CD11c^+$ subset of microglia is unique to neonatal CNS (Wlodarczyk et al., 2017).

Independently, Hagemeyer and collaborators similarly identified this amoeboid microglia subset that they called "the fountain of microglia" specifically located in myelinating regions from postnatal day (P)1 to P8, displaying a high expression of the activation marker Mac3 and dramatically collapsing at P9. Microglia found in the postnatal subcortical (with an amoeboid shape) and cortical regions (with multiple cellular processes) were found to share their early origin from the same $CX3CR1^+$ CNS endogenous precursors without any contribution from circulating blood monocytes. The pharmacological blockade of the receptor for the secreted cytokine, colony-stimulating factor 1 (CSF1), known to efficiently deplete microglia (Elmore et al., 2014), showed that early postnatal microglia is required for the proper induction of oligodendrocyte progenitors and subsequent myelination. Transcriptomic analysis of the studied subpopulation revealed genes also detected in disease models such as CLEC7a, SPP1, IGF-1, ANXA5, ITGAX, and GPNMB. An additional RNA sequencing analysis led to identify change in gene expression profile between P7 and P10, the former being related to phagocytosis, migration, priming of microglia more particularly described during aging and disease while the latter was associated with apoptosis and necrosis according to a profile further increased in adulthood. Thus, a specificity of neonatal microglia in P7 corpus callosum was the expression of SPP1, CLEC7A, and CD206 since none of them could be detected in cortical microglia (Staszewski and Hagemeyer, 2019). Importantly, functional microglia are also required for OPC homeostasis in the adult brain in agreement with the severe white matter abnormalities observed in patients suffering from microgliopathy (Hagemeyer et al., 2017).

The Extracellular Matrix at the Crossroads of Microglia and OPC Activity

The investigation of how OPCs integrate signals from the matrix and other glial cells led to a growing interest for the adhesion G protein-coupled receptor (aGPCR) family, the second-largest class of GPCRs playing crucial roles in developmental processes (Mehta and Piao, 2017). The finding that the genetic loss of one of its members, GPR56, was phenocopied by the specific deletion of the enzyme microglia-derived transglutaminase 2 (TG2) in microglia both resulting in the decrease of OPC cell division and number of mature oligodendrocytes, as well as to hypomyelination during postnatal CNS development, led to identify GPR56 as the receptor of TG2. In agreement with the ability of TG2 to bind ECM proteins to exert its crosslinking activity, the activation of GPR56 by TG2 was found to require the ECM protein laminin to promote OPC proliferation according to a mechanism leading to the dissociation of GPR56 N- and C-terminal fragments allowing the agonist to initiate G-protein signaling and subsequently RhoA activation. Thus, microglia TG2, extracellular laminin, and OPC GPR56 provide additional evidence of the critical role of microglia/oligodendrocyte communication for developmental oligodendrogenesis (Giera et al., 2015). Remarkably, laminin is also involved in OPC proliferation by promoting the metalloproteinase-mediated cleavage of dystroglycan, one of its OPC surface receptors (Leiton et al., 2015). Moreover, other ECM glycoproteins play a crucial role in myelination as, for instance, anosmin-1 characterized as an important modulator of oligodendrocyte progenitor lineage progression likely *via* FGFR1/ERK1/2 signaling activation, which results in the control of MBP expression, myelin formation and conduction velocity (Murcia-Belmonte et al., 2016). The enhanced differentiation of induced pluripotent stem cells into myelin-expressing oligodendrocytes promoted by the functionalization of culture substrates using brain ECM prepared from decellularized human brain tissue further supports the critical activity of ECM in myelination (Cho et al., 2019).

INFLUENCE OF ASTROCYTES DURING REMYELINATION

Reactive Astrocytes Provide Pro-regenerative Trophic Factors

As in other diseases, astrocytes become reactive after CNS demyelination. Astrocyte reactivity includes hypertrophy of the

cells and upregulation of intermediate filament proteins such as GFAP and vimentin. The requirement of astrocytes for oligodendrocyte-mediated remyelination has been established more than 15 years ago (Blakemore et al., 2003; Talbott et al., 2005) and more recently reported to depend on the presence of androgen hormones in male mice (Bielecki et al., 2016). The delay in astrocyte activation in mice lacking IL-1β and the finding that activated microglia release Il-1β, were the first arguments supporting the idea that microglia activation is a pre-requisite for astrocyte activation. In a consistent manner, microglia-derived IL-1β is able to upregulate CNTF. Added to astrocyte cultures, CNTF induces astrocyte activation (Herx et al., 2000; Albrecht et al., 2003), which promotes fibre myelination *in vitro* (Nash et al., 2011). *In vivo*, astrocytes derived from mice infected with the mouse hepatitis virus, a model for multiple sclerosis (MS), secrete high levels of CNTF during the remyelination phase while CNTF injection in the spinal cord upregulates the transcripts encoding FGF2, a potent OPC mitogen (Albrecht et al., 2003). The loss of astrocytic gp130 receptor, the ubiquitous signal transducer for CNTF, consistently exacerbates both demyelination and the proinflammatory T cell infiltration in the EAE model *via* apoptosis of astrocytes, decrease of CNS regulatory Foxp3-expressing T cells and increase of IL-17–, IFNγ– and TNF-producing T cells. The SHP2/Ras/ERK pathway was then identified as the involved intracellular mechanism (Haroon et al., 2011).

IL-1β also stimulates the astroglial production of LIF promoting survival of oligodendrocytes and thus decreasing disease severity in EAE mice (Butzkueven et al., 2002). In a pathological context where tumor necrosis factor (TNF) is a key component of the inflammatory response, LIF can alternatively be released upon TNFR2-mediated activation of the PI3K-PKB/Akt pathway in primary astrocytes. The selective stimulation of TNFR2 on astrocytes cocultured with OPCs promoted OPC differentiation into mature oligodendrocytes while the process was blocked in the presence of LIF neutralizing antibodies (Fischer et al., 2014). Reactive astrocytes also produce BDNF supporting oligodendrogenesis and regeneration after white matter damage. *In vitro*, conditioned medium from astrocytes restored the process of OPC maturation even under hypoxic stress known to block OPC differentiation unless the medium was specifically treated to remove BDNF. Similarly, *in vivo*, the conditional astroglial deletion of BDNF led to a highly reduced number of newly generated oligodendrocytes and thus to larger white matter damage in animals subjected to prolonged cerebral hypoperfusion (Miyamoto et al., 2015).

The multitude of factors secreted by astrocytes is proposed to induce a rapid change in the lesion environment sensed by OPCs, which thereby become activated. Upon demyelination, OPC activation is associated with a reversion of gene expression profile of adult OPCs towards a profile more closely resembling that of neonatal OPCs. A notable increase in the expression of genes associated with innate immune system functions (including IL1β and CCL2) was identified, as well, together with an increased ability of activated OPCs to migrate and differentiate compared with non-activated ones. Activated OPCs finally express transcription factors such as TCF4 and Sox2, which serve to maintain OPC in the cell cycle and to prime these cells for further differentiation (Moyon et al., 2015; Franklin and Ffrench-Constant, 2017).

Pro-inflammatory Secreted Molecules Associated With Reactive Astrocytes

All the above activities derived from reactive astrocytes are likely involved in the spontaneous regenerative process of remyelination that can occur with remarkable efficiency, not only in experimental models of demyelination but also in humans suffering from MS (Prineas et al., 1993; Patrikios et al., 2006; Patani et al., 2007; Franklin and Ffrench-Constant, 2017). However, in chronic lesions, remyelination mostly fails or occurs only in the periphery of the plaques (Prineas and Connell, 1979; Barkhof et al., 2003; Bramow et al., 2010), where OPC migration and/or differentiation are likely impaired (de Castro et al., 2013; Franklin and Ffrench-Constant, 2017). Such failure has been associated with deleterious activities of astrocytes classically related to a more severe level of reactivity and the secretion of damaging molecules (**Figure 2**). One of these molecules, TNF-α, was mainly detected in fibrous astrocytes at the periphery of chronic active MS lesions and mostly transcribed in highly demyelinated plaques (Hofman et al., 1989; Selmaj et al., 1991; Bitsch et al., 2000). Although it is not yet clear whether TNF-α is internalized or produced by astrocytes (Selmaj et al., 1991; Aranguez et al., 1995), the direct physical contact between astrocytes and oligodendrocytes was proposed to be necessary for oligodendrocyte apoptosis by TNF-α in glial cell cultures. One possibility for contact-dependent cell killing is through gap junctions known to couple astrocytes and oligodendrocytes (Nagy and Rash, 2000; Orthmann-Murphy et al., 2007) and previously suggested for the propagation of cell injury (Lin et al., 1998; Farahani et al., 2005; Froger et al., 2010; Kim et al., 2011).

In the same manner, the knockout of the IFN-γ receptor in astrocytes was found to decrease chemokine expression and inflammatory cell infiltration thus preventing demyelination and consequently lowering clinical signs, namely by alleviating both Th1- and Th17-mediated adoptive EAE (Ding et al., 2015). Together with suggesting an important role for IFN-γ signaling in astrocytes during autoimmune CNS inflammation, this phenotype was consistent with the delay in disease recovery observed in transgenic EAE mice ectopically expressing IFN-γ in astrocytes during the recovery stage and also with the dramatic reduction of oligodendroglial repopulation in the demyelinated lesions of the cuprizone model upon CNS delivery of IFN-γ (Lin et al., 2006). The detection of IFN-γ in astrocytes of active chronic MS plaques and its ability to induce class II major histocompatibility antigen HLA-DR-Ia in astrocytes *in vivo* suggested that IFN-γ may play an important role in the development of MS lesions (Traugott and Lebon, 1988; Hashioka et al., 2009).

Another cytokine secreted by astrocytes is CXCL10 that is namely detectable around active MS lesions (Ransohoff et al., 1993; Omari et al., 2005; Carter et al., 2007). Its production increases before the onset of clinical

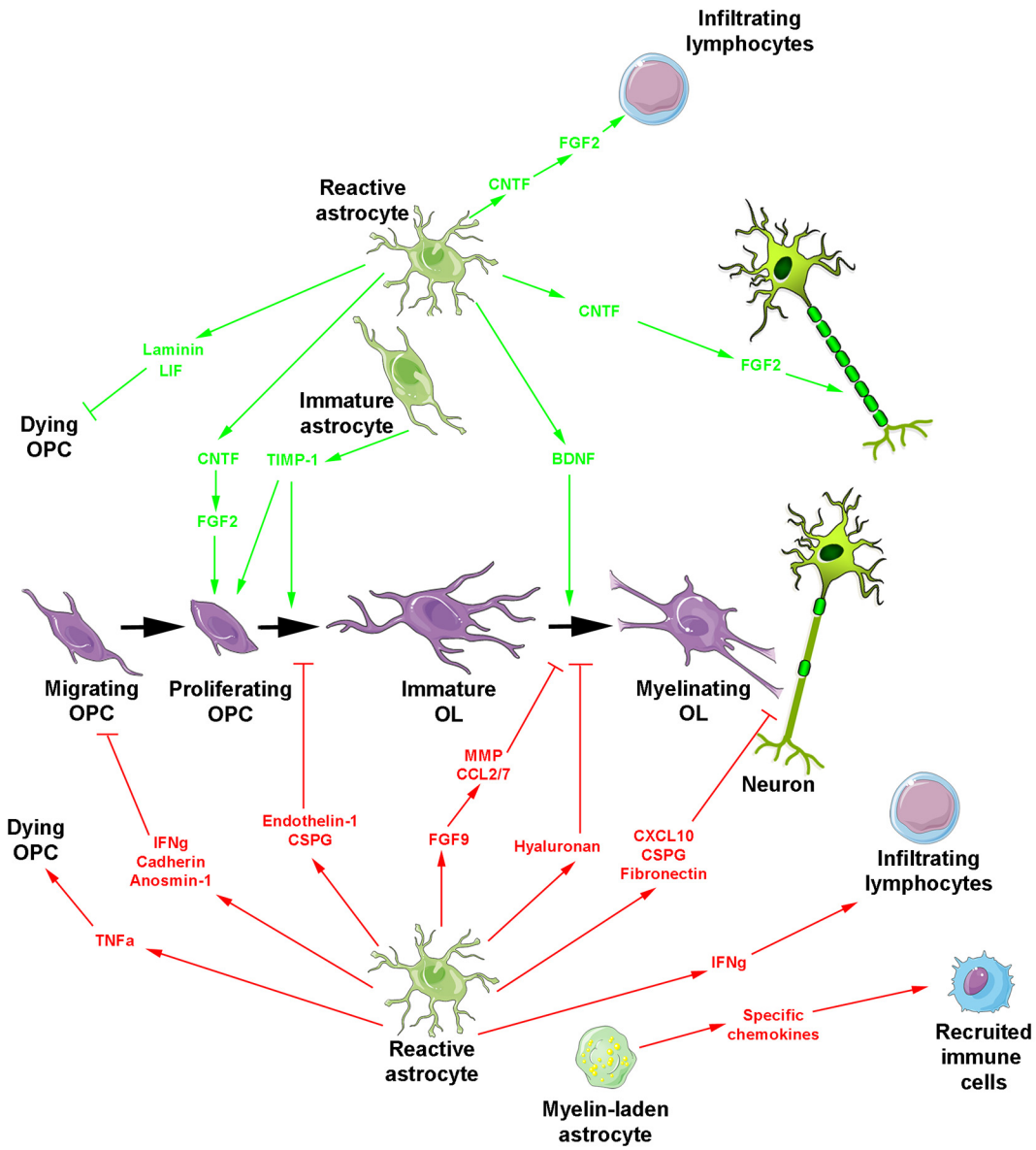

FIGURE 2 | Reactive astrocyte-derived secretion products during myelin repair. The oligodendroglial lineage is shown in purple. Reactive astrocytes generate molecules promoting (top, green arrows) or blocking (bottom, red arrows and blocking symbols) myelin regeneration. Besides reactive astrocytes, two additional forms of astrocytes are indicated including myelin-laden astrocytes endowed with a deleterious effect under inflammatory conditions and transplanted immature astrocytes which promote OPC proliferation/differentiation and additionally prevent glial scar formation.

symptoms, maintain at high levels during the peak of the disease and decreases during remission in the EAE model (Glabinski et al., 1997; Fife et al., 2001). *In vitro*, CXCL10 is upregulated in an astrocyte phenotype endowed with an inhibitory role in glial/axonal ensheathment without impairment of OPC proliferation/differentiation or process extension (Nash et al., 2011).

Among the other molecules highly expressed by reactive astrocytes, endothelin-1 also stands as a strong inhibitor of remyelination. This neuropeptide acts by promoting Notch activation in OPCs through induction of Jagged1 expression in reactive astrocytes (Hammond et al., 2014). In the same line, lesion formation was reported to be associated with increased expression of fibroblast growth factor 9 (FGF9) by astrocytes acting not *via* a direct effect on the oligodendroglial lineage, but *via* an off-target effect mediated by soluble astrocyte-derived factors inhibiting terminal differentiation of myelinating oligodendrocytes. *In vitro*, this activity is associated

with the appearance of multi-branched "pre-myelinating" oligodendrocytes that extend processes able to interact with axons without forming any myelin sheaths, reminiscent of the oligodendrocyte phenotype observed in chronically demyelinated MS lesions. Astrocyte-derived factors included metalloproteases associated with ECM remodeling and the pro-inflammatory chemokines CCL2 and CCL7 that contribute to the development of inflammatory responses in the CNS (Lindner et al., 2015).

Reactive Astrocytes Secrete Extracellular Matrix Deleterious for Remyelination

Apart from cytokines, growth factors and neuropeptides, ECM molecules are also secreted by reactive astrocytes and highly modify the lesion environment in MS, consequently influencing the behavior of OPCs (Maier et al., 2005; van Horssen et al., 2005, 2006; Clemente et al., 2011; Pu et al., 2018). Both the receptors present at the surface of OPCs and the ECM molecules secreted by reactive astrocytes are susceptible to change the environment from permissive to inhibitory for remyelination (de Castro et al., 2013). Indeed, except the interaction reported between astroglial laminin and oligodendroglial α6 β1 integrin shown to attenuate oligodendrocyte death *in vitro* (Corley et al., 2001), ECM accumulation appears as an important factor in tissue regeneration failure. For instance, the expression of N-cadherin on the surface of both oligodendrocytes and astrocytes led to the first *in vitro* evidence that this molecule was in part responsible for the poor migration-promoting properties induced by astrocytes on oligodendrocytes suggesting that the inhibition of cell-cell adhesion could improve repopulation of MS lesions by OPCs. Indeed, the blocking of cadherin function by specific peptides reduced adhesion of oligodendroglia to astrocyte monolayers, diminished the duration of the contact between oligodendrocyte processes and individual astrocytes, and increased the migration of oligodendrocytes on astrocyte monolayers (Schnadelbach et al., 2000). In the same line, astrocytes in culture produce fibronectin as fibril-like structures in inflammatory conditions, known to inhibit myelin formation in different experimental paradigms (Sisková et al., 2009). In agreement with this hypothesis, fibronectin rapidly accumulates as an acute response to demyelination in MS and disappears during remyelination, whereas astrocyte-released aggregated fibronectin persists in chronic lesions (Stoffels et al., 2013). Similarly, the ECM-associated glycoprotein anosmin-1 can also be detected in the core of chronic active and inactive MS plaques where it is suggested to prevent OPC colonization rather than inhibit their differentiation *via* a mechanism possibly involving the FGF-2 receptor FGFR1 consistently detected in the periplaque of chronic lesions (Bribián et al., 2006, 2008; Clemente et al., 2011).

Major CNS ECM proteoglycans have also been characterized in MS lesions. In active plaque edges, the chondroitin sulfate proteoglycans (CSPG) versican, aggrecan, neurocan and the dermatan sulfate proteoglycan increase in correlation with astrocytosis. In contrast, these molecules accumulate in foamy macrophages in active plaque centers, suggesting their engulfment together with myelin. In inactive lesions and normal-appearing white matter, proteoglycans are decreased and display undetectable abnormal heterogeneous aggregation, respectively (Sobel and Ahmed, 2001). The roles of CSPG during development in particular in myelination and the different localizations of CSPG according to the type of MS lesions both support the idea that these molecules comprising part of the astrogliotic scar, are critical in inhibition of OPC processes outgrowth and OPC differentiation (Siebert and Osterhout, 2011). The hypothesis is consistent with the recent design of a novel CSPG synthesis inhibitor reducing CSPG deposition into the lesion microenvironment and found to rescue OPC process outgrowth *in vitro* and to accelerate remyelination following focal demyelination in mice (Keough et al., 2016). Moreover, the glycosaminoglycan hyaluronan characterized by its ability to bind CSPG is deposited in early MS lesions in a low molecular weight form by lymphocytes and microglia / macrophages whereas it is deposited by astrocytes in a higher molecular weight form in chronic lesions. There, hyaluronan degraded by hyaluronidases expressed by OPCs inhibits OPC maturation and thus remyelination (Back et al., 2005) through a mechanism requiring the Toll-like receptor 2 expressed by oligodendrocytes and upregulated in MS lesions (Sloane et al., 2010). Evaluated in the Theiler's murine encephalomyelitis model, the spatio-temporal course of ECM alterations confirmed the correlation of matrix accumulation with astrogliosis still supporting a mainly astrocytic origin of ECM deposits. The data led to propose disturbed aggregation or post-translational modifications of matrix molecules leading to impairment of their regular degradation rather than changes in their transcription level (Haist et al., 2012).

The Dual Activity of Astrocytes in the Demyelinated Tissue

The dual activity of astrocytes described above not only reflects more or less severe levels of astrocyte reactivity, but also clearly reminds the duality that characterizes the glial scar itself, which is considered as a rearrangement of tissue structure, astrocyte proliferation, and pronounced overlap of astrocyte processes resulting in the disruption of individual astrocyte domains (Zamanian et al., 2012; Anderson et al., 2016; Adams and Gallo, 2018). While traditionally viewed as a barrier to axon regeneration, the glial scar has been nevertheless reported to be beneficial for restricting leukocyte migration outside the damaged tissue (Faulkner et al., 2004; Okada et al., 2006; Herrmann et al., 2008; Voskuhl et al., 2009). Moreover, in contrast to the initial dogma, its deleterious effect on axon regeneration was recently questioned. Indeed, genetically targeted loss-of-function approaches leading to the prevention of astrocyte scar formation, attenuation of scar-forming astrocytes or deletion of chronic astrocyte scars, failed to result in spontaneous regrowth of transected CNS axons following spinal cord injury lesions. These data were corroborated by results of RNA sequencing revealing the expression of multiple axon-growth supporting molecules namely in astrocytes (Anderson et al., 2016).

Astrocyte ability to exhibit different phenotypes participates in this heterogeneity (Zamanian et al., 2012; Liddelow and Barres, 2017). Indeed, despite a core set of genes up-regulated in different injury models such as ischemic stroke and neuroinflammation, 50% of altered gene expression is specific to a given injury. The first transcriptomic analyses provided evidence for the existence of two subtypes of reactive astrocytes (Zamanian et al., 2012) afterwards named "A1" and "A2." A1 astrocytes induced by classically activated neuroinflammatory microglia following exposure to lipopolysaccharide have been described as harmful by inhibiting OPC proliferation and differentiation. A2 astrocytes identified during the regeneration stage in an ischemia model, were found to up-regulate genes thought to be protective. In line with this observation, analysis of MS lesions reported the detection of A1 mostly in the active lesions whereas A2 was found during remyelination (Haindl et al., 2019). However, it may be more likely that the occurrence of different astrocytic phenotypes and the existence of suitable levels of various factors expressed at particular time points may achieve successful repair. In agreement with this hypothesis, investigation of the astrocytic reaction at various steps of lesion progression in the rat EAE model led to detect simultaneously in shadow plaques all features of a glial scar and densities of OPCs and mature oligodendrocytes, which were quite comparable to the densities observed in unaffected white matter (Haindl et al., 2019).

The first line response of astrocytes to myelin injury consisting of myelin phagocytosis was similarly considered to be either beneficial or detrimental to the lesion pathology, depending on the inflammatory context. Indeed, although myelin debris phagocytosis was mainly reported to be performed by microglia/macrophages, myelin-positive hypertrophic astrocytes can be observed at sites of acute myelin breakdown. The uptake was proposed to rely on receptor-mediated endocytosis and to result in astroglial NF-kB activation and secretion of specific chemokines. The latter leads to the recruitment of immune cells shown *in vitro* in rodents and validated in human disease. Although microglia and monocyte recruitment may be beneficial for myelin clearance in a non-inflammatory environment, it increases tissue damage in demyelinating conditions driven by inflammation such as MS since additional recruitment of lymphocytes and microglia/macrophages exacerbates the inflammatory process (Ponath et al., 2017).

Finally, the finding that transplanted immature astrocytes are unable to become reactive after CNS injury (Jiang et al., 2013) and that only immature (but not mature) astrocytes are neuroprotective and suppress endogenous astrocyte activation and glial scar formation presently stands as an interesting novel concept (Chen et al., 2015). Thus, transplanted human pluripotent stem cell-derived astrocytes at a defined immature stage were shown to regulate the differentiation of endogenous OPCs, to promote myelinogenesis and to improve behavioral outcome in the model of periventricular leukomalacia in neonate mice *via* a mechanism depending on the tissue inhibitor of metalloproteinases TIMP-1 (Jiang et al., 2016).

Key Studies Supporting Detrimental Roles of Astrocytes on Oligodendrocytes and Myelin

Several publications using astrocyte ablation have improved our knowledge about the main detrimental roles of reactive astrocytes in the context of CNS demyelination. For instance, OPC transplantation in ethidium bromide-demyelinated animals in the presence or absence of astrocytes indicated that astrocyte-free regions favor Schwann cell differentiation whereas the presence of astrocytes delayed the interaction of OPCs with the demyelinated axons (Blakemore et al., 2003). Similarly, the depletion of astrocytes *via* intracallosal injection of La-aminoadipate in cuprizone-treated animals revealed a notable increase in the percentage of myelinated areas, decrease in Iba-1$^+$ microglia staining and collapse in the expression of genes related to either recruitment of microglia classically triggered by astrocytes or suppression of OPC differentiation (Madadi et al., 2019). The considerable amount of fibronectin produced by astrocytes and the ability of astrocytic fibronectin to become aggregated after treatment with lipopolysaccharide led to conclude to the deleterious effects of such aggregates on oligodendrocyte differentiation and myelin regeneration *in vivo*, in agreement with the detection of a low level of fibronectin aggregates in remyelinated MS lesions (Stoffels et al., 2013). The signaling molecule endothelin-1 (ET-1) expressed by reactive astrocytes in MS and murine demyelinated lesions is a negative regulator of OPC differentiation and remyelination acting by promoting Notch activation in OPCs during remyelination (Hammond et al., 2014).

Moreover, the CCL2 chemokine produced by astrocytes plays an important role in the continued recruitment of immune cells and the activation of glial cells in the CNS during chronic EAE (Kim et al., 2014). Similarly, the adhesion molecule VCAM-1 expressed by astrocytes is essential for T cell entry into the CNS parenchyma from EAE animals (Gimenez et al., 2004). As previously mentioned, myelin uptake is an early response of astrocytes in diseases with prominent myelin injury that results in the recruitment of immune cells possibly increasing tissue damage in demyelinating conditions driven by inflammation (Ponath et al., 2017). In the same line, astrocytes constitute one of the cell types that have the capacity to express molecules required for antigen presentation under inflammatory conditions required for T cell reactivation targeting the myelin sheath-forming oligodendrocytes (Waisman and Johann, 2018). Finally, although in the healthy brain, perivascular astrocyte end-feet closely ensheath the microvasculature, activation of perivascular astrocytes may alter their involvement in the blood-brain barrier formation as suggested by the damage of perivascular end-feet reported in MS patients. The revisit of this observation in the EAE model showed that reactive astrocytes detach from the blood vessels and lose their contacts with both blood vessels and neuronal synapses, which was proposed to contribute to the neurological impairment and the cognitive decline occurring in EAE/MS as well as to the neurodegenerative disease progression (Eilam et al., 2018).

MICROGLIA IN REMYELINATION

In the context of myelin repair, microglia and their hematopoietic cell-derived counterparts, macrophages, both contribute to the regenerative process through key effects including the phagocytosis of myelin debris, the secretion of cytokines, chemokines, growth factors or soluble mediators and the recruitment/differentiation of progenitors at the lesion site. Although microglia and macrophages can now be distinguished by using a few specific markers (Bennett et al., 2018), they have been indistinguishable for a long time. Therefore, "microglia/macrophages" will be used for most presently available data.

Microglia/Macrophage Phenotypes Upon CNS Demyelination

As resident immune cells of the CNS, microglia/macrophages become activated as soon as they detect any insult. This activation implicates changes in morphology and transcriptional profiles of the cells. The failure of microglia to convert from a "resting state" with a branched-like morphology to an "activated state" with an amoeboid-like morphology was namely observed upon knocking down either the β-galactoside-binding lectin Galectin-3 (Reichert and Rotshenker, 2019) or one of the receptors of the secreted proteins Sonic hedgehog, the protein Boc (Zakaria et al., 2019).

Like astrocytes, microglia/macrophages are endowed with a dual activity supported by the existence of different phenotypes observed under various *in vitro* conditions and that have been named, respectively the classical activated state (M1) associated with pro-inflammatory activities and the alternative polarized state (M2) involved in OPC recruitment and differentiation associated with anti-inflammatory activities (**Figure 3A**). The characterization of these phenotypes performed in the model of focal demyelination induced by stereotaxic injection of lysolecithin into the corpus callosum led to the identification of a few specific markers namely including iNos, TNF, CD16/CD32 for the classical activated state and Arg1, IGF1 and CD206 for the alternative state. The switch from an M1- to an M2-dominant response was detected in microglia/macrophages at the initiation of remyelination (Miron et al., 2013). It was more recently characterized as a process requiring necroptosis of pro-inflammatory microglia and subsequent repopulation to a pro-regenerative state (Lloyd et al., 2019). *In vitro* experiments revealed the ability of M2 cell conditioned media to promote OPC differentiation whereas the depletion of M2 cells in focal demyelinating lesions prevented OPC differentiation. Also, parabiosis experiments detected a higher number of M2 cells in lesions of aged mice in which remyelination was increased by coupling to a younger mouse. Finally, the blockade of activin-A secreted by M2 cells fully prevented OPC differentiation in demyelinated cerebellar slice cultures (Miron et al., 2013).

Microglia/macrophage activation cannot be nevertheless regarded as a simple dichotomy of M1 or M2, but undoubtedly as a much broader reactive phenotype spectrum. In agreement with this observation, in the Theiler's induced demyelinating disease model, the endogenous cannabinoid 2-AG induces pro-phagocytic response of microglia and OPC differentiation, without any detectable regulation of the main markers used to identify classic and alternatively activated microglia, respectively (Mecha et al., 2019). In the same line, myelin debris was consistently proposed to promote the emergence of a novel phenotype that differs from conventional M1 and M2. Indeed, myelin-induced macrophages appear foamy, displaying a striking deficiency of the expression of the ATP-binding cassette transporter ABCA1 required for lipid efflux in response to myelin debris loading, thus suggesting that this homeostatic mechanism characterizing non-myelin-induced macrophages is overwhelmed. The impaired capacity of foamy macrophages for apoptotic and necrotic cell clearance is consistent with this hypothesis and likely contributes to the development of secondary injury (Wang X. et al., 2015).

In agreement with this observation, a growing number of recent publications support the existence of a wide spectrum of microglia activation states evidenced by single-cell and bulk RNA sequencing approaches characterizing this process not as a simple phenotypical change but as a dynamic response involving spatially and transcriptionally distinct subpopulations. High-dimensional single-cell mapping in EAE mouse CNS led to a unique activation profile comprising universal markers of microglia/macrophage activation including CD44, CD86, programmed death-ligand 1 (PDL1) as well as a decreased CD14 expression and increased major histocompatibility complex II (MHC II) and stem cell antigen-1 (Sca-1) expressions notably not detected in old mice or Alzheimer's disease models (Mrdjen et al., 2018). Single-cell RNA sequencing of microglia from LPC-mediated lesions revealed multiple gene expression profile clusters of activated microglia/macrophages sharing common genes such as apoE and unique genes such as Ccl4 or Cxcl10 according to the clusters (Hammond et al., 2019). Multiple activation states specifically reflecting microglia (and not macrophages) was also identified upon LPC-mediated demyelination suggesting that microglia can have multiple forms of activation likely identified by common or selective transcriptional programs (Plemel et al., 2020). Similarly, a specific ApoE-dependent molecular signature was identified in microglia/macrophages from different models of neurodegenerative diseases including EAE (Krasemann et al., 2017). Strikingly, one of these subsets displayed an expression profile comparable to the one found in MS microglia (Zrzavy et al., 2017). Real-time *in vivo* imaging of microglia/macrophages in EAE mice visualized the expected switch from pro-inflammatory to immuno-regulatory markers mostly in initial lesions with an increase during lesion resolution (Locatelli et al., 2018). Consistently, in actively demyelinating lesions from MS patients, some microglia/macrophages express pro-inflammatory markers while active lesions prone to re-myelinate comprise microglia/macrophages expressing anti-inflammatory markers at a higher level than chronic inactive lesions (Miron et al., 2013; Zrzavy et al., 2017).

Factors Controlling the Pro-regenerative Microglia/Macrophages

Recently, the subpopulation of CD11c-expressing microglia previously reported to be transiently expanded soon after birth

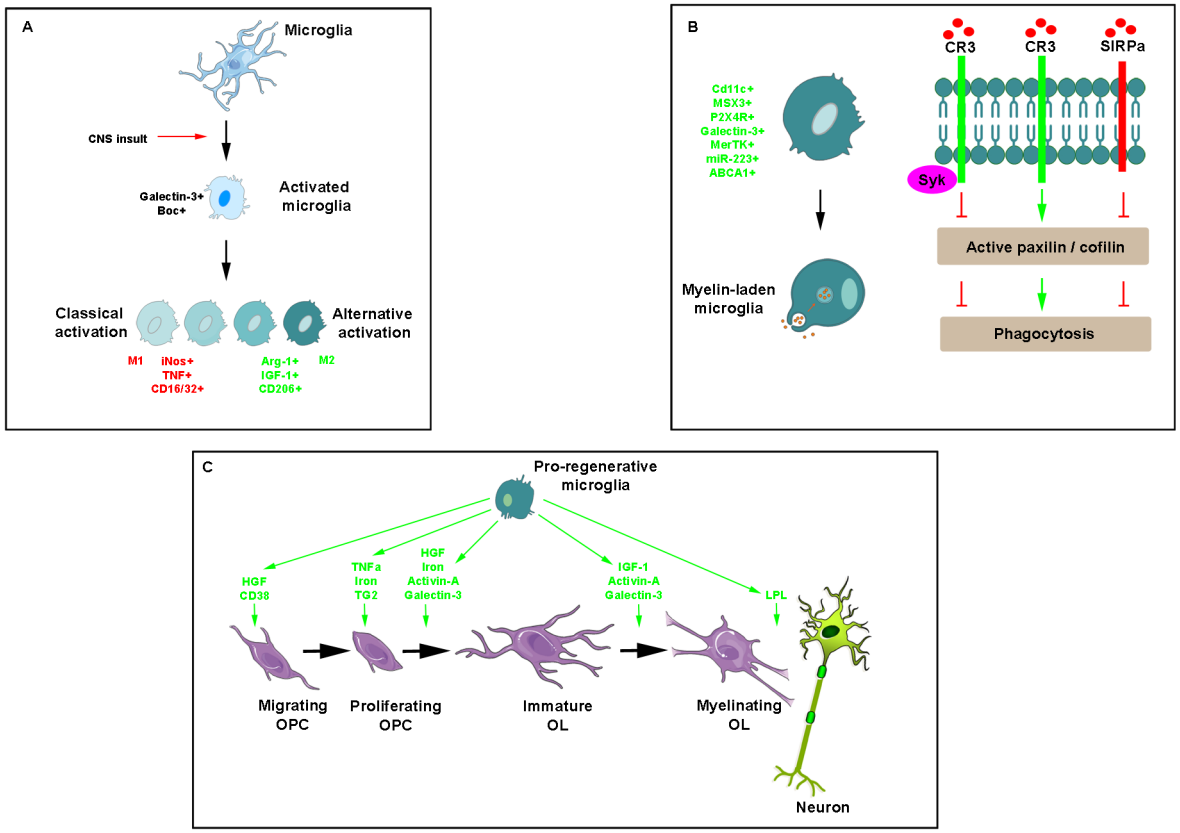

FIGURE 3 | Key effects of microglia/macrophages during myelin repair. **(A)** Illustration of morphological modifications of ramified microglia/macrophages, which adopt a rhomboid shape in the presence of any insult and become activated into a classical or alternative manner giving rise to a range of cell phenotypes expressing mostly pro- (red) or anti-inflammatory (green) molecules, respectively. **(B)** Phagocytosis of myelin debris is a critical activity of microglia/macrophages for promoting myelin regeneration. Phagocytic receptors such as CR3 advance phagocytosis by promoting the activation of paxillin and cofilin whereas the immune inhibitory receptor SIRPα, inhibits phagocytosis by promoting the inactivation of the two. Recruited by CR3, Syk downregulates CR3-mediated myelin phagocytosis protecting phagocytes from excessive phagocytosis during prolonged exposure to debris. **(C)** Pro-regenerative microglia secrete a range of molecules intervening at various steps of myelin repair.

and critical for primary myelination, was found to be remarkably increased in EAE and cuprizone models of demyelination. The expansion of this subpopulation is induced by the stimulation of CSF1R, the key transducer of CSF1 and interleukin (IL)-34 signals, essential in myeloid cell development. CSF1R activation induces the expression of the chemokine CCL2, itself leading to a dramatic increase of $CD11c^{+}$ microglia, finally resulting in improvement of EAE symptoms and lower levels of demyelination (Wlodarczyk et al., 2018). Among factors implicated in the promotion of the remyelinating phenotype of microglia, the homeobox gene Msh-like homeobox-3 (MSX3) is a pivotal regulator for microglial polarization that is induced and repressed in M2 and M1 cells, respectively. This finding is supported by the dynamic regulation of MSX3 in the EAE model as well as by the beneficial and deleterious effects observed after MSX3 gain- and loss-of-function experiments in different demyelination models. Pparg, Stat6, and Jak3 are key genes regulated by MSX3 (Yu et al., 2015). In the same line, pro-regenerative microglia express a high level of the purinergic receptor P2X4R at the peak of recovery in the EAE model. P2X4R activation increases and decreases pro-regenerative and pro-inflammatory genes, respectively, whereas its blockade prevents myelin debris clearance (Zabala et al., 2018).

The Critical Role of Microglia/Macrophages in Myelin Phagocytosis

The ability of microglia/macrophages to phagocyte myelin debris is critical for promoting OPC recruitment and differentiation into myelinating cells (Triarhou and Herndon, 1985; Kotter et al., 2001, 2005, 2006; Neumann et al., 2009). The main phagocytic receptor in microglia/macrophages is the complement receptor-3 (CR3). Binding of myelin to CR3 initiates structural changes characteristic of phagocytosis mostly reflected by filopodia-like membrane protrusions that engulf myelin as they extend and then pull myelin into phagocytes as they retract. Filopodia production depends on F-actin remodeling, which is promoted by active unphosphorylated cofilin and impeded by inactive phosphorylated cofilin. Spleen tyrosine

kinase (Syk), the non-receptor tyrosine kinase known to be recruited by phagocytic receptors including CR3, downregulates CR3-mediated myelin phagocytosis by increasing the inactive state of cofilin. This self-negative control of phagocytosis is useful in protecting phagocytes from excessive phagocytosis during prolonged exposure to particles that are fated to ingestion (Hadas et al., 2012). Remarkably, myelin debris can inhibit its clearance by using a protective mechanism whereby CD47 protein expressed by myelin debris binds the immune inhibitory receptor signal regulatory protein-α (SIRPα) on the surface of phagocytes (**Figure 3B**). The mechanism supporting such detrimental protection involves the regulators of cytoskeleton function paxillin and cofilin, which are both either activated by phagocytic receptors or inactivated by the immune inhibitory SIRPα (Gitik et al., 2014). The finding that expression of Galectin-3 correlates with myelin-debris phagocytosis in microglia led to show its implication in phagocytosis activation first by advancing cofilin activation, causing filopodia/lamellipodia to extend/engulf myelin-debris and, second, by advancing actin/myosin-based contraction through K-Ras.GTP/PI3K signaling, causing filopodia/lamellipodia to retract and thus internalize myelin debris (Reichert and Rotshenker, 2019).

The retinoid X receptor (RXR) plays an important role in monocyte/macrophage phagocytosis of myelin as suggested by pioglitazone-induced activation of peroxisome proliferator-activated receptor c, one of the permissive binding partners of RXR, shown to inhibit pro-inflammatory differentiation of MS patient-derived monocytes/macrophages and to enhance myelin phagocytosis (Natrajan et al., 2015b). Moreover, the blockade of myelin debris clearance by microglia/macrophages in cuprizone-demyelinated CX3CR1-deficient mice, revealed the critical role played by CX3CR1, the receptor controlling microglial physiology and orchestrating the crosstalk between microglia and neurons (Lampron et al., 2015). Moreover, macrophages lacking miR-223 expression have impaired ability to polarize towards the M2 phenotype thus altering myelin debris clearance and remyelination in the lysolecithin-demyelinated corpus callosum (Galloway et al., 2019). Interestingly, the use of human monocyte-derived macrophages and microglia led to demonstrate that myelin phagocytosis is significantly enhanced in cells exposed to TGF-β compared with resting basal conditions whereas it is markedly reduced in classically activated polarized cells. The transcriptional analysis of TGF-β–treated microglia revealed the tyrosine kinase receptor MerTK as one of the most upregulated among differentially expressed genes. In contrast, the enzyme and its ligands (growth arrest-specific 6 and Protein S) are down-regulated in classically activated cells (Healy et al., 2016).

Recent publications identified novel signaling pathways aimed at restoring homeostatic microglial phagocytosis in the aging CNS. Indeed, old mice fail to resolve the inflammatory response initiated after myelin damage. This observation was related to the accumulation of excessive amounts of myelin debris by aged phagocytes, which trigger cholesterol crystal formation and phagolysosomal membrane rupture, thus stimulating inflammasomes. However, stimulation of cholesterol efflux and solubility by administering 2-hydroxypropyl-β-cyclodextrin in old demyelinated mice knockout for the major cholesterol transporter ApoE was sufficient to restore the capacity to re-myelinate lesioned tissue (Cantuti-Castelvetri et al., 2018).

Combined CRISPR-Cas9 knockout screens together with RNA-seq led to discover age-related genetic modifiers of microglial phagocytosis such as the canonical B-cell receptor CD22, a negative regulator of phagocytosis that is upregulated on aged microglia. CD22 mediates the anti-phagocytic effect of α2–6-linked sialic acid. Its inhibition promotes the clearance of myelin debris *in vivo* and reprograms microglia towards a homeostatic transcriptional state leading to improvement of cognitive function in aged mice (Pluvinage et al., 2019). By up-regulating the expression of the scavenger receptor CD36, niacin (also called vitamin B3) induces myelin phagocytosis through binding its receptor, hydroxycarboxylic acid receptor 2, both *in vitro* and *in vivo* (Rawji et al., 2020).

Pro-regenerative Microglia/Macrophage-Derived Factors

Many cytokines, growth factors, and soluble factors are known to be secreted by microglia/macrophages (**Figure 3C**). Although induced by IL1β characterized as a pro-inflammatory cytokine (Mason et al., 2001), microglia/macrophage-derived IGF-1 enhances remyelination as suggested by EAE rats receiving IGF-1 subcutaneously (Yao et al., 1996) and in lysolecithin-demyelinated spinal cord of young and aged rats intrathecally infused with the growth factor (Hlavica et al., 2017). TNFα through its TNFR2 receptor promotes remyelination by increasing OPC proliferation as shown by using mouse strains knockout for the ligand or its receptor as well as by their up-regulation during the remyelination stage in the cuprizone model (Arnett et al., 2001). T helper cell-derived TGF-β induces the secretion of microglial hepatocyte growth factor (HGF), which drives OPC chemotaxis and differentiation. In agreement with this finding, spinal cord lesions from relapsing-remitting EAE mice contain both OPC and HGF-producing microglia/macrophages in the recovery-, but not in the acute phase (Lalive et al., 2005). Highly expressed in microglia, the β-galactoside-binding lectin, galectin-3, has a critical role in driving OPC differentiation and myelination in agreement with the oligodendrocyte maturation effects displayed by supernatants derived from galectin-3-expressing- but not galectin-3-deficient microglia (Pasquini et al., 2011).

Microglia/macrophages also express activin-A at onset of remyelination of focally-induced demyelination. This growth factor regulates oligodendrocyte differentiation and myelin compaction *via* the activin receptor subtype Acvr2a up-regulated during efficient myelin regeneration, in contrast to the other receptor subtype Acvr2b, which has to be down-regulated for allowing oligodendrocyte differentiation. In actively remyelinating areas of MS tissue, oligodendrocyte lineage cells expressing Acvr2a consistently outnumber cells expressing Acvr2b suggesting that an increase in Acvr2b expression may impair oligodendrocyte differentiation and myelin formation induced by Acvr2a (Dillenburg et al., 2018).

Finally, microglia is the main iron source in the form of ferritin. The latter is required for OPC proliferation and differentiation and stands as an essential cofactor for enzymes involved in myelin cholesterol and fatty acid synthesis (Schonberg et al., 2012). The cellular iron level in microglia/macrophages is controlled by hepcidin and ferroportin. The transcription of the former is regulated by the BMP/Smad and IL-6/Jak-STAT3 signaling pathways during inflammation resulting in the degradation of the only known iron exporter ferroportin, subsequently increasing intracellular iron level. Recently, the infusion of the BMP antagonist noggin in a model of ischemic stroke decreased the induction of hepcidin and ferritin proteins whereas it increased the number of myelinated axons and myelin thickness (Shin et al., 2018).

Several microglial enzymes are also clearly implicated in remyelination. As shown in development, microglia-derived TG2 signals to GPR56 on OPCs in the presence of the ECM protein laminin promote OPC proliferation and improves remyelination *in vivo* in the cuprizone model and *ex vivo* in cerebellar slices exposed to lysolecithin (Giera et al., 2015). Lipoprotein-lipase involved in lipid-processing, which plays an important role during initiation of remyelination and is considered as a feature of alternatively-activated microglia, is significantly increased in the EAE model when clinical symptoms start to decrease (Bruce et al., 2018; Kamermans et al., 2019). Finally, CD38 that catalyzes the synthesis of cyclic adenosine diphosphate-ribose (cADPR) from nicotinamide adenine dinucleotide (NAD^+), is increased in the cuprizone model of demyelination in both astrocytes and microglia and may be required for myelin clearance and oligodendrocyte repopulation (Roboon et al., 2019).

Recently, extracellular vesicles produced *in vitro* by either pro-inflammatory- or pro-regenerative microglia were proposed to be one of the mechanisms used to promote ou block myelin repair. Remarkably, exposure of cultured OPCs to inflammatory vesicles blocked OPC maturation only in the presence of astrocytes, thus implicating the latter in remyelination failure *via* a mechanism involving astrocyte conversion into deleterious cells. Moreover, biochemical fractionation revealed that the inflammatory cargo of the pro-inflammatory vesicles mainly contribute to the blockade of OPC maturation whereas surface lipid components may be mostly involved in extracellular vesicle-mediated migration and/or differentiation of OPCs likely implicating sphingosine 1 phosphate at least for OPC migration (Lombardi et al., 2019).

Therapeutic Modulation of Microglia/Macrophages

Major improvement of our knowledge of the role of microglia/macrophages during remyelination recently led to new therapeutic perspectives. One of the first papers providing evidence that increased recruitment of microglia/macrophages can support remyelination, regarded the anti-fungal amphotericin B and its association with macrophage colony-stimulating factor (M-CSF). Although using amphotericin B is precluded by its ability to stimulate TNF secretion and its toxic properties (Doring et al., 2015), the increased uptake of myelin debris induced by M-CSF in the cuprizone model remains interesting (Laflamme et al., 2018). The adoptive transfer of bone-marrow-derived M2 macrophages results in a shift of the immunological response from helper T1 to helper T2 lymphocytes through the production of anti-inflammatory cytokines, which in turn induces the polarization of microglia/macrophages to the M2 phenotype in a model of spinal cord injury (Ma et al., 2015). Preventive IL-13 gene therapy in the cuprizone model directs the polarization of microglia and infiltrating macrophages towards an alternatively activated phenotype, thereby limiting lesion severity and improving disease outcome (Guglielmetti et al., 2016).

Several molecules modulating microglia/macrophages are presently trialed in patients. For instance, the anti-muscarinic molecule Clemastine, modulates macrophage inflammatory responses *in vitro* and microglia/macrophage activation *in vivo* (Mei et al., 2014). Bexarotene, an agonist of the RXR pathway, has been identified as a positive regulator of myelin debris clearance and a key player in the age-related decline in remyelination acting *via* a yet undetermined direct or indirect effect (Natrajan et al., 2015a). The antipsychotic quetiapine fumarate promotes remyelination *in vivo* together with attenuating microglial responses to inflammatory stimuli by decreasing TNF and nitric oxide secretion and preventing activation of NF-κB (Wang H. et al., 2015).

New molecules are continuously being identified. Among them, a recombinant version of a naturally occurring human IgM, rHIgM22, has been shown to promote remyelination in Theiler's virus infection and cuprizone animal models and to stimulate myelin phagocytosis in a dose-dependent manner (Zorina et al., 2018). The CSF1R kinase inhibitor BLZ945 prophylactically and therapeutically prevents demyelination in the cuprizone model, namely in the corpus callosum. However, it increases myelin debris and axonal damage in other fiber tracts reminding the phenotype observed in cuprizone-treated TREM2 knock-out mice (Beckmann et al., 2018; Wies Mancini et al., 2019). Although TNF is a master pro-inflammatory product of activated microglia/macrophages implicated in CNS demyelination, the blockade of its soluble form did not prevent cuprizone-induced oligodendrocyte loss and demyelination, but led to efficient early remyelination due to improved phagocytosis of myelin debris and resolution of microglia/macrophage activation (Karamita et al., 2017). Finally, the allosteric modulator of P2X4R, Ivermectin, mimics P2X4R activation and improved motor function, electrical nerve conductance, myelin debris phagocytosis and remyelination in the EAE model (Zabala et al., 2018).

Key Studies Supporting Detrimental Roles of Microglia/Macrophages on Oligodendrocytes and Myelin

Besides the growing number of data supporting the beneficial consequences of promoting the pro-regenerative phenotype of microglia/macrophages, several critical works support the deleterious effects of microglia activated through the classical way. First, activated microglia synthesize a multitude of

cytokines, chemokines, cell adhesion glycoproteins or reactive oxygen radicals able to damage axons, myelin, oligodendrocytes (Minghetti and Levi, 1998; Raivich and Banati, 2004) and therefore involved in the initiation and propagation of the inflammatory cascade, which promotes demyelination in neural disorders (Perry et al., 2010). Consistently, LPS-activated microglia, polarized to pro-inflammatory status secrete tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), both known to be cytotoxic for oligodendrocytes (Selmaj and Raine, 1988). TNFα and cuprizone supplementation to rat primary cultures of oligodendrocytes consistently decreased cell viability (Pasquini et al., 2007). Moreover, a minocycline-mediated blockade of microglial activation in cuprizone-treated mice prevented demyelination while a positive correlation was shown between the production of nitric oxide and oligodendrocyte death (Merrill et al., 1993). Finally, LPS-induced secretion of the stress chaperone protein, heat shock protein 60 (HSP60), was found to initiate OPC apoptosis (Li et al., 2017).

As competent presenters of antigen, microglia activated in a classical way express molecules for antigen presentation such as MHC II and its co-stimulatory factors CD40 and CD86, characterized as classical markers of microglia activation in MS and Alzheimer's disease patients (Gobin et al., 2001; Lehmann et al., 2001; Höftberger et al., 2004). Remarkably, activated microglia/macrophage is also highly proliferative in both MS and animal models of demyelination mostly in actively demyelinating areas at the early stages of the disease but not in remyelinating lesions again attesting the major role of classically activated microglia/macrophages during the early stages of demyelinating diseases (Matsumoto et al., 1992; Schönrock et al., 1998; Ponomarev et al., 2005). Early activation of microglia is similarly observed in ischemic dementia models as shown by the increased expression of MHC-I/II or matrix metalloprotease-2 (MMP-2) 3 days after hypoperfusion whereas administration of the natural dipeptide carnosine (β-alanyl-L-histidine) able to decrease microglial activation improved cognitive function and white matter lesions (Wakita et al., 1994; Ihara et al., 2001). Finally, as previously mentioned, extracellular vesicles released by pro-inflammatory microglia block remyelination *via* a mechanism depending on the presence of astrocytes converted into harmful cells by the inflammatory extracellular vesicle cargo (Lombardi et al., 2019).

In summary, the majority of experimental evidence reported in this review, points to astrocytes and microglia/macrophages as being crucial in both developmental and repairing oligodendrogenesis and myelination. During development, astrocytes and a unique phenotype of neonatal microglia secrete different patterns of molecules controlling oligodendrocyte development and myelination. Although both cell types shape the number of OPCs with consequences for subsequent myelinogenesis, astrocytes are also implicated in a tight crosstalk between OPCs and axons making them indispensable for myelination. Also, by providing a substantial fraction of lipids incorporated into CNS myelin, astrocytes are required for the proper generation of the myelin sheath. Under pathological and demyelinating conditions, reactive astrocytes and activated microglia/macrophages both exhibit a dual activity inducing detrimental or beneficial effects, the balance of which appears to be critical for tissue regeneration. Besides the secretion of a wide range of molecules, both cell types can phagocyte myelin debris. However, in contrast to the beneficial role of microglia/macrophages-induced phagocytosis, myelin uptake occurring as an early response of astrocytes results in recruitment of immune cells possibly increasing tissue damage in demyelinating conditions driven by inflammation. As a whole, the review indicate that beyond the secretion of a multitude of cues directly controlling diverse facets of OPC biology, astrocytes and microglia/macrophages strongly modify the lesion microenvironment. Further delineation of this crosstalk should open the way towards novel therapeutic approaches aimed at recovering proper developmental myelination and relieving the obstacles on the failure of regeneration of damaged myelin characterizing CNS demyelinating diseases.

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REVIEW
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Unraveling Myelin Plasticity

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Plasticity in the central nervous system (CNS) allows for responses to changing environmental signals. While the majority of studies on brain plasticity focus on neuronal synapses, myelin plasticity has now begun to emerge as a potential modulator of neuronal networks. Oligodendrocytes (OLs) produce myelin, which provides fast signal transmission, allows for synchronization of neuronal inputs, and helps to maintain neuronal function. Thus, myelination is also thought to be involved in learning. OLs differentiate from oligodendrocyte precursor cells (OPCs), which are distributed throughout the adult brain, and myelination continues into late adulthood. This process is orchestrated by numerous cellular and molecular signals, such as axonal diameter, growth factors, extracellular signaling molecules, and neuronal activity. However, the relative importance of, and cooperation between, these signaling pathways is currently unknown. In this review, we focus on the current knowledge about myelin plasticity in the CNS. We discuss new insights into the link between this type of plasticity, learning and behavior, as well as mechanistic aspects of myelin formation that may underlie myelin plasticity, highlighting OPC diversity in the CNS.

Keywords: myelin plasticity, oligodendrocyte, oligodendrocyte precursor cell, myelin, glutamate

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INTRODUCTION

In the central nervous system (CNS), myelin is produced by oligodendrocytes (OLs) that differentiate from oligodendrocyte precursor cells (OPCs) (Fields, 2014). OPCs are distributed throughout the adult brain and represent the main self-renewing population of cells in the CNS (Dawson et al., 2003). Myelination has generally been studied in a developmental context and is often described as a process that terminates after juvenile development. However, recent work shows that myelination continues into late adulthood, with adult OPCs providing a continuous supply of new myelinating OLs (Rivers et al., 2008; Hughes et al., 2013, 2018; Young et al., 2013; Hill et al., 2018). This suggests that protracted myelination may allow for fine-tuning of neural circuits throughout life. While studies of brain plasticity mostly focus on neuronal synapses, myelin plasticity, defined as the myelination of previously unmyelinated axons or changes in the structure of already-myelinated axons (e.g., ion channel surface expression, changes in internode number and length, myelin thickness or geometry of the nodal area), is now also thought to modulate neural networks (Sampaio-Baptista et al., 2013; Gibson et al., 2014; McKenzie et al., 2014; Pajevic et al., 2014). Indeed, changes in myelin sheath stability, length and thickness can alter conduction

velocity, and therefore modulate input synchronization (Waxman, 1997; Fields, 2015). While myelin plasticity is a novel field of study, and the mechanisms underlying it are poorly understood, it is likely that several processes governing developmental myelination are applicable in the context of plasticity (**Figure 1**). In particular, developmental myelination is thought to occur either in a neuronal activity-independent or -dependent mode (Lundgaard et al., 2013). Here, we will briefly review both modes of myelination, along with the role of motor, cognitive and sensory learning, and OPC diversity, in the context of myelin plasticity.

THE DIFFERENTIAL PATH OF MYELINATION

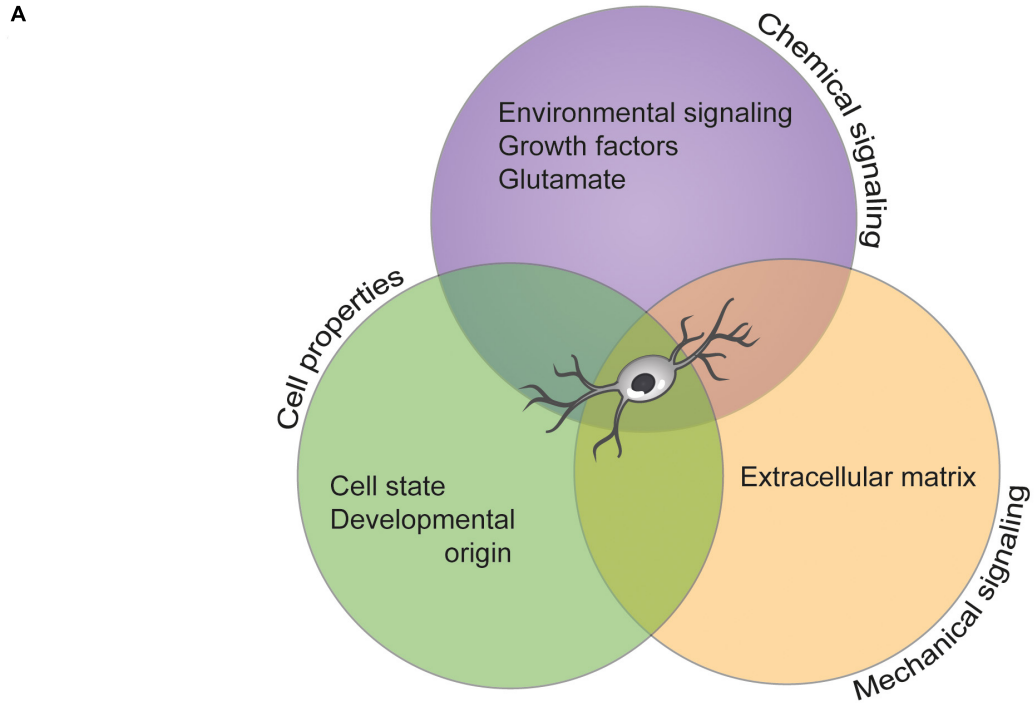
Activity-Independent Myelination

Developing OPCs are proliferative, self-renewing cells that possess the capability to differentiate into myelinating OLs (Rosenberg et al., 2008). Notwithstanding, several external cues regulate this differentiation capacity. Neuronal activity, through neurotransmitter signaling, is a regulatory signal for OPC proliferation and differentiation. However, OPCs can differentiate into OLs that wrap inert fibers with compact myelin, and have internodes of expected lengths, clearly indicating that the initiation of OL differentiation and some forms of myelination does not require neuronal activity (Rosenberg et al., 2008; Lee et al., 2012) (**Table 1**). Nevertheless, the presence of the axon, or an axon-like structure, remains a strong inductive signal for differentiation, suggesting that the biophysical characteristics of the axon, such as the shape and the caliber, regulate OPC differentiation. Axon caliber has been shown to influence myelin thickness (Voyvodic, 1989) and the internodal distribution (Trapp and Kidd, 2000). Similarly, increasing axonal diameter by knocking out *Pten* in axons induces myelination of normally unmyelinated parallel fibers (Goebbels et al., 2017), and retinal ganglion axons following enucleation (i.e., the surgical removal of one eye) (Mayoral et al., 2018). Eye enucleation in a non-degenerative mouse model reduces axonal diameter and myelination, supporting the notion that axon caliber is a main regulatory factor of myelination. Although there is a correlation between axon diameter and myelination, it is important to note that knocking out *Pten* alters growth factor signaling (Goebbels et al., 2017), and that enucleation alters spontaneous firing in the control eye (Failor et al., 2018), raising the possibility that diameter alone is not the only mechanism regulating myelination (Friede, 1972; Lee et al., 2012), or that different axons are myelinated by different mechanisms (Koudelka et al., 2016). Additionally, diameter alone does not explain how the same axon can be differentially myelinated along its length, nor how axons of the same diameter can be either myelinated or remain unmyelinated (Tomassy et al., 2014). Using a neuron-free *in vitro* system in which OPCs from either the spinal cord or cortex differentiate into OLs that ensheath inert fibers, it has been found that spinal cord OLs produced longer sheaths along the microfibers than cortical OLs, consistent with their length in the CNS (Bechler et al., 2015). These data suggest that sheath length may be intrinsic, region-specific, and programmed before differentiation. Intriguingly, Schwann cells, the myelinating glia of the peripheral nervous system (PNS), were not able to myelinate the microfibers, implying that the capacity to myelinate without axonal cues is exclusive to the CNS. However, Schwann cells usually myelinate larger diameter axons than OLs, thus another interpretation could be that the size of the fibers used in this study was insufficient to initiate Schwann cell myelination. This region-specific property points to local cues which regulate OL lineage progression and OL properties.

The physical properties of the microenvironment, such as proximity to an axon or cellular density, may induce differentiation by altering OPC size or shape, thus generating structural rearrangement within the cells (Ingber, 1997; McBeath et al., 2004). This rearrangement could allow for interactions between different effectors in a signaling pathway, and thus, promote differentiation (Boudreau and Jones, 1999). Another potential explanation is that changes in cell shape might directly modify the nuclear size or structure, inducing the transcriptional activity necessary for OPC differentiation (Maniotis et al., 1997). This possibility is supported by work showing that mechanically deforming OPCs or plating them in the presence of neurons, beads, or at high density, promotes differentiation by altering the chromatin structure (Hernandez et al., 2016).

A recent study by the Chalut group further supports the idea that the mechanical environment modulates OPC function. By mimicking the stiffness of young brains using scaffolds in culture, they demonstrated that OPCs isolated from aged rats and cultured in these softer conditions became molecularly and functionally similar to neonatal OPCs. Disrupting mechanical signaling in these aged OPCs increased their proliferation and differentiation rate, indicating that increasing brain stiffening with age downregulates the proliferation and differentiation potential of OPCs (Segel et al., 2019). During development, the maturation of the extracellular matrix (ECM) stabilizes neural networks by limiting changes in synaptic connectivity (Bikbaev et al., 2015). Conversely, removing the ECM promotes synaptic plasticity (Lazarevich et al., 2020). It is possible that ECM maturation also limits differentiation and myelination rates with aging to prevent hypermyelination and stabilize neural networks. However, local changes in the ECM may allow for local differentiation and could therefore be a mechanism underlying myelin plasticity.

Changes in the physical environment can also affect the chemical signaling by altering the extra cellular volume, for instance altering growth factor concentration. This could influence OPC development as platelet-derived growth factor (PDGF) activates the α receptor (PDGFRα) on OPCs and regulates both their proliferation and survival (Raff et al., 1988; Richardson et al., 1988; Barres and Raff, 1993). However, not all OPCs respond equally to PDGF. Although PDGFRα protein expression is similar in both gray and white matter OPCs, cells in the white matter of early postnatal organotypic slice cultures proliferate more in response to PDGF than those in the gray matter (Hill et al., 2013). Consistent with this finding, it has been shown that while all adult OPCs continue to divide, white matter cells divide at a higher rate than gray matter cells



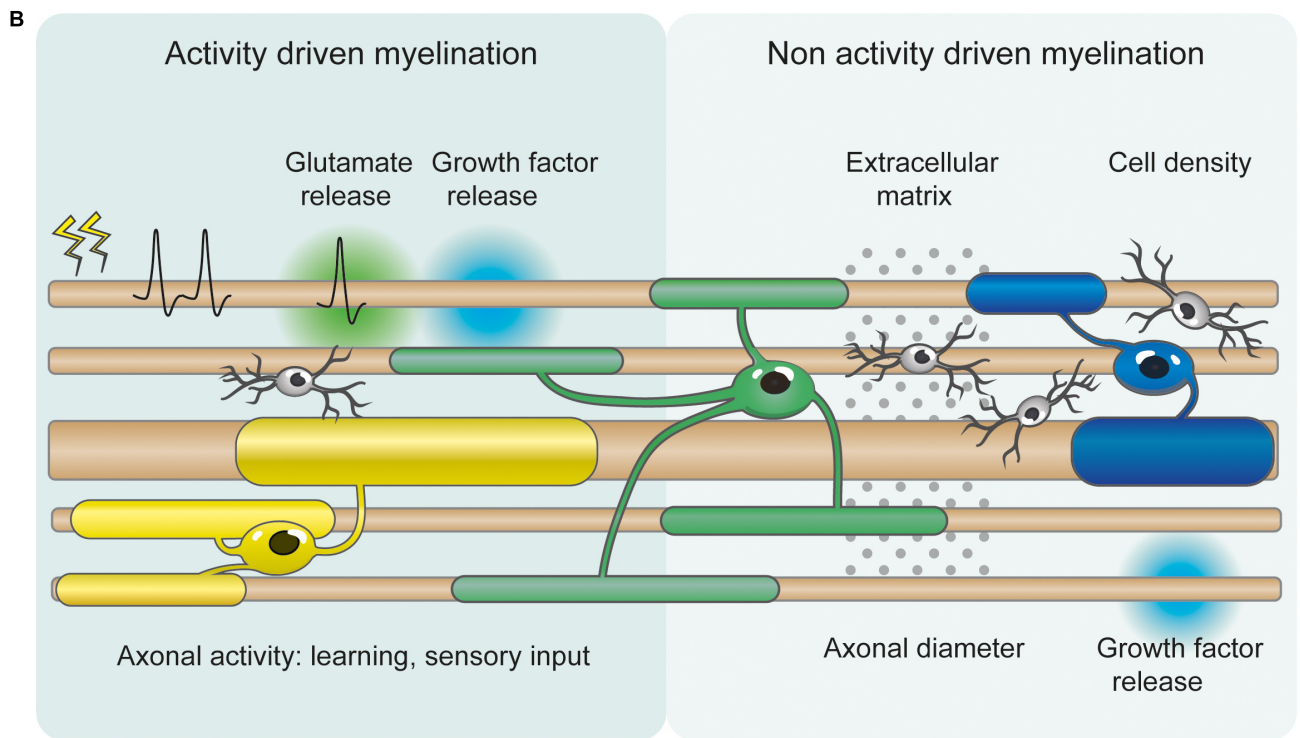


FIGURE 1 | OPC heterogeneity and axonal factors allow for differential myelination and myelin plasticity. **(A)** OPC proliferation, differentiation and myelination are orchestrated by numerous mechanical, cellular, and chemical signals. These include axonal diameter, growth factors, extracellular signaling molecules, extracellular matrix composition, cellular intrinsic deposition, neurotransmitters (such as glutamate), and neuronal activity. However, the relative importance of and cooperation between these signaling pathways is currently unclear. **(B)** Several studies indicate that myelination can be modified by activity- and experience-driven mechanisms. Glutamate and growth factor release from electrically active neurons can regulate OPC proliferation, differentiation and myelination. Additionally, motor and possibly cognitive learning, and sensory experience also influence myelination changes. However, myelination can also occur independently of neuronal activity. Non activity driven myelination could be regulated by the physical and mechanical properties of the extracellular environment, such as cellular density and extracellular matrix. OPCs are depicted in light gray. OLs are represented in different colors to illustrate the differential myelination.

TABLE 1 | Summary of current literature on activity-independent and activity-dependent myelination in the CNS.

Myelination modes	Activity-independent	Biophysical properties of the axon	Friede, 1972 Voyvodic, 1989 Fukui et al., 1991 Colello et al., 1995 Shrager and Novakovic, 1995 Trapp and Kidd, 2000 (review) Lee et al., 2012 Tomassy et al., 2014 (provides evidence that biophysical constraints alone cannot explain differential myelination) Goebbels et al., 2017 Mayoral et al., 2018
		Microenvironmental characteristics	Raff et al., 1988 Richardson et al., 1988 Maniotis et al., 1997 Rosenberg et al., 2008 Hernandez et al., 2016 Segel et al., 2019
		Cell properties	Hill et al., 2013 Bechler et al., 2015
	Activity-dependent	Neuronal regulation of OPC proliferation and differentiation, and myelination	Gyllensten and Malmfors, 1963 Tauber et al., 1980 Barres and Raff, 1993 Demerens et al., 1996 Stevens et al., 1998 Liu et al., 2012, 2016 Makinodan et al., 2012 Mangin et al., 2012 Gibson et al., 2014 Hill et al., 2014 Mensch et al., 2015 Gautier et al., 2015 Etxeberria et al., 2016 Koudelka et al., 2016 Mitew et al., 2018 Ortiz et al., 2019
		Glutamate signaling	Gallo et al., 1996 Yuan et al., 1998 Bergles et al., 2000 Karadottir et al., 2005, 2008 Micu et al., 2006 Kukley et al., 2007 Ziskin et al., 2007 De Biase et al., 2011 Wake et al., 2011 Cavaliere et al., 2012 Guo et al., 2012 Li et al., 2013 Lundgaard et al., 2013 Fannon et al., 2015 Gautier et al., 2015 Saab et al., 2016 Spitzer et al., 2016 (review) Kougioumtzidou et al., 2017 Spitzer et al., 2019

This table summarises the reviewed literature, showing selected papers which provide support (if not indicated otherwise) for the mechanisms shown.

(Young et al., 2013). It could be argued that the differential response of gray and white matter OPCs to PDGF stems from the microenvironment (physical properties) rather than a cell intrinsic process. Addressing this question, the Nishiyama group showed, by using small tissue section transplant experiments, that regional identity, and not environment, determined the proliferative response to PDGF (Hill et al., 2013). On the other hand, studies looking into differences due to the developmental origin of OPCs, with a transgenic approach (Psachoulia et al., 2009), or regional identity using cell transplantation (Vigano et al., 2013), have failed to find differences in OPC proliferation. A possible explanation of the difference in results is that using small tissue sections, instead of isolated cells, may have provided sufficient environmental signals of the original region to

influence OPCs' response to PDGF in the transplanted area, and, given a longer period of time for the section to integrate into the host slice, these experiments may have yielded a different result. These studies show that physical properties and environment influence OPC proliferation, differentiation and myelination. Although it is unclear whether these properties can mediate myelin plasticity in response to learning and sensory inputs, their contribution cannot be ruled out. Another mechanism such as neuronal activity, known to influence physical properties (Lazarevich et al., 2020), release of growth factors (Barres and Raff, 1993; Balkowiec and Katz, 2000) and regulate myelination, is perhaps more amenable to plasticity changes, as we shall discuss in the next paragraph.

Activity-Dependent Myelination (Glutamate Signaling)

Numerous studies have shown that neuronal activity can regulate myelination (**Table 1**). In addition to growth factors, glutamate release from active neurons is a likely mechanism underlying activity-dependent myelination, as OPCs receive synaptic inputs from neurons and express glutamate receptors (Bergles et al., 2000; Karadottir et al., 2005, 2008; Micu et al., 2006; Kukley et al., 2007; Ziskin et al., 2007; Lundgaard et al., 2013; Gautier et al., 2015; Spitzer et al., 2019), allowing them to monitor and respond to neuronal activity. However, it is important to note that OPCs display a range of electrophysiological profiles, with ion channel and glutamate receptor densities varying with age and brain region. OPCs acquire voltage-gated potassium channels (K_V) and sodium channels (Na_V), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), kainate receptors (KARs), and N-methyl-D-aspartate receptors (NMDARs) during development, but at different rates, which appear to be regulated by the environment (Spitzer et al., 2019).

Neuronal activity regulates OPC proliferation *in vivo* (Barres and Raff, 1993; Mangin et al., 2012; Gibson et al., 2014; Mitew et al., 2018) (**Table 1**). Numerous studies show that blocking activity blocks proliferation (Barres and Raff, 1993), while increasing activity promotes proliferation (Gibson et al., 2014; Mitew et al., 2018). Axonal activity also regulates differentiation (**Table 1**): decreasing activity by whisker removal or raising mice in social isolation impedes differentiation (Liu et al., 2012, 2016; Makinodan et al., 2012; Hill et al., 2014), although ocular deprivation leads to enhanced differentiation, albeit with reduced internode length (Etxeberria et al., 2016). However, it is important to note that ocular deprivation did not block neuronal firing, but rather altered it (Etxeberria et al., 2016), suggesting that changes in activity modulate differentiation. Similarly, enhancing activity with optogenetics, chemogenetics, receptor agonists/antagonists, or physiological manipulations, promotes differentiation (Gibson et al., 2014; Mitew et al., 2018), or enhances myelination (Tauber et al., 1980; Demerens et al., 1996; Mensch et al., 2015; Mitew et al., 2018). However, other studies using similar approaches have failed to show an effect of neuronal activity on developmental myelination (Fukui et al., 1991; Colello et al., 1995; Shrager and Novakovic, 1995). Nonetheless, blocking neuronal activity decreases myelination and prevents myelin repair after demyelination (Gyllensten and Malmfors, 1963; Demerens et al., 1996; Stevens et al., 1998; Gautier et al., 2015; Mensch et al., 2015; Mitew et al., 2018) while enhancing activity improves remyelination (Ortiz et al., 2019).

Neuronal activity not only promotes myelination through glutamate signaling, but also induces a switch to the activity-dependent mode of myelination. Indeed, activity-dependent release of neuregulin (NRG) or brain-derived neurotrophic factor (BDNF) enhances NMDAR functional expression in OPCs and switches myelination to an activity-dependent mode in neuron-OPC co-cultures (Lundgaard et al., 2013). Activity-dependent myelination occurs faster than activity-independent myelination, and crucially, blocking neuronal activity, NMDARs, or AMPARs once the activity-dependent mode is activated by NRG or BDNF significantly reduces myelination (Lundgaard et al., 2013). However, blocking activity, NMDARs or AMPARs does not affect myelination in the absence of NRG or BDNF (Lundgaard et al., 2013). In mice, blocking activity-dependent BDNF release or deleting the BDNF receptor TrkB in OPCs blocks activity-dependent myelination (Geraghty et al., 2019). Nevertheless, NRG/ErbB (the NRG receptor) signaling plays a complicated role in the CNS (Lyons et al., 2005; Brinkmann et al., 2008). Despite evidence indicating a function in OL differentiation, myelination, and survival *in vitro* (Flores et al., 2000; Kim et al., 2003; Taveggia et al., 2008), knocking out ErbB3 and ErbB4 in OL lineage cells does not prevent myelination (Brinkmann et al., 2008), although it is unclear whether there was a delay in myelination, it does seem to prevent experience-dependent myelination (Makinodan et al., 2012). The controversial actions of NRG/ErbB signaling can be explained by the ability of NRG to switch OPCs from activity-independent myelination to the activity-dependent mode through the increase of NMDAR-mediated currents in OL lineage cells (Lundgaard et al., 2013). It is likely that in the absence of ErbB3 and ErbB4, NRG could not enhance NMDAR expression in OPCs, and therefore, could not induce the switch to activity-dependent myelination. Therefore, a delay in myelination, but no myelination defect, would be expected, although this was not tested. Moreover, the release of NRG itself is activity-dependent (Ozaki et al., 2004). The resulting interaction of NRG with glutamate signaling increases myelination on active neurons, providing a mechanism by which activity plays a role in myelination and myelin plasticity (Spitzer et al., 2016).

The importance of glutamate signaling in activity-dependent myelination is revealed by studies of myelin repair following demyelinating lesions. Blocking neuronal activity, vesicular release, AMPARs or NMDARs at the lesion site impedes myelin regeneration after toxin-induced demyelination (Lundgaard et al., 2013; Gautier et al., 2015). However, the exact role of glutamate signaling *in vivo* remains elusive. *In vitro* studies report that AMPA/KAR activation reduces both proliferation and differentiation (Gallo et al., 1996; Yuan et al., 1998; Fannon et al., 2015), while activating NMDARs promotes myelination (Wake et al., 2011; Cavaliere et al., 2012; Li et al., 2013; Lundgaard et al., 2013). Nevertheless, the importance of glutamate signaling through AMPARs and NMDARs for

myelination *in vivo* is disputed due to the mild deficits observed in the respective knockouts (De Biase et al., 2011; Guo et al., 2012; Saab et al., 2016; Kougioumtzidou et al., 2017). However, both receptors may have been knocked out in OPCs prior to the activation of AMPA/KARs or NMDARs (Spitzer et al., 2019). Even if neurons released NRG or BDNF onto OPCs, myelination would not have switched to the activity-dependent mode, as OPCs did not have NMDARs or AMPARs, and therefore, NRG could not enhance NMDAR expression. If this were the case, a delay in myelination would be expected, due to compensation by the slower activity-independent mode of myelination. While this was not tested in all of the studies above, two groups found that both the AMPAR and NMDAR knockouts lead to a delay in myelination (Saab et al., 2016; Kougioumtzidou et al., 2017). As activity-independent myelination occurs slower than activity-dependent myelination, these studies indicate that it is possible that compensation may have occurred by defaulting to the slower activity-independent myelination mode. Whereas, altering AMPAR receptors, postnatally, during the peak of the myelination period increased OPC proliferation while reducing their differentiation (Chen et al., 2018), suggesting that modifying receptor properties at specific timepoints can alter OPC dynamics. These studies indicate that glutamate signaling through AMPA/KARs and NMDARs depends on a complex interplay of factors, such as the receptor subtype and density, the frequency, the amount, and probability of glutamate release from active neurons. Nonetheless, glutamate signaling remains an integral mechanism of activity-dependent myelination in the context of both normal developmental myelination and myelin plasticity.

OPC HETEROGENEITY

Myelin plasticity includes both *de novo* myelination and structural changes to existing myelin. *De novo* myelination is thought to occur through the differentiation of adult OPCs, which receive cues – presumably axon-derived – following motor, sensory or social experience. Thus, to study myelin plasticity, we must investigate how OPCs integrate these cues. This is made more complex by several groups reporting that OPCs are a heterogeneous population, with differences in their proliferation and differentiation potentials with age or between brain regions (Rivers et al., 2008; Vigano et al., 2013; Young et al., 2013; Moshrefi-Ravasdjani et al., 2017; Spitzer et al., 2019). In addition, bulk-RNA sequencing shows that OPCs exhibit age- related changes in transcriptome (Marques et al., 2018; Spitzer et al., 2019), and single-cell experiments suggest that proliferation and differentiation gene expression is altered with age (Marques et al., 2016, 2018). OPCs also display differential responses to growth factors and cytokines (Mason and Goldman, 2002; Lin et al., 2009; Hill et al., 2013; Lentferink et al., 2018). Furthermore, in zebrafish, two populations of OPCs were identified in the spinal cord: a population that mostly proliferates in response to activity, but does not differentiate, and a second population arising from the first one, which differentiates into myelinating OLs (Marisca et al., 2020). These differences must be considered, especially when attempting to study myelin plasticity through the lens of developmental myelination (**Table 2**).

Given the role of neuronal activity, via glutamate signaling, in regulating OPC proliferation, differentiation, and myelination, and its potential role in myelin plasticity regulation, it is important to understand if all OPCs display the same physiological properties. While some reports indicate that OPCs from the hippocampus and corpus callosum are homogeneous (De Biase et al., 2010; Clarke et al., 2012), differences in ion channels between gray and white matter OPCs have been described (Chittajallu et al., 2004; Spitzer et al., 2019). Furthermore, age-dependent changes in ion channels have also been described (Karram et al., 2008; Moshrefi-Ravasdjani et al., 2017; Spitzer et al., 2019) (**Table 2**). An in-depth study of mouse OPC membrane properties in different brain regions between embryonic day 13 and postnatal day 330 indicates that the density of Na_V, K_V, AMPA/KARs and NMDARs differs. Specifically, at embryonic day 13, when they first appear in the brain, OPCs have no ion channels or glutamate receptors, and acquire them with age at different rates, and differentially between brain regions (Spitzer et al., 2019). Functional expression of ion channels and glutamate receptors can be linked to the proliferation and differentiation potential of OPCs (Spitzer et al., 2019).

These data led to the identification of several OPC states. First, embryonic-like "naïve" OPCs, lacking ion channels and glutamate receptors, which cannot sense neuronal activity. Second, "highly proliferative" OPCs, with K_V, AMPA/KARs, and a high density of Na_V. Third, OPCs that are "primed"

TABLE 2 | Reviewed literature on OPC heterogeneity.

OPC heterogeneity	Differences in transcriptomics	Marques et al., 2016, 2018 Spitzer et al., 2019 Marisca et al., 2020
	Differential response to growth factors and cytokines	Mason and Goldman, 2002 Lin et al., 2009 Hill et al., 2013 Lentferink et al., 2018
	Region and age-dependent changes in physiological properties	Chittajallu et al., 2004 Karram et al., 2008 De Biase et al., 2010 Clarke et al., 2012 Moshrefi-Ravasdjani et al., 2017 Spitzer et al., 2019 Marisca et al., 2020
	Diverse proliferation and differentiation potential	Rivers et al., 2008 Vigano et al., 2013 Young et al., 2013 Marques et al., 2016 Moshrefi-Ravasdjani et al., 2017 Spitzer et al., 2019 Marisca et al., 2020

This table summarises the reviewed literature showing selected papers describing OPC heterogeneity. The references in blue suggest that OPCs are physiologically homogeneous.

for differentiation, with K_V, AMPA/KARs, a high Na_V density, and a high density of NMDARs, indicative of a high sensitivity to neuronal activity. Lastly, "quiescent" OPCs, who have lost NMDARs, and have acquired a high density of AMPA/KARs (Spitzer et al., 2019). Importantly, at every postnatal time point and brain region tested, a range of electrophysiological profiles of OPCs can be detected, although in differing proportions, suggesting that this functional diversity may represent cell states rather than heterogeneity.

Understanding OPC states is crucial for our understanding of both activity-dependent and activity-independent myelination. For instance, most embryonic OPCs are naïve, yet proliferate, and, in the spinal cord, have begun to differentiate, perhaps indicating that early developmental myelination may proceed in an activity-independent mode, presumably to ensure that critical processes like breathing are functional by birth (Foran and Peterson, 1992; Spitzer et al., 2019). In addition, the majority of OPCs are in the primed state during the first three postnatal months, at the time where differentiation and myelination are proceeding at the highest rate, and NMDAR expression is highest, suggesting that at this time, myelination is activity-dependent (Spitzer et al., 2019). Most studies on motor or sensory myelination and myelin plasticity have been performed at this time. It is therefore not surprising that activity-dependent myelination is thought to underlie myelin plasticity.

This poses the problem of what happens in mature brains, once most OPCs have become quiescent. Does myelination stop, and does myelin plasticity remain possible? Is plasticity limited to a critical window, defined by OPC ion channel expression? The majority of OPCs were described as quiescent by nine months, yet OPC differentiation and myelination have been reported to continue in the mouse cortex until 2 years of age (Young et al., 2013; Hill et al., 2018; Hughes et al., 2018; Spitzer et al., 2019). In addition, a study examining plasticity in adult mice showed that sensory enrichment increased the formation of new myelin in the somatosensory cortex of 10–14 month old mice (Hughes et al., 2018). The signaling mechanism driving this plasticity was not investigated, but sensory enrichment alters neuronal activity, which may in turn lead to the release of NRG or BDNF from neurons, promoting NMDAR functional expression in OPCs, and a shift to the primed state (Lundgaard et al., 2013; Spitzer et al., 2019). Indeed, glutamate receptors in OPCs can be regulated by growth factors (Gallo et al., 1994; Lundgaard et al., 2013). Thus, growth factors may regulate state transitions, and allow for activity-dependent myelination following major sensory events.

OPC states may also influence the different myelination strategies employed by different brain regions. For instance, in the rodent optic nerve, myelin tends to be remodeled (with changes on already myelinated axons), while in the corpus callosum, the tendency is more toward *de novo* myelination of unmyelinated axons (Young et al., 2013). It is therefore critical that we understand both OPC states and their regulators to better understand myelination, and myelin plasticity.

REGULATION OF MYELINATION BY MOTOR LEARNING, SOCIAL BEHAVIOR AND SENSORY EXPERIENCE

Recent evidence suggests that myelination may be dynamically regulated by learning and experience, and may therefore play a role in learning (**Table 3**). Structural changes in human white matter occur with learning new tasks, such as playing the piano (Bengtsson et al., 2005) or learning how to juggle (Scholz et al., 2009), though whether these changes indicate myelin remodeling remains unclear (Zatorre et al., 2012; Walhovd et al., 2014). Nevertheless, experiments combining diffusion MRI fractional anisotropy (as in human studies) and immunohistochemistry have shown that motor learning in adult mice leads to white matter structural changes which correlate with an increased myelin density (Sampaio-Baptista et al., 2013). Furthermore, the Richardson group showed that motor learning increases OPC differentiation into myelinating OLs in the motor cortex and corpus callosum, and that motor learning is in fact dependent on this (McKenzie et al., 2014; Xiao et al., 2016).

A current outstanding question in the field is whether myelination is initiated only during sensory-motor learning or in all types of learning. In human studies, changes in white matter have been observed following reading (Carreiras et al., 2009) or learning a second language (Schlegel et al., 2012), suggesting that modifications in myelin may also occur following cognitive learning. In addition to various reports demonstrating that sensory experience or neuronal activity modulate myelination in the somatosensory system (Hughes et al., 2018; Mitew et al., 2018), a recent publication suggests that myelin plasticity is important for normal cognitive function. Activity-regulated myelination fails in a model of chemotherapy-related cognitive impairment (CRCI), a syndrome characterized by deficits in attention and memory (Koppelmans et al., 2012), and this is linked to a reduced BDNF-TrkB signaling in OPCs, as demonstrated by the deficits in cognitive behavioral performance following the OPC-specific TrkB receptor loss (Geraghty et al., 2019).

TABLE 3 | Summary of reviewed literature on learning and experience.

Learning and experience	Motor learning	Bengtsson et al., 2005 Scholz et al., 2009 Sampaio-Baptista et al., 2013 McKenzie et al., 2014 Xiao et al., 2016
	Cognitive functions	Carreiras et al., 2009 Schlegel et al., 2012 Geraghty et al., 2019
	Social behavior	Liu et al., 2012 Makinodan et al., 2012
	Sensory experience	Mangin et al., 2012 Hill et al., 2014 Hughes et al., 2018

Table recapitulating the most relevant studies linking myelin modifications with learning, social behavior and sensory experience. Human studies are indicated in red, while rodent studies are indicated in black.

It seems that changes in myelination as a response to the environment have important long-term behavioral and cognitive consequences. Indeed, rearing juvenile mice in social isolation alters myelin in the medial prefrontal cortex (mPFC) (Liu et al., 2012; Makinodan et al., 2012). In one of these studies, NRG was shown to be decreased following social isolation, and the modifications in myelin were phenocopied by an OL ErbB3 receptor knockout (Makinodan et al., 2012). Together, these data indicate that social experience, presumably via neuronal activity, regulates myelination and that this is important for normal cognitive function.

Sensory experience also influences myelin plasticity. Whisker deprivation, by unilateral removal, leads to a decrease in the number of mature OLs, but an increase in OPC density and proliferation (Mangin et al., 2012; Hill et al., 2014). However, whisker deprivation also increases apoptosis of proliferating OPCs (Hill et al., 2014). Thus, these data suggest that whisker deprivation leads to a decrease in mature OL numbers, which may in turn lead to over proliferation of OPCs, although the increase in apoptosis may be a mechanism to maintain homeostasis (Hill et al., 2014).

The surprisingly rapid dynamics of OL production in response to motor learning (within 2 h) (Xiao et al., 2016) and myelin basic protein (MBP) translation in response to neuronal activity (within minutes to hours) (Wake et al., 2011) occur on a timescale that is similar to that of dendritic spine changes underlying synaptic plasticity (Xu et al., 2009). Like synaptic plasticity, myelin plasticity following motor or cognitive learning and sensory experience is thought to be regulated by activity-dependent myelination, as motor, cognitive and sensory events lead to changes in neuronal activity (although the contribution of activity-independent myelination cannot be excluded) (**Figure 1**). These data suggest that myelin plasticity and synaptic plasticity may be complementary mechanisms underlying learning and memory.

OUTLOOK AND FUTURE PERSPECTIVE

Until recently, myelination was considered a static process, and studies examining circuit function and plasticity mostly focused on synaptic plasticity. However, a number of studies described above demonstrate that myelination is far from static, and does not only change in response to injury, but also as a result of motor, sensory and cognitive events. Although some progress has been recently made, our knowledge of myelin plasticity and the mechanisms underlying it remains restricted by regional differences, OPC diversity, a lack of understanding of the neuronal dynamics required to regulate OL lineage progression, and a limited comprehension of how OL lineage cells integrate the various activity-independent and activity-dependent signals (**Figure 1**).

The study of myelin plasticity requires morphological analyses, both at the sub microscopic scale and the macroscopic scale, but also a combination of behavioral, electrophysiological and molecular analyses. This can only be achieved by combining both *in vitro* and *in vivo* experimental models.

One area that deserves major investigation is examining whether activity-dependent myelination proceeds similarly in different brain regions. From the studies reviewed in this paper, it appears that, akin to synaptic plasticity, neuronal activity-dependent myelin plasticity may be an important mechanism underlying learning and cognition. Activity-dependent release of growth factors and glutamate may be particularly important for this process, and thus, it is crucial to understand these mechanisms of myelination. Moreover, dynamic myelin changes in the hippocampus and mPFC, two regions that are comprised of both gray and white matter, are likely to have long-lasting effects on brain function. In humans, myelination of these regions continues for decades, suggesting that lifelong myelination and myelin plasticity tune neuronal networks and regulate normal brain function.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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BRIEF RESEARCH REPORT
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Longitudinal Intravital Microscopy Reveals Axon Degeneration Concomitant With Inflammatory Cell Infiltration in an LPC Model of Demyelination

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Demyelination and axon degeneration are major events in all neurodegenerative diseases, including multiple sclerosis. Intoxication of oligodendrocytes with lysophosphatidylcholine (LPC) is often used as a selective model of focal and reversible demyelination thought to have no incidence for neurons. To characterize the cascade of cellular events involved in LPC-induced demyelination, we have combined intravital coherent antistoke Raman scattering microscopy with intravital two-photon fluorescence microscopy in multicolor transgenic reporter mice. Moreover, taking advantage of a unique technique of spinal glass window implantation, we here provide the first longitudinal description of cell dynamics in the same volume of interest over weeks after insults. We have detected several patterns of axon–myelin interactions and classified them in early and advanced events. Unexpectedly, we have found that oligodendrocyte damages are followed by axon degeneration within 2 days after LPC incubation, and this degeneration is amplified after the recruitment of the peripheral proinflammatory cells at day 4. Beyond day 7, the recovery of axon number and myelin takes 3 more weeks postlesion and involves a new wave of anti-inflammatory innate immune cells at day 14. Therefore, recurrent imaging over several weeks suggests an important role of peripheral immune cells in regulating both the axonal and oligodendroglial fates and thereby the remyelination status. Better understanding the recruitment of peripheral immune cells during demyelinating events should help to improve diagnosis and therapy.

Keywords: lysophosphatidylcholine, demyelination, neurodegeneration innate immune cells, CARS, spinal glass window

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INTRODUCTION

Myelin sheath, generated by oligodendrocytes (OLs) in the central nervous system (CNS), is a multilayer membrane wrapping axons and providing electrical insulation, high-speed axonal conduction, and trophic support (Kaplan et al., 1997). Demyelination that occurs in most neurodegenerative diseases is due to the pathological destruction of myelin sheaths and subsequent degeneration of myelinating OLs. Various models of demyelination have been established in rodents, among which oligodendroglial intoxication by lysophosphatidylcholine (LPC) is the most common (El Waly et al., 2014).

Lysophosphatidylcholine is a glycerophospholipid naturally occurring in all cell membranes that is particularly enriched in the CNS white matter. Generated through the hydrolysis of membrane plasmalogens by phospholipase A_2 (Kougias et al., 2006), it can also be supplemented directly from emulsifier-rich food (Shah et al., 2017). LPC plasmatic concentration must be regulated physiologically because 100-μM doses already turn out toxic for most cell types, from OLs (Plemel et al., 2018) to endothelial cells (Akerele and Cheema, 2015). Endogenous LPC is indeed likely involved in atherosclerosis (Chang et al., 2017) and affects the occurrence of major depressive disorders (Liu et al., 2016).

At the cellular level, low-dose 1% LPC (∼10 μM) has been reported to trigger the selective death of spinal OLs but not the death of axons (Hall, 1972). Seven days after exposure, demyelination reaches a peak that is followed by a remyelination process of axons close to completion within 4 weeks after exposure (Jeffery and Blakemore, 1995). Despite its lack of direct effect on axons, LPC injection was reported to trigger inflammation and subsequent secondary Wallerian degeneration characterized by dystrophic axonal retraction bulb at the injury site and discontinuous spherical debris along their distal part (Ousman and David, 2000, 2001). From these experiments, it remains, however, unclear whether LPC itself generates an inflammatory environment because of the release of damage-associated molecular patterns from permeabilized OLs or because its injection pipette induced mechanical destruction of the parenchyma.

To address this question, we have thus established a unique murine model of focal LPC incubation that does not sever axons mechanically. Using a combination of intravital two-photon microscopy and coherent antistoke Raman scattering (CARS) microscopy, we have then simultaneously obtained access in real-time to the label-free detection of myelin (Shi et al., 2011), as well as the fate of fluorescent axonal networks in the presence of recruited inflammatory cells (Fenrich et al., 2013b). Taking advantage of the unique glass window protocol developed in our laboratory, we have thus obtained a highly time-resolved description of $LysM^+$ myeloid cell contribution to axons and myelin reorganization. We report for the first time that initial intoxication of OLs is itself responsible for the early recruitment of proinflammatory immune cell coincident with the onset of Wallerian degeneration. Soon after, it, however, converts into a prohealing inflammation that is coincident with remyelination and prior to the delayed increase of fully remyelinated axons.

MATERIALS AND METHODS

Mice

All experimental and surgical protocols were performed following the guidelines established by the French Ministry of Agriculture (Animal Rights Division). The architecture and functioning rules of our animal house, as well as our experimental procedures, have been approved by the "Direction Départementale des Services Vétérinaires" and the ethic committee (ID numbers #18555-2019011618384934 and A1305532 for animal house and research project, respectively).

Seven- to Ten-week-old fluorescent reporter mice were used for spinal cord glass window implantation (Fenrich et al., 2013a) and LPC demyelination. Thy1–CFP and/or Thy1–CFP//LysM-EGFP//Cd11C-EYFP triple-heterozygous transgenic mice were used.

Spinal Cord Glass Window Implantation and LPC Model of Demyelination

The window was applied as described in Fenrich et al. (2012) study. Prior to glass window sealing, the dura mater was opened locally to directly expose the dorsal white matter to 1% LPC (Sigma, L1381) in 0.9% NaCl that we incubated for 1 h (**Figure 1**).

Biphoton and CARS Microscopy Methods

Coherent antistoke Raman scattering imaging was done using OPO femtosecond laser source (80 MHz 150 fs; Coherent, Santa Clara, CA, United States) with excitation wavelengths λp = 806 and λs = 1,050 nm, temporally synchronized and spatially overlapped on the sample plane. The corresponding excited resonances correspond to lipid vibrations approximately 2,850 cm^{-1} with a bandwidth 150 cm^{-1}, dominated by the CH2 and CH3 stretching modes. Whereas nonlinear excitations of CFP fluorescence and EYFP fluorescence were optimal at λp = 806 and λs = 1,050 nm, respectively, wavelengths mixing between λp and λs provided the excitation of EGFP (Ricard et al., 2016). The CARS intensity was determined based on day 0 (D0); indeed, all our acquisitions were made in the same way and using the same CARS intensity.

Cells Dissociation, Sorting, and Quantitative PCR

Spinal cords were extracted 4, 7, or 14 days after LPC incubation. Spinal cords were dissected manually. Cells were dissociated using the MACs Adult Brain Dissociation Kit, mouse and rat (Miltenyi Biotec, Paris, France). GFP^+ cells were sorted using FACS (fluorescence-activated cell sorting) into lysis buffer (10:1 mix of Resuspension Buffer and Lysis Enhancer from Cells Direct one-step quantitative reverse transcription–polymerase chain reaction (RT-PCR) kit; Thermo Fisher Scientific, Waltham, MA, United States). Reverse transcription reactions were performed using 5 μg total RNA. Polymerase chain reaction reactions were performed in 20 μL of Superscript II reaction buffer (Invitrogen, Carlsbad, CA, United States) containing 0.01 M dithiothreitol, 7.5 ng/μL of dN6, 20 U of RNase inhibitor

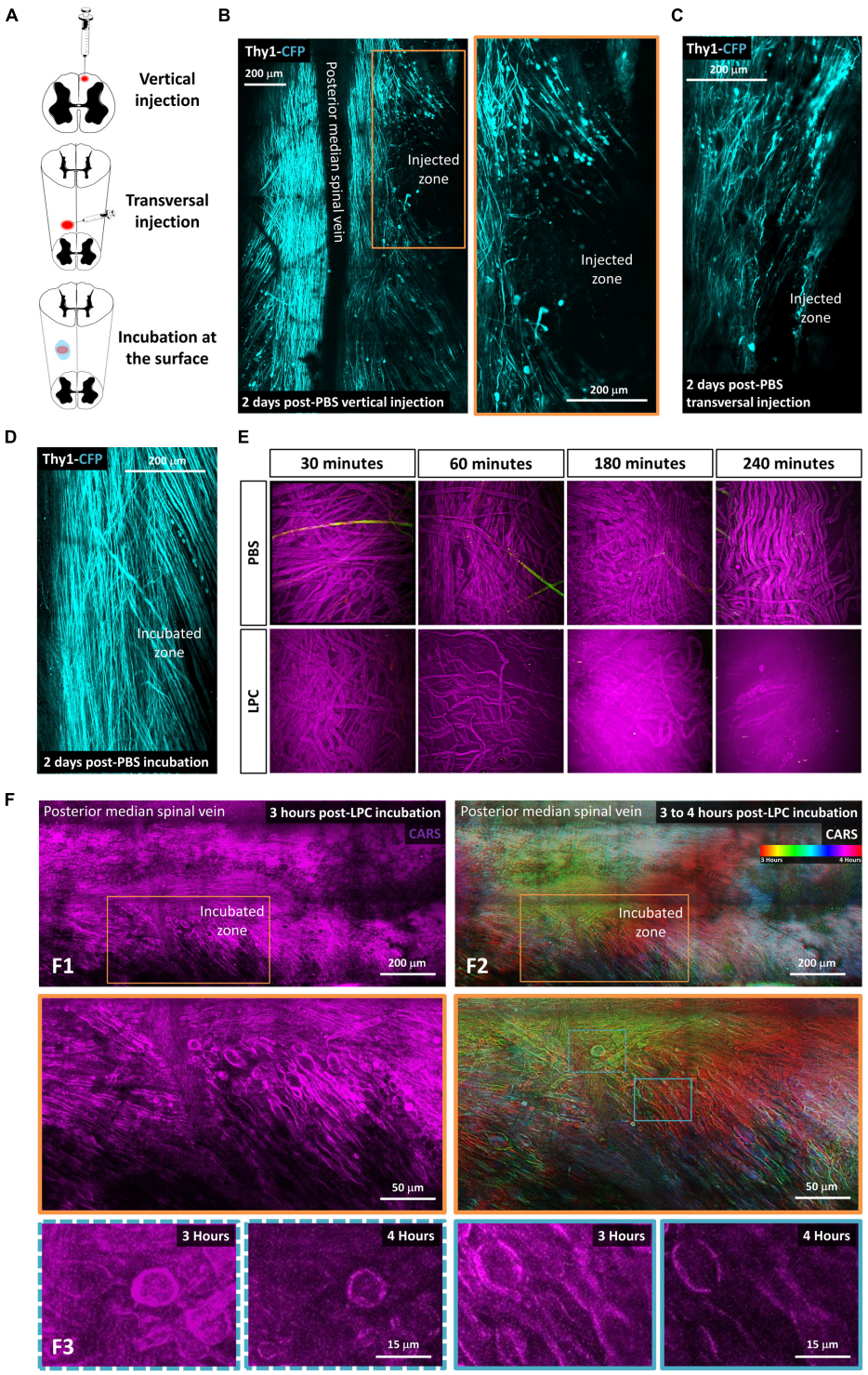

FIGURE 1 | Lysophosphatidylcholine incubation on the spinal cord surface avoids artifactual mechanical insult to axons. **(A)** Schematic description of the three different ways tested to make LPC model of demyelination: vertical injection, transversal injection, and superficial incubation. **(B–D)** *In vivo* biphoton acquisition through the dorsal implanted windows showing the dorsal Thy1–CFP axon network 2 days after vertical injection of PBS, transversal injection of PBS, and superficial PBS incubation, respectively. **(E)** *In vitro* incubation of either PBS or LPC 1% on the surface of sciatic nerve for 30 min, 1 h, 4 h, and 5 h. **(F)** *In vivo* biphoton acquisition through the implanted dorsal windows showing F1: CARS signal (magenta) starting 3 h after LPC incubation at low (top) and high magnification (bottom). F2: Average intensity projection of time-coded color images highlighting the evolution of myelin degradation over time at low (top) and high magnification (bottom). F3: CARS signals in the regions of interest outlined in F2 (bottom) are represented at two different times points, 3 and 4 h, respectively.

(Invitrogen), 10 mM dNTP and 200 U of Superscript II reverse transcriptase (Invitrogen) for 1 h at 42°C. Real-time PCR reactions on cDNA were performed using the LightCycler 480 system (Roche, Indianapolis, IN, United States) using the SYBR Green I Master Kit (Eurogentec, Seraing, Belgium) with 2 μL of cDNA and 200 nM of each PCR primer. Each reaction was performed in triplicate.

Immunolabeling on Spinal Cord Sections

Mice were transcardially perfused with 4% paraformaldehyde. The spinal cords were removed, postfixed overnight, and cut into 100-μm coronal and sagittal sections using a vibratome (Leica Microsysteme, Rueil-Malmaison, France). Immunofluorescent labeling was performed on sections fixed with paraformaldehyde 4%. The following antibody was used: anti-MBP (rat, 1/500; BioRad MCA409S, CA, United States). The sections and cells were incubated with appropriate Alexa-conjugated secondary antibodies and then counterstained with Hoechst 33258 (1/1,000; Sigma-Aldrich, St. Louis, MI, United States).

Image Analysis, Quantification, and Statistical Analysis

Images were handled using ZEN 2.1 (Zeiss, Oberkochen, Germany) and ImageJ software (ImageJ, NIH, United States). Axons and cells were counted manually. All the presented values are means ± SEM unless otherwise stated. Data were statistically processed with nonparametric Mann–Whitney *U* tests for independent two-group comparison.

RESULTS

Development of LPC Model of Focal Demyelination in the Spinal Cord Without Mechanically Induced Axonal Damages

Our aim was to take advantage of innovative intravital imaging modalities to describe the direct and indirect effects of LPC on spinal myelin and axons. In earlier studies (Jeffery and Blakemore, 1995), the vertical needle used to inject LPC in the spinal cord likely produced significant Wallerian degeneration, which occurs even in absence of chemical injection (Dray et al., 2009; Fenrich et al., 2013b). Therefore, we first tried to minimize nonchemical contaminating sources of axonal death in this focal demyelination protocol by changing the penetration angle of the injection pipette from vertical to transversal (**Figure 1**). Two days after phosphate-buffered saline (PBS) 1 × injection (0.7 μL), we then compared the densities of suffering axons in these two conditions based on the presence of retraction bulbs, spherical debris, or wavy morphologies that precede Wallerian degeneration. Although transversal injection improved axonal sparing, PBS delivery through the capillary still resulted into significant axonal degeneration (31% ± 3%, $n = 3$ in transversal injection, vs. 57% ± 4.0%, $n = 3$ in vertical injection; **Figure 1C**). Noteworthy, this degeneration was totally avoided when replacing the intraparenchymal injection by PBS incubation of the spinal cord surface following the calibrated opening of the dura mater. One-hour PBS incubation left axons virtually undisturbed (3% ± 1.5%, $n = 3$), and axonal networks remained stable after 2 days (3.9% ± 0.7%, $n = 3$, **Figure 1D**).

Taking advantage of a simple *in vitro* sciatic nerve preparation, we next established the incubation conditions required to visibly destabilize the myelin sheath. Fresh sciatic nerve slices were incubated in 1% LPC for 30 min to 4 h prior to paraformaldehyde fixation and subsequent imaging by CARS microscopy. Coherent antistoke Raman spectroscopic microscopy is a nonlinear imaging technique revealing the endogenous contrast of lipids based on the vibrational signature of their CH2 bond. The high density of phospholipid chains in myelin sheath is responsible for a significant CARS contrast compared to unmyelinated regions of the CNS tissue. Unhealthy ruffling of myelin sheath was thus observed on 6% of axons after 30-min incubation. It was observed in 85% of axons following a 60-min incubation, and myelin was finally completely destroyed after 3- to 4-h incubation (**Figure 1E**).

A 1-h incubation time was then chosen to conduct intravital experiments in the spinal cord. This exposition produced detectable effect while limiting the overall surgery time prior to the final glass window implantation and the subsequent longitudinal imaging. In brief, LPC 1% was rinsed with PBS after 1 h, and the glass window was sealed before time lapse imaging of the lesion site over 4 h. Our focal incubation model conclusively triggered demyelination over a region that progressively spread to cover a surface that doubled over time (192% ± 13% $n = 4$) (**Figure 1F**) and reaching a depth of 156 μm (±18 μm) below the surface. As highlighted in **Figure 1F2**, lesion surrounding whitish myelin represents the stable myelin whose density is conserved in all time-coded color images. Red areas represent the initial demyelination zone, where myelin was present in the initial images but had disappeared at the time coded in green and blue. A second wave of degeneration was obvious in the green areas were myelin density had strongly declined at the time of blue–pink coding of the images. Because the intensity of CARS signal correlated almost perfectly with the level of MBP labeling on postmortem immunohistological staining (**Supplementary Figure S1**), we concluded that the combination of glass window and CARS imaging was ideal to follow the evolution of myelin degradation over time *in vivo*.

Demyelination-Induced Wallerian Degeneration Following LPC Intoxication

The average CARS signal in a region of interest reported the lipid density irrespective of their distribution into structured myelin sheath or into degenerative myelosomes (Gasecka et al., 2017). The long-term consequences of a 1-h exposition to LPC were next characterized over weeks by 2 Photon-CARS microscopy. The same volume of interest was repeatedly acquired on the same animal at various postincubation times, and we compared the evolution of myelin coverage, with regard to the changes of fluorescent axon densities (**Figure 2**). Whereas 100% of Thy1–CFP axons are usually myelinated in the dorsal spinal cord region, LPC incubation immediately triggered demyelination

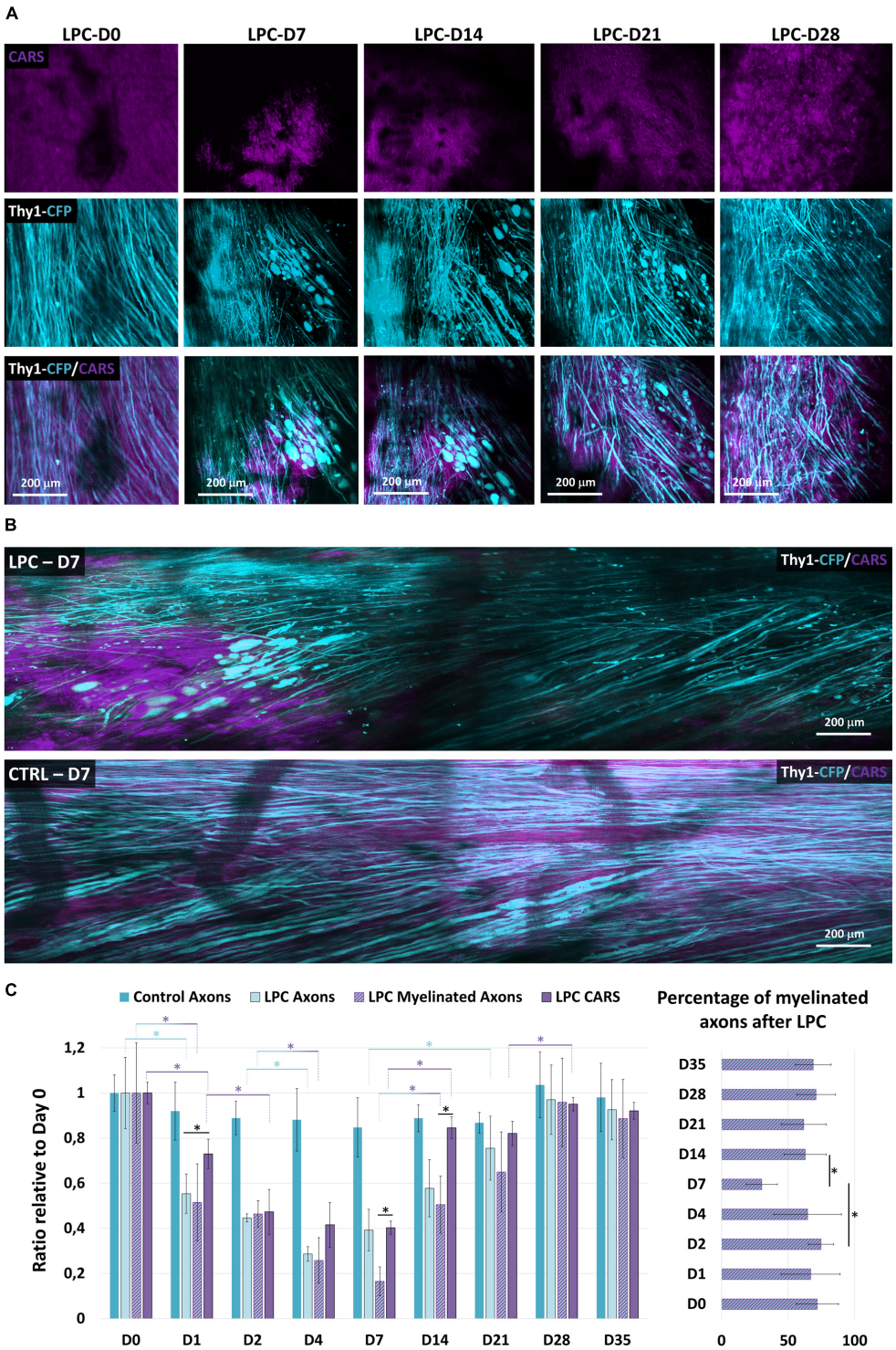

FIGURE 2 | Longitudinal imaging reveals LPC-mediated myelin and axon degenerations and rescue. **(A)** Follow-up of the same LPC incubated zone through the dorsal implanted windows showing myelin content at days 0, 7, 14, 21, and 28 as detected with CARS in myelin sheath (magenta, top) and Thy1–CFP fluorescence (cyan, middle) and overlay (middle). **(B)** Intravital images of dorsal spinal cord 7 days following incubation of LPC or PBS. Note the localized disappearance of CARS signal and of axons selectively for LPC incubation. But not PBS. **(C)** Bar graphs presenting the evolution of the axonal density and the CARS signal both normalized to their value on the first day at days 0, 1, 2, 4, 7, 14, 21, 28, and 35 (we used at least five mice for each condition and for each time; total number $n = 22$). Bar graph showing the evolution of the percentage of myelinated axons over total axons in the receiver operating characteristic curve on days 0, 1, 2, 4, 7, 14, 21, 28, and 35 after LPC incubation. Asterisk indicate statistical significance $p < 0.05$.

that further extended during the following days. On D4, CARS intensity reached a minimal value that represented 42% of the initial intensity (**Figure 2C**). After quantifying the Thy1–CFP axons in a volume of interest representing 400 by 400 μm over a depth of 100 μm, we found that LPC exposure also triggered a brutal axonal degeneration in the first 2 days that left only 44% of the initial axonal density on D2. This initial loss of axons was followed by a second wave of degeneration that brought axonal density to 28% of its initial value on D4 (**Figure 2C**). Axonal degeneration was thus faster and larger than the demyelinating events highlighted by CARS and resulting either from the loss of myelin coverage or the clearance of myelin debris.

Conversely, axons regenerated linearly from D4 to D14, although CARS signal remained stuck at its minimal value in the first week and then promptly recovered during the second week (**Figure 2C**). On D14, however, axonal density represented only 57% of its initial value, whereas CARS signal exhibited an 80% recovery. Both myelin and axons were finally fully recovered by D28 (**Figure 2C**). The delay between the two phenomena suggested that the fate of neuronal and oligodendroglial networks is regulated by independent mechanisms. This idea was further supported by the observation of the delayed remyelination of axons that were already regenerated at D7 (**Supplementary Figures S2A,B**).

Sequential Disruption of Myelin Sheath and Delayed Degeneration of Axons

To clarify the sequence of cellular events responsible for the brutal loss of axons despite a progressive decline of CARS signal, we next looked for subtle changes of myelinated axon morphologies as readouts of the effect of LPC on OLs and their axonal counterpart. Eight typical morphological patterns of degeneration were established from the pool of images acquired from five animals (**Figure 3A**). (1) Both axon and myelin were straight and smooth, two markers of cellular health; (2) axon and surrounding myelin presented a wavy appearance; (3) axon and myelin formed bubble-like swellings; (4) disconnected axonal bubbles inside apparently healthy myelin sheath; (5) small axonal bubbles surrounded with myelin sheath; (6) discontinuous axon bubbles surrounded by myelin sheath; (7) pure myelin debris interleaved with mixed myelin/axons debris; and (8) debris of mixed composition. Immediately after LPC incubation, approximately 33% of axons exhibited a pathological spring-like wavy shape that suggested the likely occurrence of mechanical constraints on otherwise linear healthy axons sheaths (**Figure 3A2**); 14.5% of axons presented neuronal swelling apparently resulting from presumed lipidic constrictions (**Figure 3A3**). One day later, the incidence of spring-like shapes declined in favor of neuronal swelling (**Figure 3A3**), whereas Wallerian-like features (**Figure 3A6**) became the most represented pattern (**Figure 3B**). Wallerian degeneration further proceeded until D2 when fluorescent degenerative axonal bodies became sparser (**Figures 3A7,B**) and when large areas were covered with high background CARS signal overlaid with scattered nonfluorescent liposomes (**Figure 3A8**). The set of morphological patterns mainly observed in the earliest postincubation hours progressively evolved toward a set of patterns mainly observed on the second day postincubation (**Figures 3A,B**) as expected if the described degenerative stages in fact resulted from a morphological continuum of axonal shapes driven by biomechanical constraints.

Time-lapse images over hours conclusively demonstrated the possible evolution from one type of pattern to the next (**Figure 3C**), suggesting a gradual remodeling. Time-coded images of myelin and axons, respectively, highlighted a distortion of the myelin sheath that subsequently induced axonal deformation.

Most axons were in advanced degenerated states (**Figures 3A6–8**) by D2 (**Figure 3B**), a time when the background CARS signal was high despite the sparseness of structured myelin sheath segments (**Figure 3A**). Such degenerative events remained predominant until D7 when they started to decline massively, leaving space to the progressive recolonization by healthy axons (**Figure 3D**). Our results altogether support that early disorganization of myelin structure could trigger axonal fragmentation and neurodegeneration. Axonal recovery finally occurs even before the remyelination process starts.

Inflammatory Cell Infiltration Concomitant With Axon Degeneration in LPC Model

Demyelination has been associated with the activation of phagocytic inflammatory cells (Chu et al., 2019; McMurran et al., 2019). Whether these inflammatory cells contribute to the degenerative processes was investigated by the application of our demyelination model on Thy1–CFP//LysM-EGFP mice. In these mice, monocytes, granulocytes, and macrophages are labeled with EGFP throughout their lifetime in the CNS unless they differentiate into monocyte-derived dendritic cells (Caravagna et al., 2018). We thus quantified in real time the density of these inflammatory cells in the dorsal spinal cord and found that inflammation is significantly increased 4 days after LPC incubation (**Figures 4A,B**). A significant reduction was then transiently observed on D7 prior to a large second accumulation of GFP^+ cells that peaked on D14 and completely resorbed by D28, time of full axonal recovery (**Figure 4B**). Whereas inflammatory response presented significant intersubject variability, we found at all time points that the axonal densities observed in every subject correlated linearly with the corresponding GFP^+ cell densities (**Figure 4C**). Noteworthy, though, axonal densities were all the more preserved in animals for which GFP^+ cell densities were low during the first week; axons were all the more regenerated in animals for which GFP^+ cell densities were high when considering the second and third week (**Figure 4C**). These results therefore suggested that the two peaks of GFP^+ cell densities observed, respectively, on D4 and D14 (**Figure 4B**) corresponded to inflammatory responses with different functional phenotypes.

To further characterize this phenotypic switch, we tested by RT-qPCR on GFP^+ FACS-sorted cells the expression of six proinflammatory genes [tumor necrosis factor α (TNF-α), CD86,

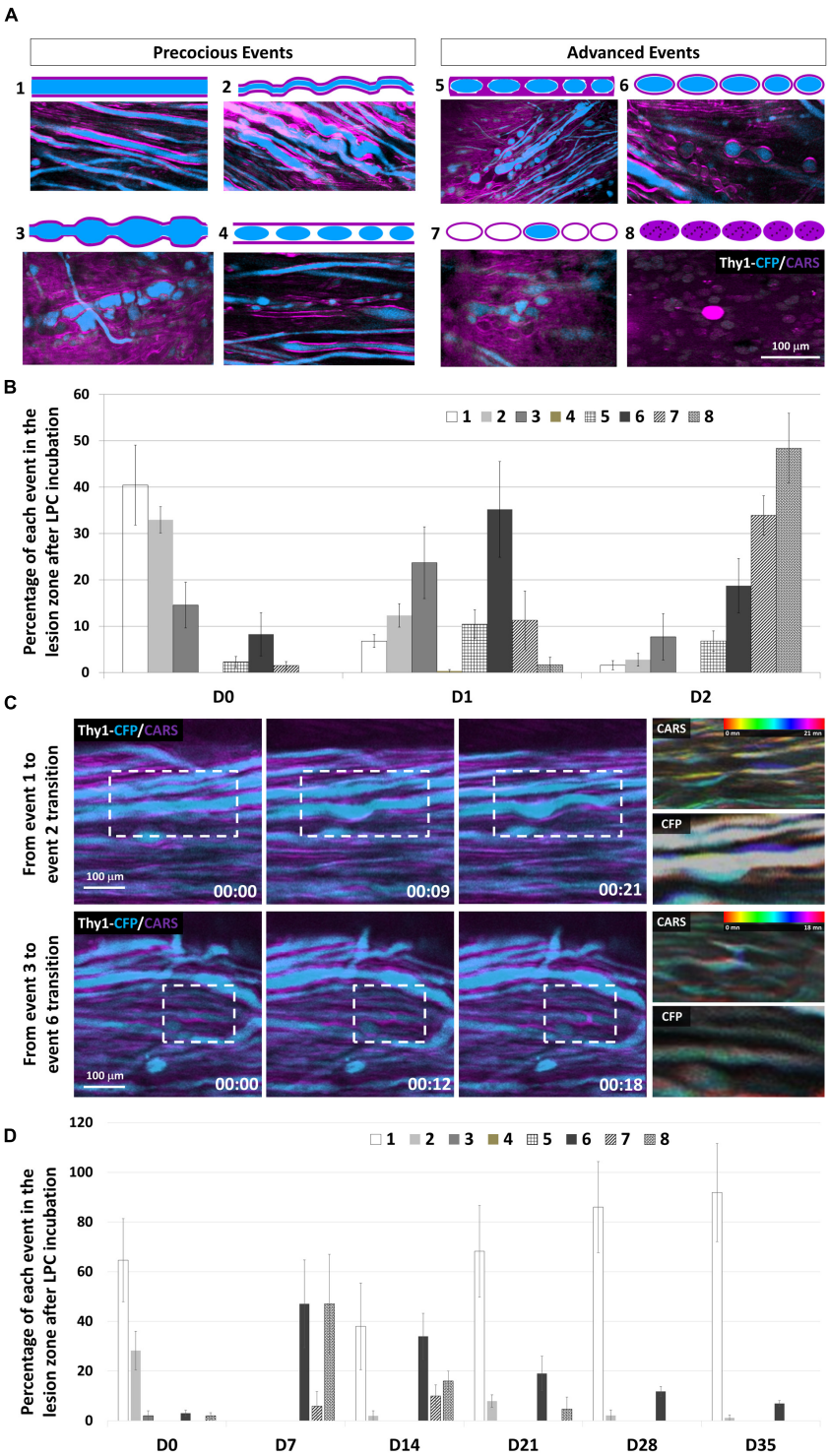

FIGURE 3 | Subcellular effects of LPC incubation on myelin sheath and axons classified according to eight canonical features. **(A)** Intravital images and schematic representations of typical morphological features encountered in the lesion area. CARS signals (myelin, magenta) and Thy1–CFP fluorescence (axon, cyan). **(B)** Graph showing the relative occurrence of each event numbered from 1 to 8 in the lesion zone at days 0, 1, and 2 after LPC incubation (n = 5 mice for each time). **(C)** Outlining intravital time lapse images showing the transitions from event 1 to event 2 (top) and from event 3 to event 6 (Bottom). For each transition, temporal-color coding of the CARS channel and its corresponding CFP channel showing the subcellular morphological evolutions. **(D)** Graph showing the percentage of each event from 1 to 8 in the lesion zone at days 0, 7, 14, 21, 28, and 35 after LPC incubation (we used at least five mice for each condition and for each time; total number n = 13).

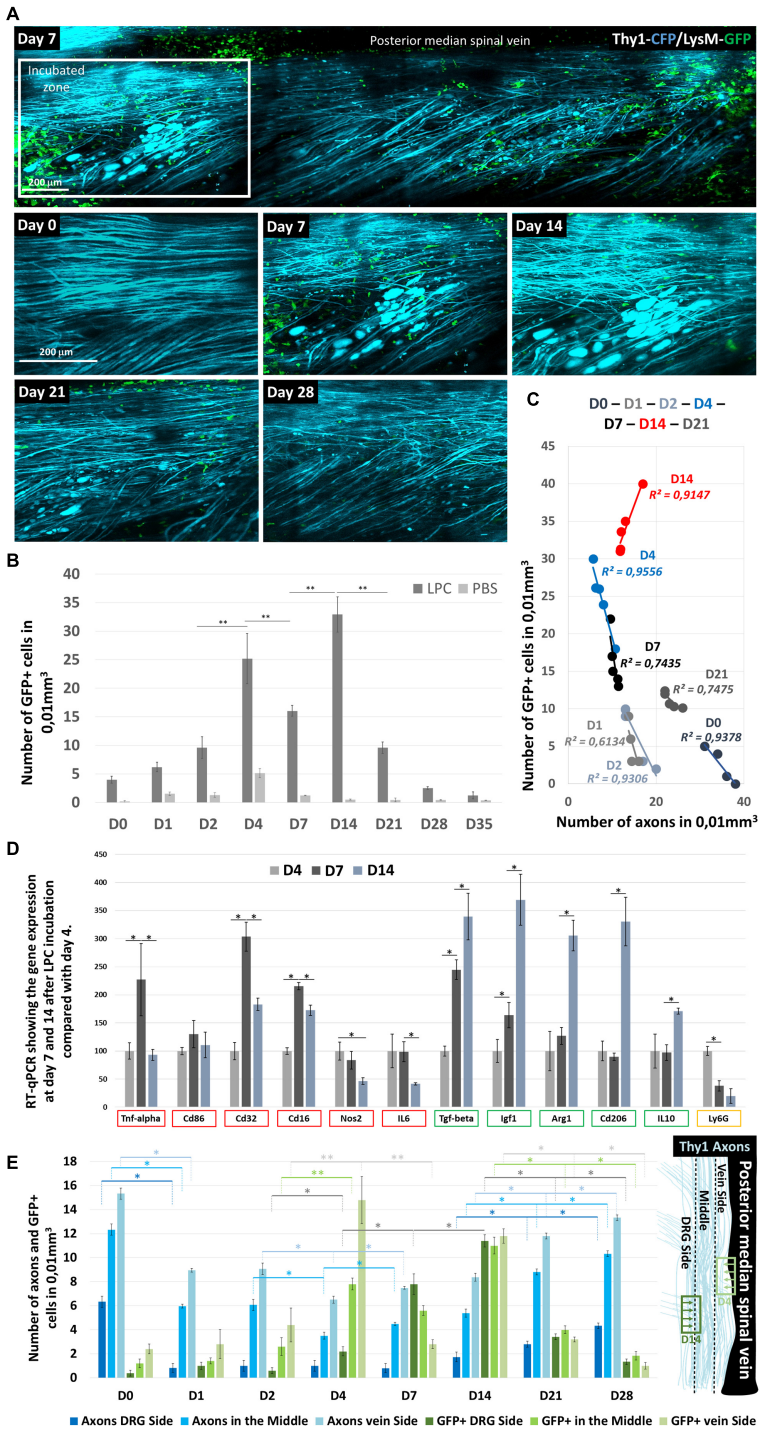

FIGURE 4 | LysM-GFP$^+$ cell infiltration in the Spinal cord and their contribution to axon degeneration. **(A)** Follow-up of the same zone showing LysM-GFP$^+$ cells (green) and Thy1–CFP axons (cyan) at days 0, 7, 14, 21, and 28 after LPC incubation **(B)**. Graph comparing the number of GFP$^+$ cells infiltrating the spinal cord at days 0, 1, 2, 4, 7, 14, 21, 28, and 35 after LPC or PBS incubation (n = 5 mice for each time and each condition). **(C)** Graph showing the correlation between the number of axons counted in a given mouse and the number of GFP$^+$ cells present in the same field of view at days 0, 1, 2, 4, 7, 14, and 21. Each point represents one mouse. Note the reversal of the correlation between D4 and D21 (n = 5 mice for each time and each condition). **(D)** Reverse transcription–qPCR quantifications of proinflammatory gene expression (TNF-α, CD86, CD32 CD16, NOS2, and IL-6), anti-inflammatory gene expression (TGF-β, IGF-1, Arg1, CD206, and IL-10), and neutrophil marker (Ly6G) gene expression, respectively, 7 and 14 days after LPC incubation when normalized to the gene expressions observed on D4. Asterisk indicate statistical significance $p < 0.05$ (n = 3 mice for each time). **(E)** Graphs representing the number of cells in subregions of the ipsilateral spinal cord and the corresponding number of axons counted in the same regions as a function of day after LPC incubation. Regions are defined on the anatomical scheme. Asterisk indicate statistical significance $p < 0.05$; two asterisks indicate statistical significance $p < 0.01$ (we used at least five mice for each condition and for each time; total number n = 22).

CD32 CD16, NOS2, and interleukin 6 (IL-6)] and five anti-inflammatory genes [transforming growth factor (TGF-β), IGF-1, Arg1, CD206, and IL-10], as well the expression of Ly6G as a marker of granulocytes and neutrophils. Proinflammatory genes (**Figure 4D**, red squares) presented a maximal transcription on D7, followed by a significant decline at D14 in particular for TNF-α, CD32 CD16. A significant and continuous decrease over time was instead observed for NOS2 and IL-6. Conversely anti-inflammatory genes presented a systematic increase over time (**Figure 4D**, green squares), whereas Ly6G expression declined over time (**Figure 4D**, yellow square).

The idea of an inflammatory switch was further supported by the existence of two different patterned distributions of cells during the first week (**Figure 4E**). GFP^+ cells mostly spread in the vicinity of the dorsal vein until D4 despite a location of the lesion site close to the dorsal root. On D7, however, most GFP^+ cells were observed close to the dorsal root, to finally distribute themselves equally all over the ipsilateral spinal surface. Interestingly, axon losses between D2 and D4, the peak of GFP^+ infiltration, were all the more important close to the dorsal vein where cells were located. Conversely, the percentage of regenerated axons between D7 and D14 was more important on the side of the dorsal root where GFP^+ cell density was highest (**Figure 4E**).

DISCUSSION

Lysophosphatidylcholine model of demyelination is among the most used model for studying demyelination, the myelin repair, and the way to improve it. So far, LPC was always administered into the brain or spinal cord parenchyma by capillary injection (Hall, 1972; Arnett et al., 2004; McMurran et al., 2019). Localized axonal degeneration was thus mainly attributed to mechanical lesion by injection pipette and hence LPC effect considered as specific to myelin and OLs. In this study, we have developed an LPC model without mechanical injection and monitored cellular interactions at the lesion site *in vivo* over time. Implantation of a unique dorsal glass window on multicolor fluorescent mice (Fenrich et al., 2012) offered longitudinal imaging access to axonal networks, myelin sheath, and immune cells after LPC lesion. Thanks to simultaneous two-photon and CARS microscopies, we evidenced that LPC that initially targets OL is also responsible for axonal degeneration.

We already showed in fixed tissue that CARS microscopy is beneficial for detecting the early changes of myelin in an experimental autoimmune encephalomyelitis (EAE) model of demyelination (Gasecka et al., 2017). We have here implemented intravital CARS microscopy and further showed that the dynamics of myelin coverage contains crucial information to elucidate the mechanism by which LPC triggers axon degeneration as early as D0. This axonal degeneration was unlikely because of direct action of LPC because neurotoxicity was not reported in culture (Vereyken et al., 2009) and because Thy1–CFP^+ axons in the imaged zones are in fact anatomically shielded from extracellular fluids by OLs *in vivo*. Time-coded images of the demyelinating events indicated a clear coincidence between the chemically induced insults to the myelin sheath and the changes in axonal morphology. The mechanical constraints exerted by OLs on axons likely explained the formation of spring-like axonal shapes and subsequent Wallerian degeneration of axons. Degeneration indeed started in the first few hours after exposition and progressed in a two-step process during the following 4 days.

Myelosomes detection by CARS microscopy (Gasecka et al., 2017) contributed to the overall CARS intensity until their complete elimination from the parenchyma. Thus, at late degenerative stages, the strong CARS background was likely explained by the lipid spreading from the degenerated membranes of these myelosomes. As a result, an apparent uncoupling between the kinetics of CARS signal and the kinetics of axonal losses in the lesioned area was observed until D4. Whereas both axonal density and CARS signal recovered to prelesion values within 4 weeks, a similar decorrelation was observed during the recovery phase of these two parameters: recovery was early but slow for axonal density whose recovery started on D7; it was delayed but rapid for CARS signal whose recovery started on D14. Such delayed recovery of the CARS signal might be explained by the requirement for proliferation, recruitment, and differentiation of OPC to replace apoptotic OLs whose GPR17 receptor had been activated (Mayo et al., 2012; Seyedsadr and Ineichen, 2017). In the case of neurons instead, recruitment of progenitor cells is unlikely (Mothe and Tator, 2005). The fact that axons regenerated so early after LPC lesion therefore suggested that neuronal death was probably not involved and that the regenerative axonal sprouting instead occurred as soon as their oligodendroglial and inflammatory environment stopped from being deleterious.

Individual axon imaging confirmed that axonal regeneration started between D4 and D7, whereas, at D7, myelin coverage remaining at the minimal lipid density was present in the environment. Indeed, the ratio of myelinated axons was transiently low at D7, and it increases at D14. Our results therefore confirmed that the regenerative processes were differently and independently regulated by the postlesion physicochemical environment in the case of neurons and OLs. Different chemokines (Balabanov et al., 2007) and cytokines (Cannella and Raine, 2004; Ramesh et al., 2012) are released in the environment at the first sign of OLs suffering. These participate to the activation of microglia and astrocytes and subsequent recruitment of innate immune cells (Domingues et al., 2016). These immune cells can be harmful through the disruption of glutamate hemostasis or the production of nitric oxide and reactive oxygen species (Peferoen et al., 2014; Domingues et al., 2016). Yet they can also have a beneficial role through the release of TGF-b and IL-10, which are anti-inflammatory and prohealing growth factors (Peferoen et al., 2014).

Our results outlined two waves of innate immune cell infiltrations with different phenotypes, the first between D2 and D4 and the second between D7 and D14. The negative correlation between axons and innate immune cell densities observed on

D4, as well as the significant secondary decrease of axon density between D2 and D4, supported the idea that the first wave of infiltration could be harmful to the axons. Similar conclusion was indeed made in an EAE model of inflammation where the initial infiltration of $EGFP^+$ neutrophils triggered the fast degeneration of axons (Caravagna et al., 2018). On the other hand, the significant positive correlation between axons and innate immune cell densities observed among mice on D14, as well as the significant increases of axons between D7 and D14, supported the idea that the second wave of $EGFP^+$ cell infiltration could be beneficial for axonal regeneration. Noteworthy, this second wave was coincident with the fast recovery of CARS signal, as expected from a beneficial impact on remyelination, and despite the only weak impact observed on demyelination for the first wave. As the clearance of myelin debris is a prerequisite for remyelination (Peferoen et al., 2014), we propose that the second wave is responsible for an amplification of the environment cleaning and therefore mainly composed of phagocytic immune cells with rather anti-inflammatory phenotype.

Two characteristic distribution patterns were evidenced for these two waves supporting the existence of at least two different subpopulations. Indeed, $EGFP^+$ cells of the first wave expressed *Nos2* and *IL6* significantly more than $EGFP^+$ cells at D14, and they were significantly more present medially next to the dorsal spinal vein despite a lateral location of the lesion. However, cells of the second wave expressed significantly more anti-inflammatory genes such as *Tgf-beta*, *Igf1*, *Arg1 cd206*, and *IL10*, and they were preferentially found on the DRG side and progressively invaded the lesion. A DRG resident pool of macrophages (Krishnan et al., 2018) was likely recruited in a second stage, while initial inflammatory response was led by vascular neutrophils (Neirinckx et al., 2014).

CONCLUSION

In conclusion, this study shows that LPC exposition can itself cause axon degeneration first as a consequence of oligodendroglial suffering and second due to innate immune cell infiltration. It emphasizes the importance of multimodal nonlinear optical microscopies to characterize the subcellular substrate of myelin plasticity. By pointing out the crucial role of dynamic inflammatory processes, it highlights the requirement of intravital studies to unravel multicomponents physiological response involved in neurodegenerative diseases. On the same animal over time, it finally illustrates the ambivalence of innate immune responses, thereby paving the way to immunomodulatory therapies as a strategy to improve assistance to neurodegenerative patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Comité d'éthique à l'expérimentation animale no 71.

AUTHOR CONTRIBUTIONS

FD and BE designed the animal model, the imaging experiments, interpreted the results, and wrote the manuscript with inputs of all the authors. SB aligned and maintained the multimodal microscope. All authors contributed to image acquisition. BE, EB, and CK did the experiments, analyzed the images, and quantified all the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00165/full#supplementary-material

FIGURE S1 | Comparison of MBP immunolabeling and CARS microscopy. **(A)** CARS signal detection and MBP Immunolabeling on the same coronal sections of the spinal cord at LPC incubation site on D7. Four rectangle areas (dotted lines) were defined on each section: 1, 2, and 3 are outside the LPC lesion while 4 is inside the LPC lesion. **(B)** Graph showing the positive correlation between CARS and MBP signal intensities (n = 3 mice). **(C)** High magnification image in the white matter of the dorsal spinal cord. Scale bars represent 50 μm for **(A)** and 10 μm for **(C)**.

FIGURE S2 | Longitudinal imaging of the fate of individual myelinated axons after LPC incubation. **(A)** CARS and Thy1–CFP signals collected from the same area over 14 days showing individual axon bundle at D0. Note that axonal degeneration was faster and more extensive than the loss of CARS signal. Axonal regeneration was already significant on D7 when myelin coverage was still minimal. Myelination was restored by D14. **(B)** High magnification of the white square in the **(A)**. Scale bars represent 10 μm. **(C)** Multicolor images of the mouse presented in **Figure 2** showing the superimposition of the following channels: CARS signal (Purple), Thy1–CFP^+ axons (Blue), and LysM-GFP^+ cells (Green) 7, 14, and 21 days after LPC incubation.

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Slit1 Protein Regulates SVZ-Derived Precursor Mobilization in the Adult Demyelinated CNS

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Slit1 is a secreted axon guidance molecule, also involved in adult neurogenesis. In physiological conditions, Slit1 loss promotes ectopic dispersal of SVZ-derived neural precursors (SVZ-NPCs) into periventricular structures such as the corpus callosum. Demyelination of the corpus callosum triggers SVZ-NPC migration to ectopic locations and their recruitment by the lesion, suggesting a possible role for Slit1 in SVZ-NPCs ectopic dispersal regulation in pathological conditions. Here, we have investigated the function of Slit1 protein in the recruitment of SVZ-NPCs after CNS demyelination. We find that the dynamics of oligodendrogenesis and temporal profile of developmental myelination in *Slit1*$^{-/-}$ mice are similar to *Slit1*$^{+/-}$ controls. SVZ micro-dissection and RT-PCR from wild-type mice, show that Slits and Robos are physiologically regulated at the transcriptional level in response to corpus callosum demyelination suggesting their role in the process of SVZ-NPC ectopic migration in demyelinating conditions. Moreover, we find that the number of SVZ-NPCs recruited by the lesion increases in *Sli1*$^{-/-}$ mice compared to *Slit1*$^{+/-}$ mice, leading to higher numbers of Olig2^{+} cells within the lesion. Time-lapse video-microscopy of immuno-purified NPCs shows that Slit1-deficient cells migrate faster and make more frequent directional changes than control NPCs, supporting a cell-autonomous mechanism of action of Slit1 in NPC migration. In conclusion, while Slit1 does not affect the normal developmental process of oligodendrogenesis and myelination, it regulates adult SVZ-NPC ectopic migration in response to demyelination, and consequently oligodendrocyte renewal within the lesion.

Keywords: Slit1, neural precursor, oligodendrocytes, recruitment, migration, myelin

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INTRODUCTION

The discovery of on-going neurogenesis throughout life in the majority of mammals (Hinds, 1968; Altman, 1969) as well as the identification of neural stem/precursor cells (NPCs) in the adult rodent (Reynolds and Weiss, 1992), non-human primate (Kornack and Rakic, 2001; Pencea et al., 2001) and human CNS (Kirschenbaum et al., 1994) opens new perspectives for self-repair of brain damage (reviewed in Picard-Riera et al., 2004; Nait-Oumesmar et al., 2008; Okano and Sawamoto, 2008; Kaneko and Sawamoto, 2009; Spigoni et al., 2014). Multipotent and self-renewable precursors are located in the hilus of the hippocampus and the subventricular zone (SVZ) of the

lateral ventricles of the forebrain, as well as in the spinal cord ependyma (Morshead et al., 1994; Weiss et al., 1996; Ernst et al., 2014). *In vitro*, the SVZ-NPCs, self-expand in response to epidermal growth factor (EGF) and fibroblast growth factor (FGF), and differentiate into neurons, astrocytes and oligodendrocytes upon growth factor retrieval (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993). *In vivo*, they retain the capacity to divide and migrate in chain through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they become peri-glomerular and granular neurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994). They are repelled from the SVZ by the choroïd plexus and CSF flow, which triggers their migration toward the OB where they disperse and differentiate into olfactory neurons (Hu, 1999). Within the RMS, SVZ-NPCs follow blood vessels and are surrounded by an astrocytic channel, which guides them from the SVZ to the OB (Snapyan et al., 2009; Whitman et al., 2009; Kaneko et al., 2010). In both focal and multifocal demyelinating models, SVZ neuroblasts (type A) and transit amplifying cells (type C cells) proliferate and are mobilized from their normal pathway of migration to be recruited by the lesion where they generate new oligodendrocyte progenitors (OPCs) to participate with parenchymal OPCs, in myelin repair (Nait-Oumesmar et al., 1999; Decker et al., 2002a,b; Picard-Riera et al., 2002; Menn et al., 2006; Aguirre et al., 2007; Jablonska et al., 2010; Tepavcevic et al., 2011; Xing et al., 2014; Brousse et al., 2015). Demyelination induced ectopic migration is preceded by SVZ proliferation and ependymal modifications suggesting a role for ependymal cells in SVZ stem cell signaling in inflammatory-demyelinating conditions (Pourabdolhossein et al., 2017). Unlike in rodents, in multiple sclerosis, NPCs accumulate in the SVZ suggesting their reactivation but failure of recruitment by nearby lesions (Nait-Oumesmar et al., 2007; Tepavcevic et al., 2011). While little is known about the molecular machinery regulating ectopic migration of the adult SVZ-NPCs, gaining insights into this process could promote their involvement in brain repair.

During development and adulthood, NPC migration from the SVZ is under the control of a variety of growth factors (reviewed in García-González et al., 2010), extra cellular matrix components, cell adhesion and guidance molecules (reviewed in Spigoni et al., 2014). While some of these molecular cues differ between the developmental and adult stage reflecting the maturation of the RMS glial tube (Peretto et al., 2005), the Slit proteins remain major regulators of the SVZ-NPC directed migration toward the OB. The Slits are secreted proteins discovered for their role in axon guidance and cell migration (Blockus and Chédotal, 2016). Three Slits, Slit1, Slit2, and Slit3, were identified in mammals and are all expressed in the developing and adult CNS (Brose et al., 1999; Nguyen-Ba-Charvet et al., 1999, 2004; Plump et al., 2002). Slits bind to transmembrane receptors called roundabout (Robo). In mammals, four Robo receptors were identified, but only two of them, Robo1 and Robo2, bind Slits (Brose et al., 1999; Koch et al., 2011; Zelina et al., 2014). Interestingly, SVZ/RMS NPCs express Robo2 (Marillat et al., 2002; Kaneko et al., 2010) and type A and C cells express Slit1 (Nguyen-Ba-Charvet et al., 2004; Pennartz et al., 2004).

Slit/Robo proteins have multiple functions in non-neural and neural tissues (Blockus and Chédotal, 2016). In the CNS, they regulate midline crossing (Nguyen-Ba-Charvet et al., 1999; Plump et al., 2002; Unni et al., 2012), and are involved in SVZ-NPC migration. Namely, Slits repel SVZ-derived NPCs *in vitro* (Wu et al., 1999; Nguyen-Ba-Charvet et al., 2004; Kaneko et al., 2010), and a gradient of Slit2 in cerebral ventricles, guides NPCs along the RMS toward the OB *in vivo* (Sawamoto et al., 2006). Moreover, Slit1 is likely to facilitate the migration of SVZ-derived cells by reorganizing the RMS astrocytic tunnels (Kaneko et al., 2010, 2018). Finally, the phenotypic analysis of *Slit1* knockout mice ($Slit1^{-/-}$) showed that SVZ-derived NPCs migrate more caudally and radially in Slit1-deficient animals and that $Slit1^{-/-}$ neurosphere-derived cells migrate in a dispersed, rather than in a chain-like pattern (Nguyen-Ba-Charvet et al., 2004).

Although these studies suggest that Slit proteins regulate the direction and modality of migration of the SVZ cells in normal conditions and in a stroke model, their regulation and role in demyelinating conditions are still unknown. $Slit2^{-/-}$ mice and *Slit1/Slit2* double mutant mice die at birth. However, $Slit1^{-/-}$ mice are viable and fertile. Here, we analyzed the function of Slit1 in the recruitment of SVZ-derived NPCs after CC demyelination. In parallel, we analyzed the effects of Slit1 loss on PSA-NCAM purified NPC cell migration *in vitro*. Our data show that Slit/Robo proteins are physiologically regulated in response to demyelination. Moreover, the loss of Slit1 promotes SVZ-NPCs migration *in vivo* and *in vitro* in a cell autonomous manner. These observations indicate that Slit1 plays a crucial role in regulating negatively the SVZ-NPC ectopic mobilization in response to demyelination, and could be a therapeutic target of interest to promote myelin repair by SVZ-derived progeny.

MATERIALS AND METHODS

Animals

Slit1 knockout mice were created by replacing a portion of an exon containing the second leucine-rich repeat domain, located in the 5′ region of the gene, with a targeting cassette containing an internal ribosome entry site (IRES), a tau-green fluorescent protein (GFP) fusion protein and a neomycin resistance gene (Plump et al., 2002). $Slit1^{+/-}$ mice, which NPCs have the same migratory properties than in wild-type animals, were used as controls (Nguyen-Ba-Charvet et al., 2004). Mice were bred at the Institut de la Vision and ICM animal facilities. All experiments were performed in accordance with the European Community regulations, ICM and INSERM ethical committee (authorization 75-348; 20/04/2005).

Lysolecithin (LPC)-Induced Demyelination

To avoid any confounding immune mediated effects, we chose the well-characterized model of LPC-induced demyelination. This model leads to acute demyelination within 2 days post-injection (dpi), SVZ-derived OPC proliferation and recruitment by the lesion peaking at 7 dpi, followed by ongoing differentiation into mature oligodendrocytes at 12 dpi and remyelination within

30 dpi (Nait-Oumesmar et al., 1999; Decker et al., 2002b). To induce demyelination, adult animals (P90) were anesthetized with 100 mg/kg body weight Ketamine (Alcyon) and 10 mg/kg body weight Xylazine (Alcyon) dissolved in 0.9% sterile saline, and positioned in a stereotaxic frame. Animals were injected unilaterally into the CC, using appropriate coordinates (1.5 mm anterior to Bregma, 1 mm lateral and 1.8 mm deep from the skull surface) with 2 µl of a 1% LPC solution (Sigma-Aldrich) in 0.9% NaCl as previously described (Caillava et al., 2011). PBS injected animals served as controls. Animals (3/4 per condition) were sacrificed 4, 6, 12 and 21 days after LPC injection.

Real Time (RT) PCR

Adult female C57BL/6J (P90) were divided in three groups: without lesion, PBS or LPC injected (n = 16 of each phenotype). Seven days after PBS or LPC injection, the lateral SVZ were carefully dissected on coronal forebrain slices and dissociated. RNA was extracted using a Qiagen kit according to manufacturer recommendations (RNeasy Lipid Tissue Mini Kit Qiagen). RNA quantity and quality were evaluated using Nanodrop spectrophotometer (Thermo scientific) and a bioanalyzer, respectively (Bioanalyzer 2100, Agilent Technologies). RNA was then retro-transcribed using Invitrogen kit (Thermoscript RT PCR 11146-016) to obtain cDNA stored at −80°C. RT-qPCR was performed using Taqman method (Platinium PCR Supermix UDG, Invitrogen) according to the manufacturer instructions on ABI Prism 7000 (Applied Biosystems). Expression of *Slit1*, *Slit2*, *Slit3*, *Robo1*, and *Robo2* were quantified using corresponding primers (TaqMan Gene Expression Assays Applied Biosystems) and normalized to TBP level as a reference gene. Quantifications were conducted in triplicate for each gene and repeated in two independent experiments.

In vivo SVZ Mobilization Assay

To assay ectopic mobilization of SVZ-derived progeny into the demyelinated and control corpus callosum, animals were pulse labeled with seven intraperitoneal injections of Bromodeoxy-Uridine (BrdU; 75 mg/kg body weight; Sigma-Aldrich) at 2 h intervals, the day before the stereotaxic injections of LPC/PBS as previously described (Picard-Riera et al., 2002; Tepavcevic et al., 2011).

Tissue Processing for Immunohistochemistry

Lesioned and unlesioned animals were perfused trans-cardially with 4% PFA in cold 0.1 M phosphate buffer (PBS, pH 7.4). Brains were post-fixed in the same fixative for 1 h. Fixed tissues were then cryoprotected in 20% sucrose overnight at 4°C and frozen at −60°C in isopentane cooled in liquid Nitrogen. Twelve µm sagittal sections comprising the SVZ were cut with a cryostat (Leica, CM 3050S) and stored at −20°C for immunohistochemistry.

For immunohistochemistry, sections were incubated at room temperature for 20 min in blocking solution (PBS containing 0.2% Triton X-100 and 4% BSA). Primary antibodies were diluted using the same carrier solution and incubated overnight at 4°C. Primary antibodies were rabbit polyclonal anti-GFP (Merck Millipore) to label GFP expressing cells in *Slit1* mice, rabbit polyclonal anti-Olig2 (Merck Millipore) and anti-Sox10 (R&D System) to label the oligodendrocyte lineage, anti-platelet derived growth factor receptor alpha (PDGF-Rα, Santa Cruz Biotechnology) to label OPCs, mouse monoclonal anti-CC1 (Calbiochem) and rabbit anti-MBP (Sigma-Aldrich) to label differentiated oligodendrocytes, mouse monoclonal anti-PSA-NCAM to label NPCs (AbCys SA), mouse monoclonal anti-Ki67 (BD, Pharmingen), to label cycling cells and anti-Caspase-3 (Cell Signaling) to label cell death. Sections were washed and incubated for 1 h with species-specific secondary antibodies and counterstained with Hoechst (Sigma-Aldrich). Finally, tissue sections were washed in PBS and mounted under coverslips using Fluoromount (SouthernBiotech). Immunohistochemistry for BrdU was performed treating sections for 30 min at 37°C with HCl 2N in PBS containing 0.1% Triton X-100, then rinsed abundantly with PBS. Sections were incubated overnight at 4°C with rat monoclonal anti-BrdU antibody (AbCys SA). For double labeling, sections were first immuno-labeled with antibodies as described above, then post-fixed for 15 min in PFA 4% before BrdU pre-treatment and labeling.

Tissue Processing for Electron Microscopy

Animals (P15 and P90, n = 3 of each group age and phenotype) were intra-cardially perfused with 4% PFA and 2.5% glutaraldehyde (Sigma-Aldrich) in phosphate buffer (PB, NaH_2PO_4, 0.2 M; Na_2HPO_4 0.08 M). Brains were post-fixed in the same solution for 1 h and cut into 100 µm sections with a vibratome (Leica). Sections were then fixed in 2% osmium tetroxide (Electron Microscopy (EM) Sciences) for 30 min. After dehydration, slices were flat embedded in Epon. Ultrathin sections of the area of interest were cut using an ultra-microtome (Ultra-cut E; Reichert-Jung).

Western Blotting

The CC of P90 brains were carefully micro-dissected. Protein extraction was performed using RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 0.1% 2-mercaptoethanol, 1% Triton X-100). A protease inhibitor cocktail (5µl/mL, Sigma-Aldrich) and the reducing agent dithiothreitol (DTT, 0.5 µl/mL, Sigma-Aldrich) were also added. Protein concentration was determined in each sample. Protein extracts were boiled for 5 min before loading onto a 10% polyacrylamide gel (Mini-Protean Precast Gels, Bio-Rad, 40 µg of protein *per* lane). Gels were then electro-transferred to a 0.2 µm PVDF membrane (Bio-Rad). Blots were blocked in 5% milk in Tris–Buffered Saline buffer (TBS, Bio-Rad) for 1 h, and then incubated at 4°C overnight with one of the following antibodies: rabbit-anti-MBP (1:500, Millipore) and rabbit-anti-α-Tubulin (1:3000, Abcam). Bands were detected with the appropriate horseradish peroxide–conjugated secondary antibodies, reacted with chemo-luminescent ECL substrate (GE Healthcare). Band intensities were measured using the ImageJ 1.37c software

(National Institutes of Health, United States). Western blots were performed with the CC from *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$ mice (n = 3/group). Data were averaged and presented as means ± SEM.

Cell Culture

Cerebral hemispheres of P1 homozygous (n = 5) and heterozygous (n = 5) *Slit1*-deficient mice were dissected free of meninges and enzymatically dissociated using trypsin 0.05%. The reaction was stopped with fetal bovine serum (10%) and DNase (5 mg/ml). After centrifugation at +4°C, for 5 min, cells were collected and re-suspended in DMEM/F12 medium (1:1) supplemented with N2 supplement (1%), B27 (0,5%), insulin (25 μg/ml), glucose (6 mg/ml), Hepes (5 mM), bFGF (20 ng/ml), and EGF (20 ng/ml, Peprotech). Cells were sorted by anti-PSA-NCAM magnetic immuno-panning (Miltenyi Biotec) selection and plated on poly-ornithine/laminin. Immuno-selected PSA-NCAM + NPCs were grown for 5 days in floating conditions to generate neurospheres, or as dissociated cells for immuno-characterization or cell tracking.

In vitro Cell Migration Assay

To study migration, neurospheres were plated on a poly-ornithine/laminin substrate (Sigma-Aldrich, 100 μg/ml and 10 μg/ml, respectively). NPC migration was followed by time-lapse video-microscopy (Zeiss AXIOVERT 200) for 12 h (frame every 15 min). At least 10 spheres or 30 cells were followed. Analysis of image stacks was performed using the *Metamorph* software (Ropert Scientific). Cell tracking was performed using Image-J software (National Institutes of Health, United States). Speed of migration, number of directional changes and changes in cell orientation were evaluated for each group. Changes in cell orientation were measured based on the variation of the absolute angle performed by the NPCs and defined as $\Delta\alpha$ [deg] or the angular change between the in-plane components of the most recent displacement vector (pointing from the previous point to the current point of the track) and the preceding displacement vector.

In vitro Immunocharacterization

PSA-NCAM immuno-selected cells of each genotype were plated on poly-ornithine in EGF/FGF supplemented medium to assay purification efficacy. After short-term adhesion (12 h), cells were incubated at RT for 20 min in blocking solution (PBS containing 0.2% Triton X-100 and 4% BSA) fixed with PFA 4%, washed and finally incubated with primary antibodies. Mouse monoclonal anti-A2B5 (mouse IgM hybridoma from ATCC), and anti-NG2 (Chemicon) were used to identify oligodendrocyte progenitors, anti-Nestin (Merck Millipore) to identify stem/precursor cells, anti NeuN (Millipore) to identify neurons, anti-GFAP (Dako) to identify astrocytes. For A2B5 and NG2 immunostaining, cells were labeled before PFA fixation. Primary antibodies were diluted in PBS and incubated 1 h at RT. After primary antibodies, cells were rinsed and incubated for 1 h with species-specific secondary antibodies and counterstained with Hoechst (Sigma-Aldrich). Finally, cells were washed in PBS and mounted using Fluoromount (Southern Biotech).

Imaging and Quantification

For immunohistochemistry, tissue sections were scanned with a Zeiss Axio Scan slide scanner microscope. Images of specific details were acquired using a Zeiss fluorescence microscope equipped with ApoTome 2. The number of Slit1-GFP^{+} cells expressing Caspase-3, or Ki67, or Olig2 combined with CC1 was quantified in defined areas of the SVZ, RMS and CC lesion at 4, 6, and 12 dpi. Quantification of Olig2 and CC1 in the non-injured brain (P15 and P90) was performed in the core of the CC. For each animal, 5,6 serial sections at 180 μm intervals were analyzed.

For electron microscopy, images were taken with a Philips CM 120 electron microscope. The percentage of myelinated axons over total axons and total number of axons at P15 and P90 was determined with the ImageJ software at a magnification of 62 000 for a minimum of 500 axons per animal (n = 2 mice/group).

Statistics

Each n represents one animal or cell sample in the experiment. *In vivo* experiments were performed on a minimum of three mice per group. For the *in vitro* analysis, experiments were performed at least three times with NPC obtained from different dissections and dissociations. Statistical analysis was carried out using GraphPad Prism six software. All values were expressed as mean ± SEM. Normality in the variable distributions was assessed by the D'Agostino&Pearson omnibus test and Grubbs' test was used to detect and exclude possible outliers. For Normality test, means were compared by two-tailed Student's t-test. When one or both groups did not follow a normal distribution, means were compared by two-tailed Mann-Whitney U-test. When different independent groups were compared, we performed a one-way ANOVA plus Tukey's multiple comparison tests. P-values lower than 0.05 were used as a cut-off for statistical significance.

RESULTS

Slit1 Loss Does Not Alter Oligodendrocyte Numbers nor Myelination in the Postnatal and Adult Corpus Callosum

As during post-natal development, CC OPCs arise mainly from the SVZ (Kessaris et al., 2006), Slit1 loss could affect the developmental process of myelination *in vivo*. To identify a possible requirement of Slit1 in the progression of CC myelination, we first quantified, in the intact CC (core) at postnatal day 15 (P15) and 90 (P90), the number of cells expressing Olig2 to identify cells of the oligodendrocyte lineage, CC1, to identify oligodendrocytes, or expressing Olig2 but not CC1, to identify OPCs. This showed no significant difference in the number of the various oligodendroglial populations between the two genotypes with, at P15: 6.8 ± 0.8 vs 5.6 ± 1.2 Olig2^{+}/CC1^{-} OPCs, and 50.4 ± 16.5 vs 50.8 ± 25.7 Olig2^{+}/CC1^{+} oligodendrocytes, for *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$, respectively, and at P90: 2.2 ± 0.6 and 2.2 ± 0.4 Olig2^{+}/CC1^{-} OPCs, and 102.23 ± 15 and 123.70 ± 7.72

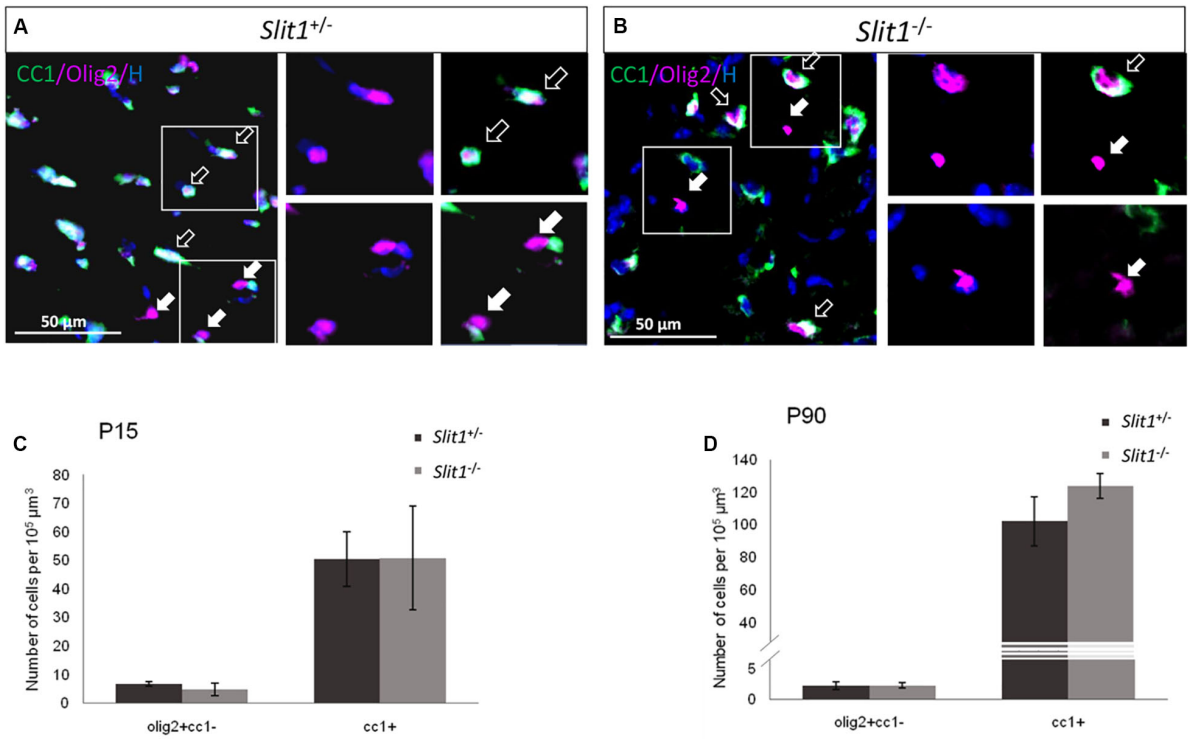

FIGURE 1 | Oligodendroglial differentiation in developing and mature white matter at P15 and P90. **(A,B)** Representative illustrations of Olig2 (red) and CC1 (green) expressing cells (Hoechst, blue), in the corpus callosum of *Slit1*$^{+/-}$ **(A)** and *Slit1*$^{-/-}$ **(B)** mice, at P90. **(C,D)**. Full arrows point to examples of Olig2^{+} OPCs and empty arrows to Olig2^{+}/CC1^{+} oligodendrocytes, Quantification shows no significant difference in the number of the different cell types between *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$ mice at P15 **(C)** or P90 **(D)**. Scale bar, 50 µm. Results are expressed as means ± SEM and analyzed with a Mann Whitney test.

Olig2^{+}/CC1^{+} oligodendrocytes for *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$, respectively (**Figure 1**) suggesting that Slit1 deficiency did not alter oligodendrogenesis. Since oligodendrocytes are responsible for myelin sheath synthesis, with each cell myelinating up to 40 independent axons (Matthews and Duncan, 1971), loss of Slit1 could impact myelin synthesis. To investigate this possibility, we assessed the expression of the myelin basic protein (MBP), a major constituent of myelin (Norton and Cammer, 1984), at P15, during active myelination, and at P90, when myelination is completed. No difference in MBP immuno-reactivity was detected between *Slit1*$^{-/-}$ mutants and controls (**Figures 2A–D**). We further used electron microscopy to analyze myelin fine structure and quantify the number of myelinated axons in the core of the corpus callosum, at P15 and P90. Myelin appeared normal in the absence of Slit1 (**Figures 2E–H**) and the percentage of myelinated axons over total axons in the CC was equivalent between heterozygous and homozygous mice (P15: *Slit1*$^{+/-}$, 79 ± 11%; *Slit1*$^{-/-}$, 79 ± 5%; P90: *Slit1*$^{+/-}$, 92 ± 4%; *Slit1*$^{-/-}$, 89 ± 0.5%) (**Figures 2J,L**). The absence of difference in myelination between the two phenotypes was confirmed at P90, by Western Blot quantifying MBP expression levels and using alpha-tubulin as control (*Slit1*$^{+/-}$, 0.58 ± 0.14; *Slit1*$^{-/-}$, 0.59 ± 0.08; $P = 0.82$) (**Figure 2I**). As Slits also regulate the development of CC axons (Unni et al., 2012), we also examined whether the total number of axons at P90 was modified, but found no difference between *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$ mice (*Slit1*$^{+/-}$, 911.027 ± 32.12 axons/mm^2; *Slit1*$^{-/-}$, 882.551 ± 15.25 axons/mm^2; $P = 0.16$; results were analyzed with a *t*-test).

Transcriptional Regulation of Slits and Robos After LPC-Induced Demyelination of the Corpus Callosum

Previous reports indicated that transcripts/proteins for *Slits* and their receptors *Robos,* are highly expressed in the periventricular and ventricular areas of the adult brain in non-pathological conditions with *Slit1* and *Robo2* and *Robo3* expressed in the adult SVZ/RMS (Nguyen-Ba-Charvet et al., 2004; Kaneko et al., 2018). On the other hand, LPC-induced demyelination activates the SVZ and enhances ectopic migration of SVZ-derived NPCs to the lesion with maximal recruitment by 7 days (Nait-Oumesmar et al., 1999; Decker et al., 2002b). Since the absence of Slit1 induces ectopic migration of SVZ-NPCs in unlesioned animals, we hypothesized that Slit1 could be physiologically regulated in response to LPC-induced demyelination. To investigate this possibility, we first analyzed the expression of Slits and Robos transcripts in the adult SVZ in response to LPC induced demyelination of the corpus callosum at 7 dpi. To this end, the lateral SVZ from non-injected, PBS or LPC injected wild-type mice were carefully micro-dissected from coronal brain sections for RNA extraction (**Figure 3A**). RT-PCR showed that the levels of expression of *Slit1* and *Slit3* transcripts, but not *Slit2*, were

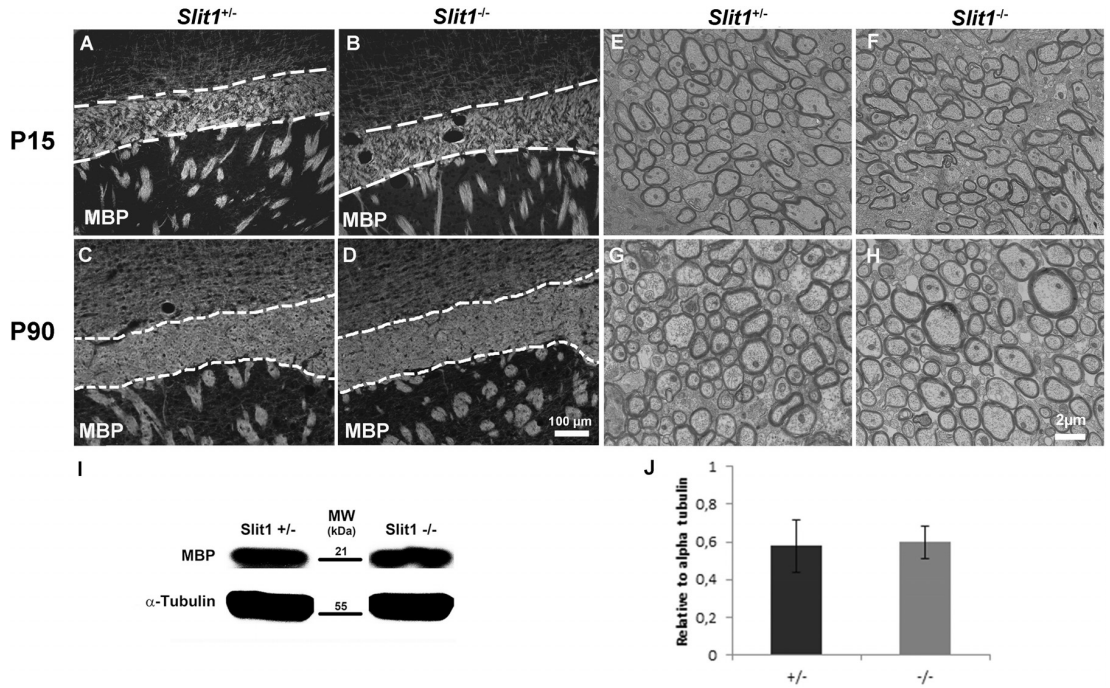

FIGURE 2 | Time-course analysis of developmental myelination. **(A–D)** No difference in MBP immunodetection between *Slit1*$^{+/-}$ **(A,C)** and *Slit1*$^{-/-}$ **(B,D)** mice on corpus callosum (broken lines) frozen section of P15 **(A,B)** and P90 **(C,D)**. **(E–H)** Electron microscopy reveals no difference in the density of myelinated axons or myelin structure between *Slit1*$^{+/-}$ **(E,G)** and *Slit1*$^{-/-}$ **(F,H)** at P15 **(E,F)** and P90 **(G,H)**. Scale bar, 100 µm **(A–D)** and 2 µm **(E–H)**. **(I–J)** No difference in MBP levels by Western blot at P90. Results are expressed as means ± SEM and analyzed with a Student's *t*-test, $^{*}P < 0.05$.

significantly repressed in SVZ cells harvested from LPC injected animals compared to non-injected, and PBS injected animals (**Figure 3B**). This was correlated with a significant decrease in the expression of *Robo2 and Robo3* transcripts, *Robo2* being the preferential partner of *Slit1* in the SVZ (**Figure 3C**).

Slit1 Loss Enhances Ectopic Dispersal and Recruitment of SVZ-NPCs Into the Demyelinated Corpus Callosum

Previous observations indicated that in *Slit1*$^{-/-}$ mice, GFP is expressed by type A (neuroblasts) and type C cells (intermediate progenitors) and that the loss of Slit1 in physiological conditions, promotes SVZ and RMS derived cells into the periventricular structures and especially in the CC (Nguyen-Ba-Charvet et al., 2004, and above results). Mobilized GFP^{+} cells are known to express PSA-NCAM (Nguyen-Ba-Charvet et al., 2004), and GFP expression is down-regulated in SVZ-derived progeny when maturing in glial cells in the corpus callosum, or neuronal cells in the OB (Nguyen-Ba-Charvet et al., 2004; Kaneko and Sawamoto, 2009). To investigate the consequence of *Slit1* deletion on SVZ-progeny ectopic mobilization and recruitment in response to demyelination, and avoid only partial tracking of SVZ-derived progeny due to GFP down-regulation P90 *Slit1*$^{-/-}$ and *Slit1*$^{+/-}$ mice were injected intra-peritoneally with BrdU 1 day before the LPC injection and their brain collected 4, 6, 12 dpi. This method of LPC-induced demyelination of the CC is commonly used to assess SVZ-derived progeny recruitment in lesion sites (Nait-Oumesmar et al., 1999; Decker et al., 2002a,b; Picard-Riera et al., 2004). Immuno-detection of BrdU on sagittal sections at 6 dpi, confirmed the more dispersed migration of PSA-NCAM^{+}/BrdU^{+} cells in *Slit1*$^{-/-}$ animals compared to controls (**Figure 4**). In both genotypes, the majority of GFP^{+} cells expressed BrdU but not all BrdU^{+} cells expressed GFP, supporting the down-regulation of GFP with maturation of mobilized cells.

To further understand the role of Slit1 in SVZ-NPC recruitment by the lesion, we next evaluated the number of GFP^{+}/BrdU^{+} cells in the SVZ, RMS (**Figures 5A,B,E**) and the lesion (**Figures 5C,D,E**) at 4, 6 and 12 dpi into the CC. The recruitment of GFP^{+}/BrdU^{+} cells was maximal at 6 dpi, as previously described (Nait-Oumesmar et al., 1999; Caillava et al., 2011) with a two-fold increase of GFP^{+}/BrdU^{+} cells in the lesions of *Slit1*$^{-/-}$ mice (399 ± 16 cells/mm^2) compared to *Slit1*$^{+/-}$ mice (199 ± 21 cells/mm^2) and a corresponding differential depletion in the SVZ and RMS of *Slit1*$^{-/-}$ mice (SVZ: 685 ± 38 and RMS: 580 ± 15) compared to *Slit1*$^{+/-}$ mice (SVZ: 900 ± 66 and RMS: 720 ± 29) (**Figure 5E**, $P < 0.05$, *t*-test). The enhancement of the SVZ-NPC recruitment into the lesion was specific of demyelinating conditions as recruitment of lower amplitude was observed in the corpus callosum of PBS injected (*Slit1*$^{+/-}$, 0.5 ± 0.002 cells/mm^2, *Slit1*$^{-/-}$, 0.7 ± 0.001 cells/mm^2, $P = 0.22$, *t*-test) and non-injected mice (Nguyen-Ba-Charvet et al., 2004). Because SVZ-derived cells can be recruited by the LPC-induced focal demyelination and differentiate into cells of the oligodendrocyte lineage, we

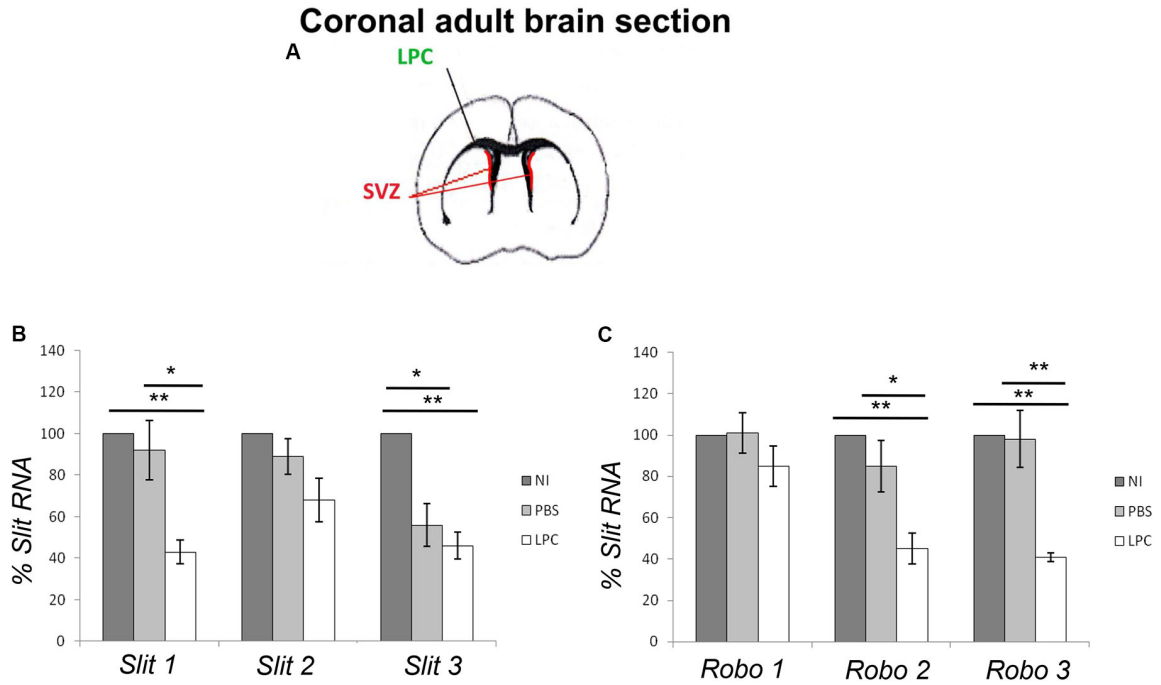

FIGURE 3 | Slit and Robo real-time PCR quantification. **(A)** Representative scheme of a coronal section of the adult brain highlighting the SVZ area selected for the study. **(B)** *Slit1* and *Slit3* expression levels are significantly repressed in SVZ cells from LPC injected animals. **(C)** Slits down regulation is correlated with a significant decrease in *Robo2 and Robo3* expression levels. NI, not injected, LPC, lysolecithin, SVZ, subventricular zone. Results are expressed as means ± SEM, analyzed with one-way ANOVA plus Tukey's multiple comparison tests $*P < 0.05$, $**P < 0.01$ and are reported to the percentage of non-injected controls.

investigated the impact of Slit1 deficiency on these events. Triple detection of BrdU, GFP and Olig2, indicated that parenchymal Olig2$^+$ cells of the CC did not express GFP. Moreover, oligodendroglial cell recruitment was enhanced significantly by two-folds at 6 dpi in response to demyelination in the absence of Slit1 (81.2 ± 2.9 cells/mm^2) compared to *Slit1*$^{+/-}$ mice (41 ± 4.9 cells/mm^2; $P < 0.05$, *t*-test; **Figure 5F**). These findings further imply that Slit1 is an important negative modulator of ectopic migration and recruitment by the lesion of SVZ-derived progeny including those committed toward the oligodendrocyte lineage.

Slit1 Loss Does Not Affect NPC Proliferation, nor Cell Death in the Adult Corpus Callosum After LPC-Induced Demyelination

In the LPC model, demyelination is completed within 2 days after toxin injection, and is followed by increased cell proliferation in both the adult SVZ/RMS and the lesion during the first week after LPC injection (Nait-Oumesmar et al., 1999; Caillava et al., 2011). Although the sole absence of Slit1 does not affect proliferation in the adult SVZ, in normal conditions (Nguyen-Ba-Charvet et al., 2004; Borrell et al., 2012), we investigated the possible role of Slit1 in the proliferation in the adult anterior SVZ, and RMS of adult *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$ mice, in response to demyelination. To this end, we quantified the number of GFP/Ki67 double-positive cells at 4, 6 and 12 dpi (**Supplementary Figure S1**). As expected, proliferation in the SVZ/RMS was maximal at 4 dpi with a two-fold reduction of the total number of GFP$^+$/Ki67$^+$ cells proliferating in the SVZ, RMS and lesion, at 6 dpi and five to six-folds reduction at 12 dpi compared to 4 dpi of both groups. However, there was no statistical difference between *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$ mice for each location, at all time points analyzed (**Supplementary Figures S2M–O**). These results suggest that adult SVZ-NPC proliferation after CC demyelination is either Slit1 independent or efficiently compensated by other Slits.

Since the increase in ectopic migration, observed at 6 dpi, in *Slit1*$^{-/-}$ brains could also have resulted from altered cell survival in response to demyelination, we assessed the potential contribution of Slit1 to apoptosis. Caspase-3$^+$ cells were quantified in the SVZ, RMS, and lesion at 6 dpi of LPC (**Supplementary Figure S2**). The number GFP$^+$/Caspase-3$^+$ cells was very low and not different in both groups (<0.01% of cells).

These observations rule out a possible contribution of altered proliferation or survival in the enhanced recruitment of SVZ-derived NPCs by the lesion resulting from Slit1 loss.

Slit1 Function in NPC Migration Is Cell Autonomous

It has been suggested that the role of Slit1 in SVZ/RMS cell migration is primarily non-autonomous (Kaneko et al., 2010). We previously demonstrated *in vitro* that neurospheres obtained

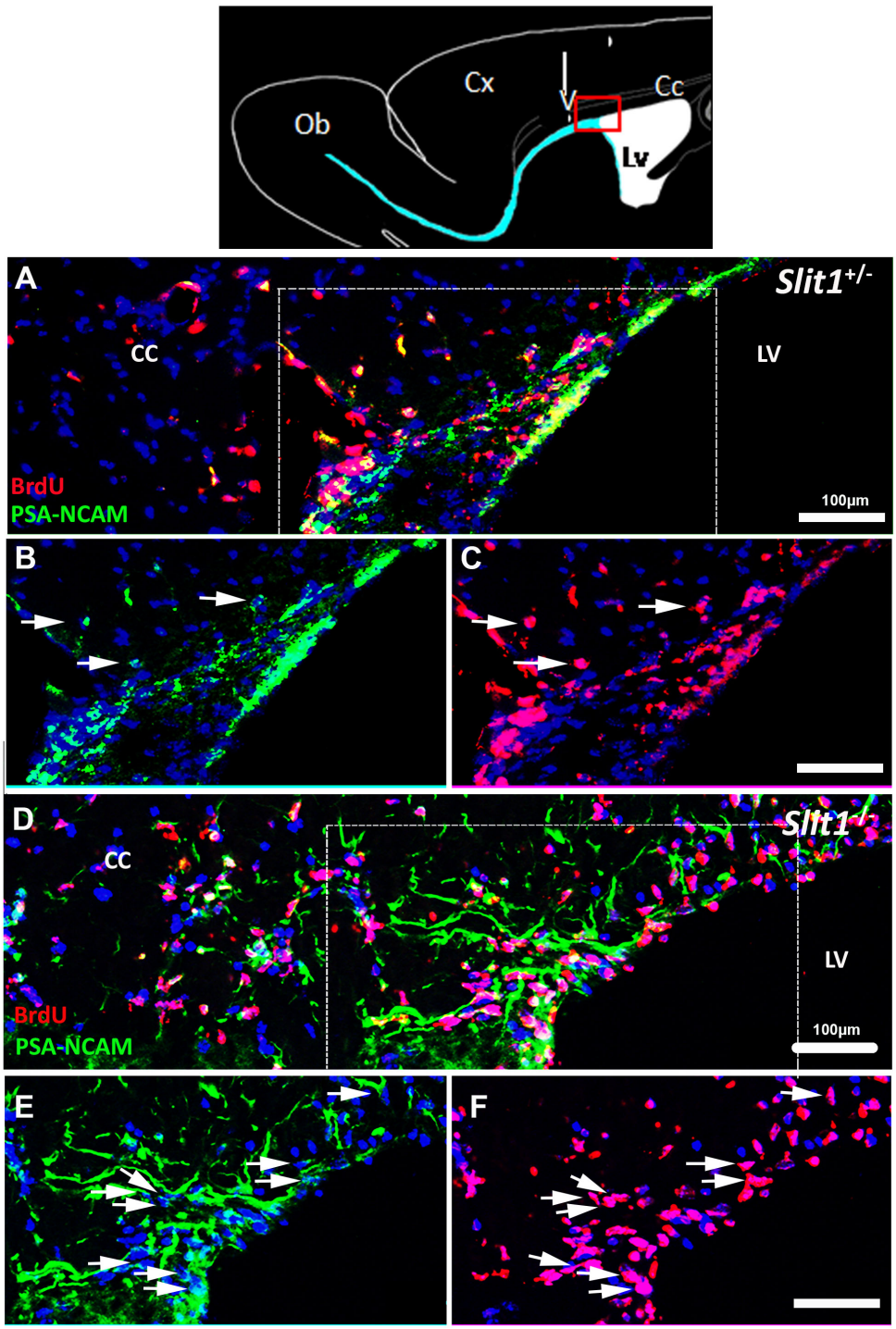

FIGURE 4 | Dispersal of SVZ-derived progenitors into the corpus callosum in response to LPC- induced demyelination of the CC. The top scheme represents a forebrain sagittal section with the area of interest (red box), and the area of LPC injection (arrow). **(A–F)** The lesion is out of the field, and localized in the upper left corner of the images. The SVZ-derived progeny was identified by double labeling for BrdU and the NPC marker, PSA-NCAM at 6 dpi. $BrdU^{+}$/$PSA\text{-}NCAM^{+}$ cells are more dispersed in the demyelinated corpus callosum in *Slit1*$^{-/-}$ **(D–F)** compared to *Slit1*$^{+/-}$ **(A–C)** mice. **(B,C)** and **(E,F)** are single channels and enlarged views of A and D, respectively, illustrating that the majority of BrdU traced cells (red) express PSA-NCAM (green). Scale bar, 100 µm. LV, lateral ventricle; CC, Corpus Callosum.

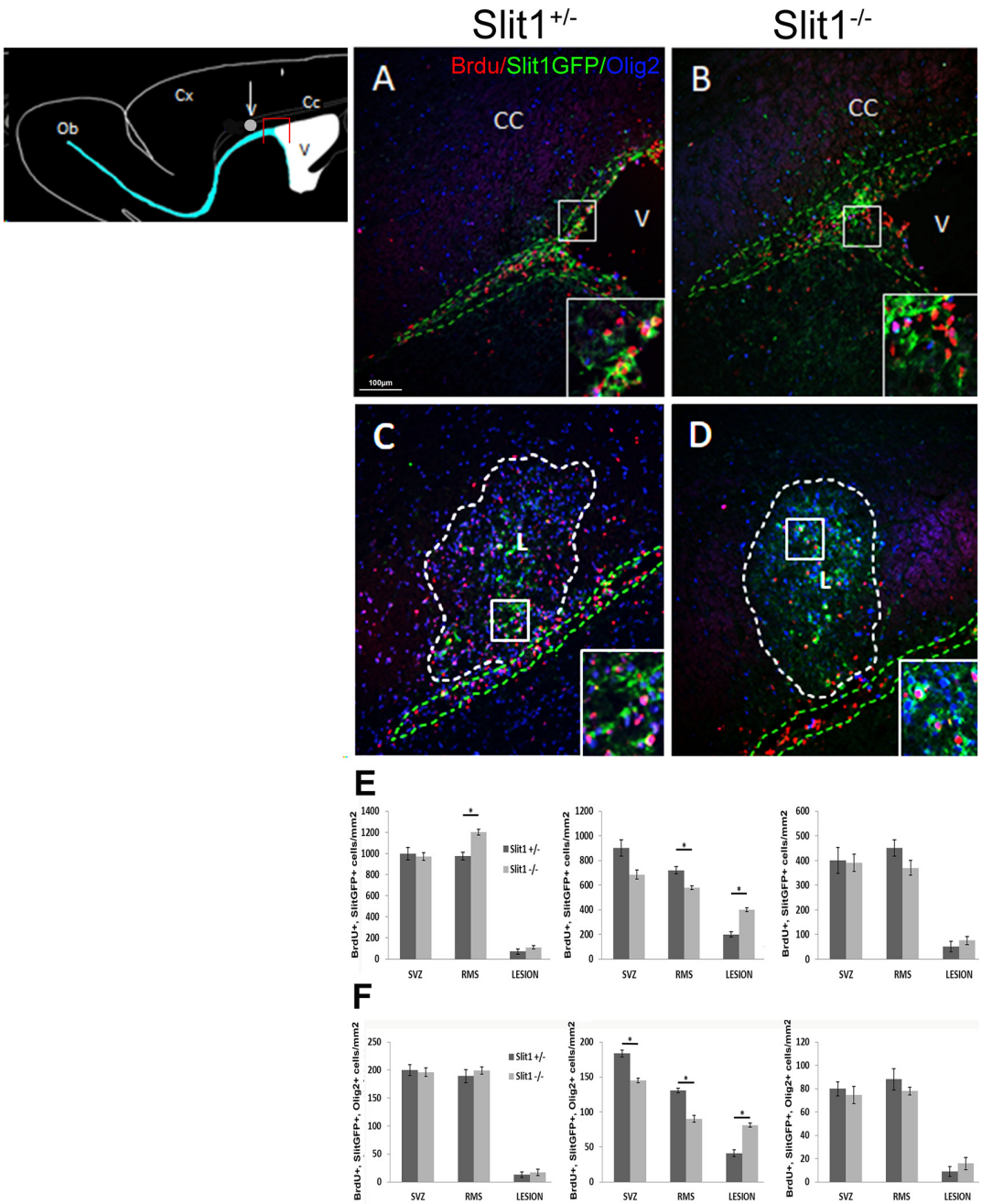

FIGURE 5 | Recruitment of the SVZ-derived progeny by the LPC-induced lesion of the corpus callosum at 6 dpi. The scheme represents the areas of interest: the lesion (white circle) and the SVZ (red box). **(A–D)** illustrates the presence of BrdU$^+$ and Slit-GFP$^+$ cells on sagittal forebrain sections at the levels of the **(A,B)** SVZ and **(C,D)** corpus callosum lesion of *Slit1*$^{+/-}$ **(A,C)** and *Slit1*$^{-/-}$ **(C,D)** mice. Insets are higher magnifications illustrate Slit-GFP$^+$ cells expressing BrdU. **(E,F)** Quantification at the level of the SVZ, RMS and lesion at four (left graphs), six (middle graphs) and 12 dpi (right graphs) indicates that BrdU$^+$/Slit1-GFP$^+$ **(E)** and BrdU$^+$/Slit-GFP$^+$/Olig2$^+$ **(F)** cells colonize the lesion more efficiently in *Slit1*$^{-/-}$ animals, than in *Slit1*$^{+/-}$ controls. Note that parenchymal Olig2$^+$ cells including adult OPC, do not express GFP. Insets illustrate BrdU$^+$/GFP$^+$ cells at higher magnifications. Broken lines in green delineate the SVZ **(A,B)** and RMS **(C,D)** and in white, define the lesion **(C,D)**. V, lateral ventricle; CC, Corpus Callosum, L, lesion. Scale bar, 100 µm. Results are expressed as means ± SEM and analyzed with a Student's *t*-test, * = $P < 0.05$.

from *Slit1*$^{-/-}$ newborn mice migrate abnormally, when seeded on poly-ornithine/collagen, for 5 days, in medium containing or not, EGF and FGF-2 (Nguyen-Ba-Charvet et al., 2004). However, neurospheres are a mixture of stem/progenitor cells, which render the data interpretation complex with respect to cell autonomy. Since Slit1 expression is prominent in

PSA-NCAM$^+$ progenitors (Pennartz et al., 2004), and in order to use an homogeneous cell population, we re-addressed the function of Slit1 in NPC migration using purified preparations of immuno-selected PSA-NCAM$^+$ NPCs. We first examined the antigenic profile of the purified PSA-NCAM$^+$ NPCs after short-term culture, using the NPC markers, Nestin, and PSA-NCAM, the combined OPC markers NG2/A2B5, the astrocyte marker GFAP, and neuronal marker NeuN. The majority of *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$ PSA-NCAM sorted NPCs (identified by Hoechst staining) expressed the Nestin (91 ± 1.6 vs 90 ± 0.5%) and PSA-NCAM (94 ± 5.5 vs 90 ± 2.1%), with few cells expressing NG2/A2B5, and none expressing GFAP and NeuN. Migration was followed by time-lapse video-microscopy plating either PSA-NCAM$^+$ spheres or dissociated cells on a poly-ornithine/laminin substrate. *In vivo* analysis of sphere-derived cells indicated a greater dispersion of NPCs in the SVZ/RMS of *Slit1*$^{-/-}$ compared to controls suggesting that they were miss-oriented. To gain insights into the pattern of migration, we analyzed the number and angular amplitudes of directional changes performed by the NPCs. Spheres were used to induce radial chain-like migration (**Figures 6A,B**). Quantification of migration speed of dissociated PSA-NCAM$^+$ NPCs showed that *Slit1*$^{-/-}$ NPCs, migrated significantly faster than *Slit1*$^{+/-}$ cells at all times tested (**Figure 6C**). NPCs derived from *Slit1*$^{-/-}$ spheres changed their direction more frequently (6.5 ± 1.5 times per cell) for *Slit1*$^{+/-}$, vs 8 ± 2.0 for *Slit*$^{-/-}$; $P = 0.02$, *t*-test) and had significantly larger angular amplitudes ($\Delta\alpha$ deg of *Slit1*$^{+/-}$, 30.47 ± 4.93; *Slit1*$^{-/-}$ 46.35 ± 2.98, $P = 0.01$, *t*-test) compared to *Slit1*$^{+/-}$-derived NPCs (Movie, **Figure 6D**). These data indicate that Slit1 function in NPC migration is cell autonomous.

DISCUSSION

Remyelination failure may result from defects in proliferation, migration and/or differentiation of cells of the oligodendroglial lineage. Therefore the identification of extrinsic and intrinsic factors governing progenitor cell proliferation, migration and differentiation into oligodendrocytes is crucial to elucidate the mechanisms of postnatal neurogenesis and gliogenesis. Because adult SVZ-OPCs and NPCs contribute to myelin repair in the mammalian forebrain (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002; Menn et al., 2006; Aguirre et al., 2007; Jablonska et al., 2010; Xing et al., 2014; Brousse et al., 2015), identifying these factors is also essential to understand spontaneous remyelination, which in diseases such as MS is insufficient to ensure clinical recovery. Although our knowledge of signals that regulate SVZ-derived OPC and NPC cell proliferation and differentiation is quite extensive, those that regulate SVZ cell migration/recruitment are less well understood. More specifically, the role of Slit proteins in SVZ ectopic cell migration in response to demyelination has not been addressed.

Here, we used *Slit1* knockout mice to investigate the functional role of this axon guidance molecule in NPC/OPC migration in myelination and in response to demyelination. Combining *in vitro* and *in vivo* experiments, we provide evidence that Slit1 loss alters adult NPC/OPC migration *in vitro* and *in vivo* under pathological conditions without affecting NPC cell proliferation, survival or developmental myelination.

Corpus callosum OPCs arise mainly from the SVZ during post-natal development (Kessaris et al., 2006). We first show that Slit1 loss has no effect on oligodendrocyte differentiation during early postnatal development since the relative proportions of OPC/mature oligodendrocytes were identical in control and *Slit1*$^{-/-}$ mice. This is further supported by the normal levels of the MBP protein, number of myelinated axons and g ratios in *Slit1*$^{-/-}$ mice. This absence of developmental myelination defects in *Slit1*$^{-/-}$ mice might result from compensatory mechanisms as other Slits are expressed during forebrain development (Marillat et al., 2002; Plump et al., 2002).

Slits/Robos proteins can act as repellents and are expressed by NPCs. We hypothesized that the expression of these proteins could act as a brake in type A and type C cells slowing down their exit from the SVZ/RMS. Our previous work showed that in physiological conditions Slit1 disruption disorient SVZ precursors from their olfactory bulb destiny (Nguyen-Ba-Charvet et al., 2004). The present findings strengthen the hypothesis that disruption of Slit1 function has a positive impact on NPC ectopic migration into the corpus callosum, leading to enhanced cell recruitment by the lesion. This effect was specific, as the lack of Slit1 did not affect cell proliferation or survival. Moreover, it was specific of SVZ-derived progeny as Slit-GFP is not express by parenchymal OPCs (see **Figure 5**). This is in contrast with molecules such as Anosmin1, which are expressed by SVZ-NPCs and OPCs, and, which modulation affects the development of both populations including oligodendrocyte differentiation and myelination (Murcia-Belmonte et al., 2016). The role of Slit1 in preventing NPC migration from the SVZ in normal conditions was further supported by the fact that under pathophysiological conditions, *Slits* and *Robos* are down-regulated in the SVZ at the transcriptional level, 7 days post demyelination, and thus concomitantly with NPC recruitment at the lesion. Of particular interest was the specific down regulation of *Slit1* and *Robo2*, which act as partners within the SVZ/RMS system (Kaneko et al., 2018). These observations are consistent with the idea that Slit1 in normal conditions prevents SVZ cell mobilization to ectopic locations and that, consequently, they are down regulated to allow NPC ectopic dispersal from the SVZ in response to demyelination.

In the absence of Slit1, SVZ-derived NPC migrate in a more dispersed mode toward the lesion than control NPCs suggesting that Slit1 plays a role in NPC directed migration. This was confirmed by the time-lapse analysis of MACS-sorted PSA-NCAM$^+$ NPC cell migration as changes in both cell orientation and direction were observed in the absence of Slit1. As these purified NPC cultures were devoid of astrocytes or any other cell types, our data imply that directed migration/orientation of NPCs acts through a cell-autonomous mechanism involving possibly the interaction between Slit1 and Robos expressed by

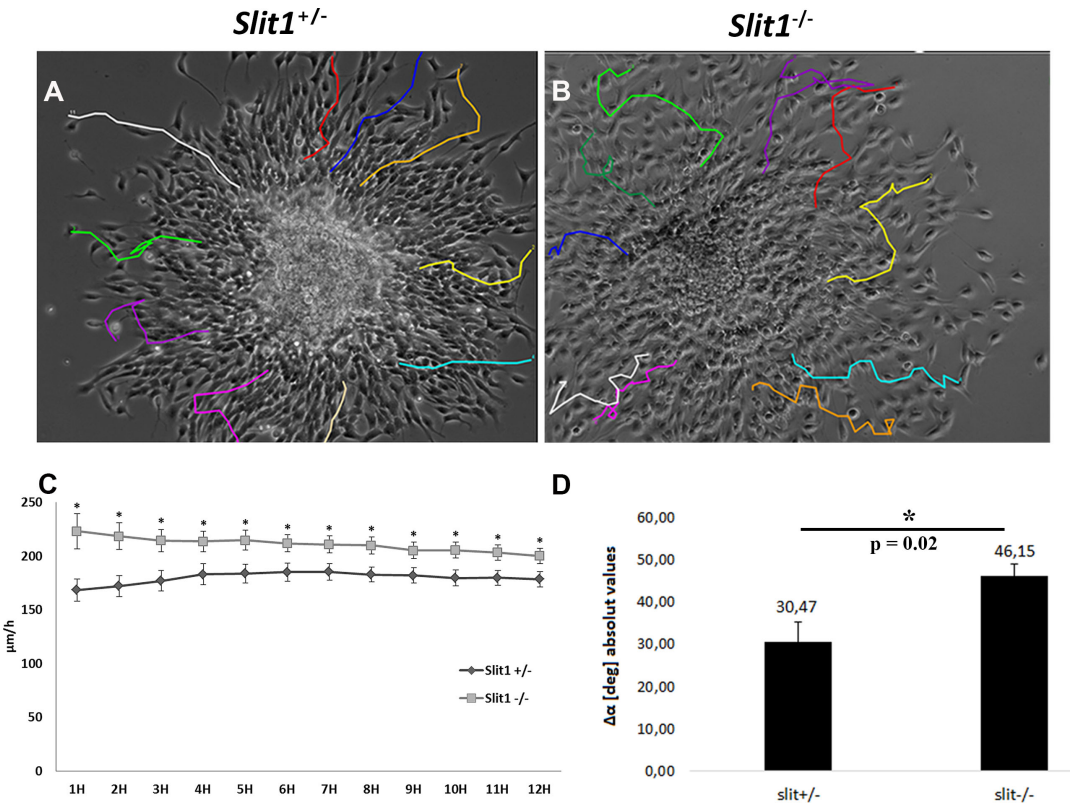

FIGURE 6 | *Slit1*$^{-/-}$ Migration modalities of NPCs by time-lapse video recording. PSA-NCAM immuno-selected precursors were plated as neurospheres **(A,B,D)** or as dissociated cells **(C)** on Poly-ornithine/laminin. Cell migration was followed by time-lapse video microscopy during a period of 12 h. **(C)** NPCs from *Slit1*$^{-/-}$ animals showed a higher speed of migration than controls. **(D)** NPCs escaping from neurospheres show significant changes in amplitude direction. Results are expressed as means ± SEM and analyzed with a Student's *t*-test, **(C)****P* < 0.05, **(D)****P* = 0,02.

sister cells (Nguyen-Ba-Charvet et al., 2004). Our results thus challenge those provided by Kaneko and colleagues, suggesting that NPC migration in the adult brain acts via a non-autonomous mechanism resulting solely from the interaction of neuroblasts with astrocytes expressing Robo and Slit, respectively (Kaneko et al., 2010). Our *in vitro* data provide additional support for a role for Slits as intrinsic regulators of NPC directed migration, without excluding an additional role as an extrinsic regulator of NPC migration across astrocytes. Recent data indicate that Slit-Robo signaling, mediates rapid and dynamic changes in the actin cytoskeleton of reactive astrocytes to maintain the route for neuronal migration toward a lesion induced by stroke (Kaneko et al., 2018). While astrocyte reactivity is also triggered by LPC-induced demyelination, lesions are acute, and far less severe than stroke, inducing a minor scar after NPC/OPC recruitment, suggesting a minor impact of the astrocyte Slit-Robo regulation during NPC recruitment by the LPC-lesion.

Several studies unraveled the presence of cellular and molecular cues regulating ectopic migration/recruitment of SVZ-derived NPC in pathological conditions (Spigoni et al., 2014). Such cues are mainly present in the environment (extrinsic cues) and include sequentially, disruption of the astrocyte furrow liberating neural progenitors from their physical constrain (Nait-Oumesmar et al., 1999), lesion-induced vascular remodeling via Netrin1, guiding and promoting progenitor emigration to the demyelinating site (Cayre et al., 2013), and glial-derived neurotrophins such as CNTF (Vernerey et al., 2013) and chemokines such as SDF1 via CXCR4 (Imitola et al., 2004) attracting the emigrating progenitors to the lesion. Only few reports indicate the role of intrinsic cues. Polysialylated residues (PSA) on NCAM by facilitating homophilic NPC interactions (Hu et al., 1996), enhances NPC migration to the olfactory bulb and prevents their efficient recruitment by the lesion (Decker et al., 2002a). In physiological conditions, Slit1 orients NPCs toward the OB, refraining (negative regulator) their dispersion away from the SVZ/RMS niche. Here we show, that their genetically programmed down-regulation in demyelinating conditions in the presence of PSA-NCAM expression, is sufficient to disorient NPCs and contribute with vascular remodeling and lesion-derived chemo-attractants, to their efficient recruitment by the lesion.

Very little is known about the molecular mechanisms down-regulating Slit and Robo expression in response to demyelination. These may include unknown factors of the environment and/or

the SVZ-progeny maturation status, decreasing Slit1 expression with cell differentiation (Kaneko et al., 2018). One possibility could be that the lesion changes the local expression of molecules such as FGF and Wnts both of which influence oligodendrocyte production in the SVZ (Chavali et al., 2018; Kang et al., 2019) and can modulate the expression of *Robo* and *Slit* genes (Borrell et al., 2012). The lesion might also perturb neuronal activity locally and it is known that for example, during development, neuronal activity can also modulate Robo expression (Mire et al., 2012).

Slit proteins could play redundant roles. Our analysis by RT-PCR shows that all *Slit* and *Robo* transcripts were down regulated in response to corpus callosum demyelination. *Slit2*, which is expressed by periventricular tissue (septum and choroid plexus) was not significantly down regulated in response to LPC. *Slit3* was significantly repressed. However, it is minimally expressed by SVZ precursors and does not bind the major SVZ-NPC *Robo2* partner. These observations suggest that *Slit2* or *Slit3* are unlikely to act as redundant partners in the regulation of SVZ-ectopic migration in response to LPC. Despite the potential redundant function of the Slit/Robo family during forebrain development, *Slit1* and *Robo2* transcripts, which proteins are exclusively expressed by the adult SVZ (Nguyen-Ba-Charvet et al., 2004; Kaneko et al., 2010, 2018) were significantly down-regulated. Therefore, the present study highlights for the first time a crucial role for Slit1 as a regulator of adult NPC ectopic migration in response to demyelination. Although future studies should investigate the regulation of Slits/Robos in a more relevant animal model of MS and MS tissue, the present findings could contribute to a better understanding of remyelination failure in MS and be crucial to design novel therapeutic approaches targeting Slit/Robos to enhance the SVZ contribution to CNS remyelination in demyelinating diseases.

DATA AVAILABILITY STATEMENT

The Western blot data used to support the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

Experiments were performed according to European Community regulations and INSERM Ethical Committee (authorization 75-348; 20/04/2005) and were approved by the local Darwin Ethical Committee.

AUTHOR CONTRIBUTIONS

CC, GS, BG-D, CB, and AB-V contributed conception and design of the study. GS and CC organized the database. CD, GS, BG-D, and NS performed the statistical analysis. AY expanded and provided transgenic animals, AB-V wrote the first draft of the manuscript. CC, GS, BG-D, NS, AY, and AC wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00168/full#supplementary-material

FIGURE S1 | Proliferation of Slit1-GFP cells in response to demyelination. The orientation of SVZ and lesion is similar to the scheme of Figure five but tilted at 90° to the right. **(A–L)** Representation of double labeled Ki67, Slit1GFP at 6 dpi **(A–F)** in the SVZ, and **(G–L)** in the lesion. Insets are enlargements of Ki67/GFP double-labeled cells in C, F, I, and L, respectively. **(M–O)** Quantification of double-labeled Ki67/Slit1GFP cells indicates an increase of proliferation in the SVZ and the lesion at 4 dpi compared to 6 and 12 dpi but no differences in proliferation between *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$ mice. Broken lines delineate the SVZ in **(A–F)**, and the lesion in **(G–L)**. V, lateral ventricle; CC, Corpus Callosum; L, lesion. Scale bar 50 μm. Results are expressed as means ± SEM and analyzed with a Student's *t*-test.

FIGURE S2 | NPC cell death in response to demyelination in the adult brain. The orientation of SVZ and lesion is similar to the scheme of **Figure 5** but tilted at 90° to the right. **(A–D")** At 6 dpi, apoptotic cells identified by Caspase three immuno-labeling, were not found in the adult SVZ/RMS **(A–B")** nor in the demyelinated CC **(C–D")** of *Slit1*$^{+/-}$ and *Slit1*$^{-/-}$ mice. **(E–F")**, enlarged view of Caspase3^{+} cells in the olfactory bulb of the same mice. Broken lines delineate the SVZ **(A–B")**, and the lesion **(C–D")**. LV, lateral ventricle; CC, Corpus Callosum; L, Lesion. Scale bar 100 μm **(A–D")** and 10 μm **(E–F")**.

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REVIEW
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That's a Wrap! Molecular Drivers Governing Neuronal Nogo Receptor-Dependent Myelin Plasticity and Integrity

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Myelin is a dynamic membrane that is important for coordinating the fast propagation of action potentials along small or large caliber axons (0.1–10 μm) some of which extend the entire length of the spinal cord. Due to the heterogeneity of electrical and energy demands of the variable neuronal populations, the axo-myelinic and axo-glial interactions that regulate the biophysical properties of myelinated axons also vary in terms of molecular interactions at the membrane interfaces. An important topic of debate in neuroscience is how myelin is maintained and modified under neuronal control and how disruption of this control (due to disease or injury) can initiate and/or propagate neurodegeneration. One of the key molecular signaling cascades that have been investigated in the context of neural injury over the past two decades involves the myelin-associated inhibitory factors (MAIFs) that interact with Nogo receptor 1 (NgR1). Chief among the MAIF superfamily of molecules is a reticulon family protein, Nogo-A, that is established as a potent inhibitor of neurite sprouting and axon regeneration. However, an understated role for NgR1 is its ability to control axo-myelin interactions and Nogo-A specific ligand binding. These interactions may occur at axo-dendritic and axo-glial synapses regulating their functional and dynamic membrane domains. The current review provides a comprehensive analysis of how neuronal NgR1 can regulate myelin thickness and plasticity under normal and disease conditions. Specifically, we discuss how NgR1 plays an important role in regulating paranodal and juxtaparanodal domains through specific signal transduction cascades that are important for microdomain molecular architecture and action potential propagation. Potential therapeutics designed to target NgR1-dependent signaling during disease are being developed in animal models since interference with the involvement of the receptor may facilitate neurological recovery. Hence, the regulatory role played by NgR1 in the axo-myelinic interface is an important research field of clinical significance that requires comprehensive investigation.

Keywords: nogo receptor, Nogo-A, Caspr, paranode, myelin, PrPc, reelin

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INTRODUCTION

Central nervous system (CNS) myelination is a developmentally regulated process governed by key molecular events that integrate dynamic changes at axonal and oligodendroglial cell membranes. Myelination ensures efficient propagation of action potentials along axons. CNS myelin-forming oligodendrocytes initially contact axons that they subsequently may ensheath depending on their electrical activity (Foster et al., 2019). Myelinated axons of the adult CNS demonstrate a substantial degree of plasticity at the axo-glial and axo-myelinic membrane contacts, that are now known to be dynamically modified according to patterns of neural activity (Mitew et al., 2018; Hughes and Appel, 2019). These specific axo-myelinic contacts are coordinated by the myelin-associated glycoprotein (MAG) in cooperation with contactin 1 and Caspr paranodal adhesion molecules to structure the integral myelin domains (Djannatian et al., 2019). Modifications of axonal, and oligodendroglial membranes are regulated by these integral adhesion molecules, being arranged according to structural domains for appropriate morphometry, that are required for axonal propagation through saltatory conduction. A new hypothesis that may shed light on the molecular organization of the structural subdomains of the CNS axo-myelinic unit, is derived from evidence that the Reticulon 4 receptor (RTN4R), known as the Nogo-66 receptor (NgR1), can modify the integral paranodal protein, Caspr, preventing its cleavage and turnover (Lee et al., 2017). This molecular inhibition of Caspr cleavage may ensure the tight segregation of key voltage-gated ion channels, preventing their lateral diffusion from the node of Ranvier and the juxtaparanode through the barrier established by the septate-like junctions at the paranodal domains.

During disease and trauma, NgR1 has been reported to be upregulated in neurons that exhibit axonal transection or are undergoing degeneration, thereby governing neurite outgrowth inhibition in an extracellular milieu rich in myelin-associated inhibitory factors (MAIFs) or astroglial-derived chondroitin sulfate proteoglycans (CSPGs; for review see Petratos et al., 2010; Lee and Petratos, 2013). During neuroinflammation, NgR1 is also increased in neurons that may potentiate axonal degeneration through downstream signaling that can destabilize or disassemble the axonal cytoskeleton following Nogo-A-dependent ligation (Petratos et al., 2012; Lee et al., 2019). Of functional importance, is that during the development of the CNS visual system, NgR1 is strongly expressed in parvalbumin-positive interneurons to restrict the critical period of ocular dominance plasticity based on a disinhibitory microcircuit (Stephany et al., 2016). This raises the possibility that the expression of NgR1 throughout the neuronal soma, dendrites, and axons can be variable and inducible to control activity-dependent plasticity that may include axo-glial synapse-like structures. With recent evidence implicating that NgR1 can coordinate plasticity and memory formation in specific cortical regions (Karlsson et al., 2016) and the evidence for myelin plasticity to be center stage of human learning and cognition (Sampaio-Baptista et al., 2020), the investigative path to NgR1-dependent myelin plasticity (see **Figure 1**) is an integral open question in neurobiology that requires elucidation due to the numerous therapeutic strategies being developed for individuals with mainly acquired neurological diseases.

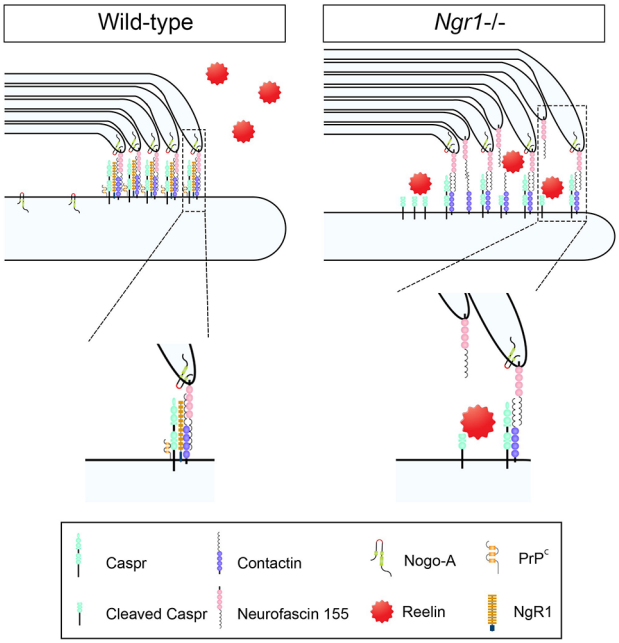

FIGURE 1 | Axo-glial paranodal junctions and a potential regulatory role for Nogo receptor 1 (NgR1): evidence of a disrupted paranode in *ngr1*$^{-/-}$ mice. Schematic representation of the proposed unstructured paranodal septate junction in the central nervous system (CNS) myelinated fibers of *ngr1*$^{-/-}$ mice. The absence of NgR1 expression in neurons limits the capacity for cellular prion protein (PrPc) and contactin associated protein (Caspr) to interact, leaving Caspr as a substrate for intramembranous cleavage by Reelin. Expedited Caspr cleavage may promote the decompaction of myelin form the paranodal junctions altering the electrophysiological signature and potentially triggering the continuous turnover of myelin (adapted from Coman et al., 2006).

These questions can only be addressed when we investigate the molecular dynamics of the axo-glial and axo-myelinic interfaces during development and disease. From an ultrastructural vantage point, key subdomains are present at paranodal regions that flank the nodes of Ranvier, enriched in voltage-gated sodium channels (Caldwell et al., 2000). The establishment of the node is essential for the fast propagation of action potentials by conduction through the internode. Paranodal regions contain high concentrations of proteins such as contactin-1 and contactin associated protein (Caspr), which play an important role in establishing the neuronal membrane anchoring point for the oligodendroglial neurofascin 155 (NF155) protein (Gollan et al., 2003). Mice, deficient in the Caspr gene *(cntnap1)* exhibit abnormal formation of CNS nodes, since Caspr regulates the binding of NF155 to contactin 1 through direct interaction and processing for its membrane localization (Gollan et al., 2003). Genetic knockout models of contactin, Caspr, or neurofascin 155 display a similar pattern of paranodal disorganization (Bhat et al., 2001). This paranodal disruption involves the misdistribution of

juxtaparanodal voltage-gated K^+ channel 1.2 ($K_v1.2$) close to and encroaching into the nodal gap (Coman et al., 2006; Howell et al., 2006), thereby implicating a disruption to axonal-myelin membrane integrity (**Figure 2**). This disruption is manifest as a loss of transverse bands and increased intermediate distance with everted paranodal loops in *cntnap1* (Caspr) mutant mice that although display normal myelination but exhibit disrupted paranodal septate-like junctions, resulting in reduced axonal conduction velocity (CV). Therefore, it is plausible that in the presence of axonal degeneration without prominent demyelination observed in some MS lesions (Bjartmar et al., 2001), such paranodal disruption, leading to axonal degeneration, may precede overt demyelination (Desmaziàres et al., 2012).

Evidence gathered from auditory processing fibers within the brainstem has shown that elevated CVs occur in the large diameter myelinated axons that respond to low-frequency sound waves, with reduced internodal distances (Ford et al., 2015). The morphometric variability in low frequency-respondent auditory processing fibers highlights that there exists a non-canonical array of myelinated segments along axonal fibers to tune axonal conduction in time and space. The investigations that proposed this more rational theory of tunable conduction in the CNS, initially showed that variable diameters and internodal distances can occur along the length of axons to modify action potentials and conduction at anatomically relevant junctures when capacitance modulation can be tuned to regulate synaptic transmission outcomes and plasticity (Ford et al., 2015). These investigators then identified that the conduction speeds generated through computer simulations of the morphometric parameters measured in the mammalian auditory cochlear nucleus globular bush cells (GBC) processing fibers, predicted that action potential propagation speeds are modified to confer the simultaneous arrival times at the giant calyx of Held presynaptic terminal, establishing a precise temporal association of input signals for binaural recognition. These structural variances, therefore, seem integral to how CNS axons can integrate information post-synaptically in disparities of time and space. It would be of great significance if we could identify the molecular drivers that diversify the proximal and distal myelinated segments that filter the current flow since this can occur during demyelinating disease and re-establishment of CNS fiber capacitance may limit the deficits manifest in conditions such as MS (Ortiz et al., 2019).

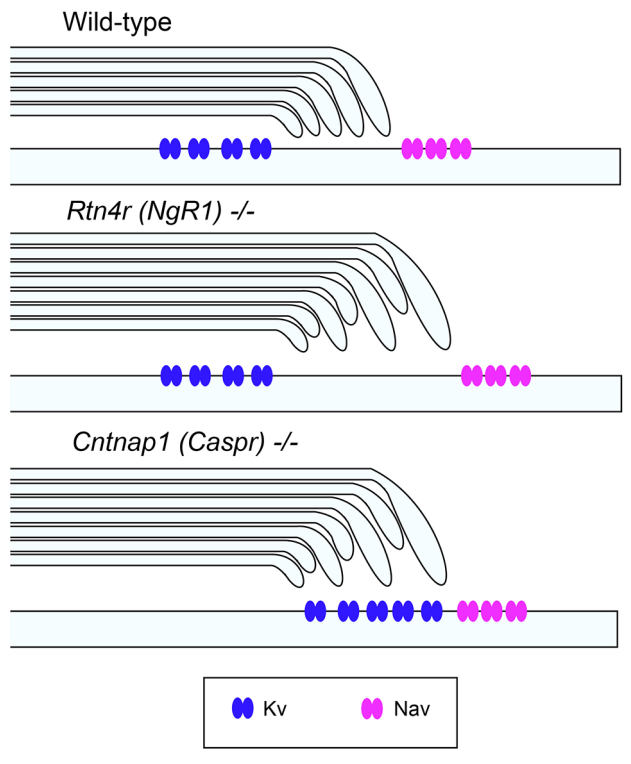

FIGURE 2 | Unaltered ion channel distribution in the axo-myelinic junction of the CNS of *ngr1*$^{-/-}$ mice. Schematic representation of the proposed ion channel distribution in the CNS myelinated fibers of wild-type, *ngr1*$^{-/-}$ and *caspr*$^{-/-}$ mice. In *caspr*$^{-/-}$, juxtaparanodal Kv channels are displaced and distributed throughout the paranodal region, whereas in *ngr1*$^{-/-}$, although lengthening of paranodal Caspr was found, ion channel distribution could not be identified (Lee et al., 2017).

NOGO RECEPTOR 1 (NgR1) REGULATES NEURONAL MORPHOLOGY AND SYNAPTIC PLASTICITY

The NgR1 is a high-affinity receptor for the Nogo-66 extracellular C-terminus domain of Nogo-A, an integral oligodendroglial and myelin membrane protein. NgR1 regulates the experience-dependent turnover of dendritic spines and limits synaptic plasticity in the cortical gray matter (Akbik et al., 2013). Such modifications in neuronal axodendritic architecture are dependent on myelin ligands that bind avidly to the leucine-rich repeat region (LRR) of NgR1 (Dickendesher et al., 2012; Akbik et al., 2013). We have recently proposed a novel NgR1-dependent mechanism regulating myelin plasticity governed at paranodal regions of the CNS that is disinhibited in mice lacking the Ngr1 allele (**Figure 1**). Indeed, our investigations have identified further ultrastructural changes in the prefrontal cortex of *ngr1*$^{-/-}$ mice whereby the cell bodies of projection neurons in the cortical region were enlarged with elongated apical dendrites (**Figures 3E–G**). This profile was in opposition to the width of cortical Layer I but in-line with that observed in Layers II-V (**Figures 3B–D,H–J**, respectively). Cortical layer I (molecular layer) in *ngr1*$^{-/-}$ mice, showed a decrease in thickness (**Figures 3B–D**) suggesting reduced density of myelinated fibers or reduction in the unmyelinated axonal densities residing in this layer. A plausible explanation for this reduced fiber density may be that the dense perineural nets surrounding cortical neurons can exhibit an altered composition of chondroitin sulfate proteoglycans (CSPGs), modifying the connectivity in the cortex of the *ngr1*$^{-/-}$ mice, particularly since NgR1 can be an alternate receptor for CSPGs (Ye and Miao, 2013). However, at first glance, these observations do not align with the concept of modified neuronal architecture among the projection neurons since no such alterations could

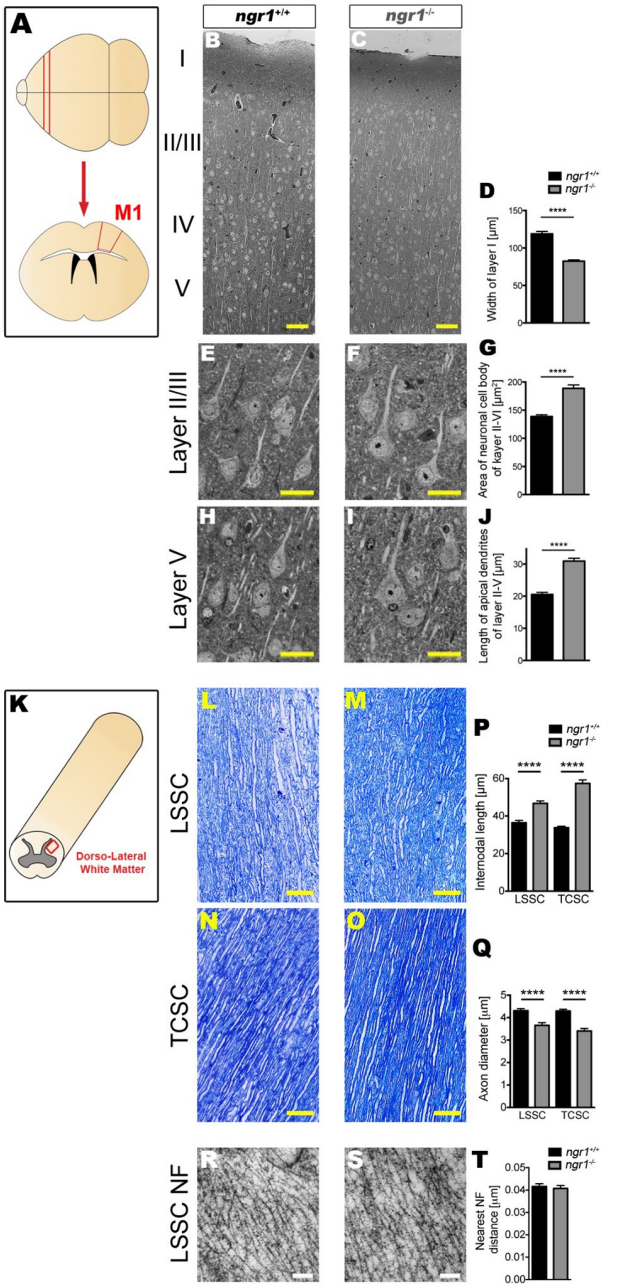

FIGURE 3 | Altered neuronal architecture within the frontal cortex and altered axonal and myelin architecture in the spinal cord of *ngr1*$^{-/-}$ mice. For detailed methods please see the **Supplementary Information**. **(A)** Illustration of the M1 frontal motor cortex region from which samples were imaged. **(B–J)** Semi-thin sections from the M1 regions of *ngr1*$^{+/+}$ and *ngr1*$^{-/-}$ mice revealed a different neuronal morphology. **(B,C)** Light microscopic images of the M1 cortical layer from layer I to V in *ngr1*$^{+/+}$ and *ngr1*$^{-/-}$ mice (scale bar = 50 μm). **(D)** Measurements of the molecular layer width; layer I, was significantly shorter in *ngr1*$^{-/-}$ compared to *ngr1*$^{+/+}$ mice. **(E–I)** Representative high magnification images of pyramidal neurons from **(E,F)** Layer II/III **(H,I)** and layer V. Both **(G)** area and **(J)** length measurements obtained for the apical dendrites of neurons within the cortical layers II-V were found to be increased in *ngr1*$^{-/-}$ compared to *ngr1*$^{+/+}$ mice. **(K)** Illustration of the dorsolateral white matter region of the spinal cord from which samples

(*Continued*)

FIGURE 3 | Continued
were imaged. Representative image showing how internodal length and axonal diameters measured is shown below. **(K–Q)** Toluidine blue-stained semi-thin (1 μm) dorsolateral white matter sections of lumbosacral (LSSC) and thoracic-cervical (TCSC) spinal cords (red rectangle region) of both adult *ngr1*$^{+/+}$ and *ngr1*$^{-/-}$ mice (scale bars = 50 μm). **(P)** Internodes of *ngr1*$^{-/-}$ were significantly longer in both LSSC and TCSC when compared with *ngr1*$^{+/+}$. **(Q)** Axonal diameters of *ngr1*$^{-/-}$ mice were significantly smaller in both LSSC and TCSC when compared with *ngr1*$^{+/+}$ mice. **(R,S)** Ultra-thin (100 nm) electron micrograph longitudinal sections of LSSC from adult **(R)** *ngr1*$^{+/+}$ and **(S)** *ngr1*$^{-/-}$ mice showing normal ultra-structure of neurofilaments in both genotypes (scale bar = 100 nm). **(T)** Quantification of nearest neighbor distances between neurofilaments in myelinated axons of descending fiber tracts in *ngr1*$^{+/+}$ and *ngr1*$^{-/-}$ LSSC (****$P < 0.0001$, $n = 8$ for both genotypes).

be detected and neurofilaments were evenly spaced in the cortex of both *ngr1*$^{-/-}$ and *ngr1*$^{+/+}$ mice (**Figures 3R–T**; Lee et al., 2017). Nevertheless, this apparent contradiction can be reconciled when the plasticity of adult cortical neurons of *ngr1*$^{-/-}$ mice is taken into account (Dickendesher et al., 2012). In the M1 and V1 cortex of *ngr1*$^{-/-}$ mice, the gains and losses of dendritic spines are approximately double that of control mice, with potentiated turnover implicating synaptic plasticity (Dickendesher et al., 2012). This suggests that a gate in plasticity is lacking in the *ngr1*$^{-/-}$ mice since the potent Nogo-A neurite outgrowth inhibitor expressed in mature oligodendrocytes in adulthood cannot limit the membrane-dependent dendritic or axonal varicosity formation. Therapeutics are being developed to target NgR1-dependent membrane interactions in various disease paradigms (for review see Petratos et al., 2012; Lee and Petratos, 2013; Lee et al., 2014; Kim et al., 2018). Therefore, it is important to dissect and define the precise mechanisms in which NgR1 can regulate plasticity at the axo-glial synapse.

We know that NgR1 may be important in ocular dominance plasticity within the visual cortex of naïve mice (McGee et al., 2005; Stephany et al., 2014). We also know that acute electrophysiological plasticity can be regulated by Nogo-A-NgR1 signaling (Raiker et al., 2010). Moreover, it has been shown that adult synaptic plasticity and dendritic architecture, can be regulated by NgR1 (Lee et al., 2008; Raiker et al., 2010; Zagrebelsky et al., 2010; Delekate et al., 2011; Wills et al., 2012; Akbik et al., 2013). This regulatory role of NgR1 in synaptic plasticity has been linked to a neuropsychological phenotype that mimics schizophrenia (Budel et al., 2008). Together, these results indicate that NgR1 has a distinct role in the regulation of neural architecture. However, whether there are ultrastructural differences within CNS white matter tracts, related to axo-glial connectivity and dynamics, has not been investigated to date and is a valid line of investigation. This is so, since myelin plasticity is now well documented in the enhancement of cognitive function, an established role in NgR1-dependent physiology.

An alternate hypothesis that may explain the morphometric variability in the cortical architecture observed in our *ngr1*$^{-/-}$ mice may involve the level and or dynamics of intracortical myelination, regulating dendritic arborization and hence maturation. It has been demonstrated that the

cognate high-affinity ligand for NgR1, Nogo-A, is not only localized to oligodendroglial plasma membranes but has been shown that its neuronal expression to be specifically observed during development and can limit dendritogenesis (Petrinovic et al., 2013). These investigators demonstrated that Nogo-A knockout mice exhibited elaborate Purkinje cell dendritic trees, greater synaptic strength between parallel fiber terminals, and Purkinje cell post-synaptic densities with potentiated excitatory presynaptic current (EPSC). The data suggest neuronal Nogo-A expression limits the development and synaptic strength, at least of the cerebellar cortex (Petrinovic et al., 2013). Moreover, it has been established that both oligodendroglial-specific and neuronal-specific Nogo-A can regulate the dendritic arborization with distal vs. proximal dendrites influenced respectively (Zemmar et al., 2018). However, the stabilization of dendritic synaptic fields demonstrated by Nogo-A in dendrites in the hippocampal cortex cannot be replicated in axons, which is governed by NgR1/Nogo-A suggesting a key signaling mechanism driving axo-dendritic synaptic plasticity (Zagrebelsky et al., 2010). However, whether these fundamental receptor/ligand interactions occur at axo-oligodendroglial synapses that govern the dendritic maturation in simplistic intracortical regions (Glasser et al., 2014), is yet to be elucidated.

NgR1 REGULATES MYELINATED FIBER STRUCTURE AND FUNCTION

We have recently performed systematic ultrastructural analyses of the dorsolateral spinal cord white matter tracts in the thoracic and lumbosacral segments of the *ngr1*$^{-/-}$ mice that have revealed longer mean internodal lengths when compared to wildtype littermate controls. Mean axon diameters were also reduced in the *ngr1*$^{-/-}$ compared to the wild type littermate controls (**Figure 3**). Along with the observed reduced axonal caliber with commensurate thinner myelin exhibited by mice mutant for the *ngr1* allele, we also demonstrated that these mice had increased numbers of thin fibers in spinal cord fascicles (Lee et al., 2017). Indeed, these ultrastructural changes may explain the altered functional and locomotor performance exhibited by these mice (**Figure 4**).

Our gait analysis of naïve wildtype and *ngr1*$^{-/-}$ animals has identified altered paw angles and the rate of deceleration during the braking phase of the gait cycle. Forepaws of naïve *ngr1*$^{-/-}$ mice were more internally rotated (narrower absolute paw angles), and their hind paws more externally rotated (wider absolute paw angles) than naïve wild-type mice (**Figure 4**). These observations suggest *ngr1*$^{-/-}$ mice may have difficulty in balancing themselves during a stance. Another feature of abnormal gait in the *ngr1*$^{-/-}$ mouse is a significant increase in the maximal rate of change of paw area (MAX dA/dT), a measure of how rapidly the animal decelerates during the braking phase. These observations need to be further investigated to elucidate the precise neurophysiological role/s of NgR1 in gait regulation, and further dissect how changes in axon and myelin properties observed in the *ngr1*$^{-/-}$ mice can potentiate such neurobehavioral outcomes.

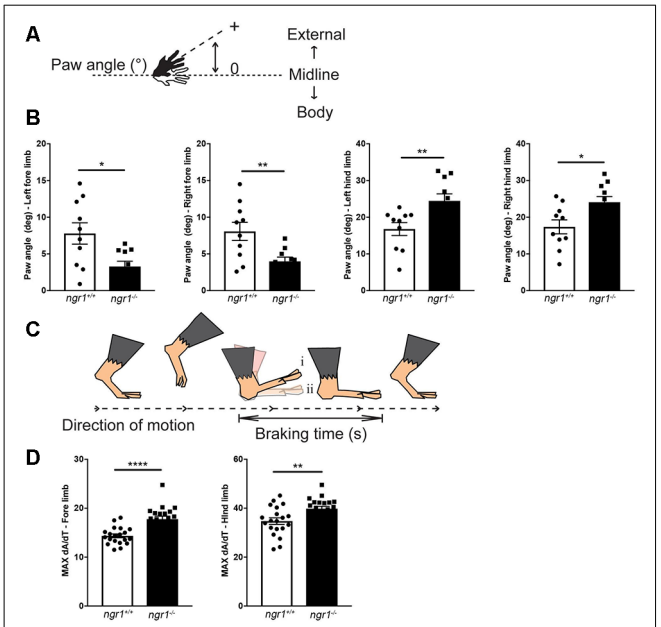

FIGURE 4 | Altered paw angle and rate of deceleration during the braking phase in naïve *ngr1*$^{-/-}$ mice. For detailed methods please see the **Supplementary Information**. **(A)** Absolute paw angle is the angle that the paw makes with the long axis of the direction of motion (midline). Higher absolute paw angles represent greater degrees of external rotation. **(B)** The forepaws of naïve NgR1-KO (*ngr1*$^{-/-}$) mice are less externally rotated than naïve wildtype mice (*ngr1*$^{+/+}$), while NgR1-KO hind paws are more externally rotated than WT mice. **(C)** The maximal rate of change of the paw area (MAX dA/dT) provides a measure of how rapidly the animal decelerates during the braking phase. An increased MAX dA/dT represents a greater change in paw area over the same amount of time; therefore, compared to its littermate (i), the animal is placing its paw down faster (ii; modified from Vincelette et al., 2007). **(D)** KO mice had an increased MAX dA/dT compared to WT mice for both fore- and hind-paws. Data are presented as mean ± SEM. Unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

NEURONAL NgR1 AND THE AXO-MYELIN INTERFACE

So how can NgR1, a high-affinity pleiotropic receptor that inhibits neurite outgrowth, regulate the plasticity of the axo-glial unit? The dynamics of integral adhesion proteins regulating axo-myelinic membrane interactions are related to the rate of activity in axonal conduction (Mensch et al., 2015; Almeida and Lyons, 2017; Hughes and Appel, 2019; Ortiz et al., 2019; Saifetiarova et al., 2017). A thorough analysis of the cellular and molecular ultrastructure of axo-glial units within the white matter of *ngr1*$^{-/-}$ mice shows an altered adhesion of paranodal myelin with the axolemma that corresponds with a preserved expression of Kv1.2 ion channels but distributed (or the diffusion of) contactin-related protein throughout the node and internode regions (Lee et al., 2017). Also, the expression of NgR1 is dependent on neuronal activity and postsynaptic density formation, limiting hippocampal neuron dendritic growth and plasticity (Wills et al., 2012). It is established that neurons of the visual system lacking the expression of NgR1 exhibit increased levels of excitatory synaptic input and

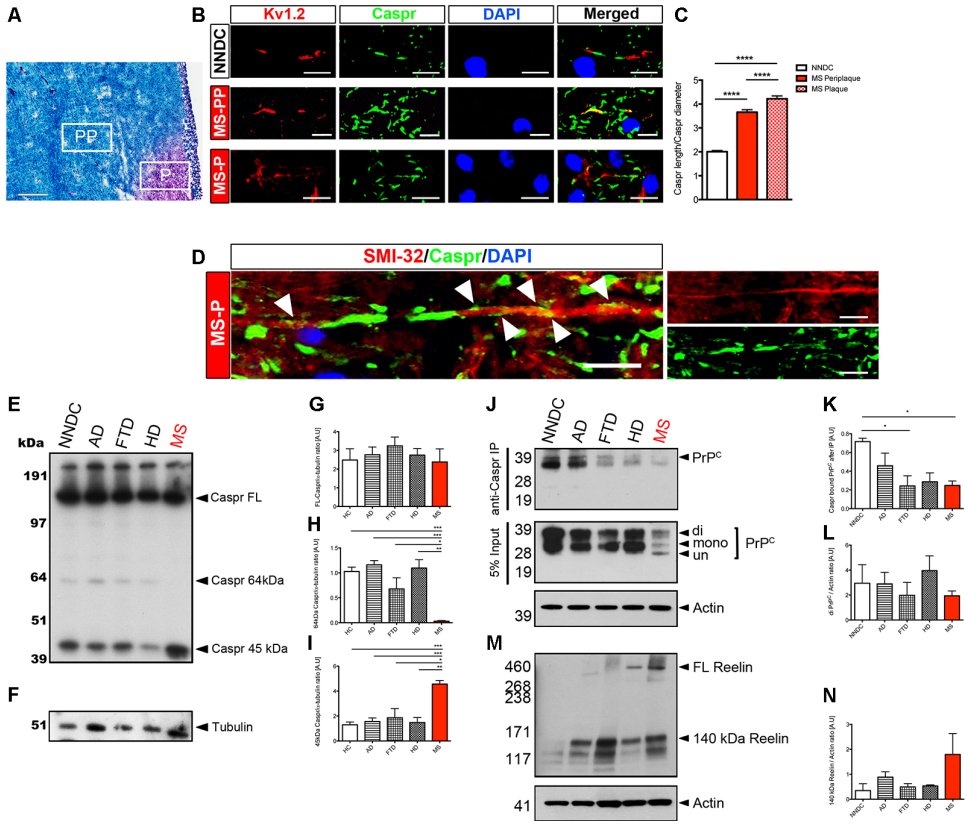

FIGURE 5 | Chronic active MS lesions show Caspr re-distribution, increased NgR1 and axonal damage. For detailed methods please see the **Supplementary Information**. **(A)** Representative LFB-PAS stained images obtained from progressive MS patient brain tissues. From these, we selected periplaque (PP) and plaque (P) regions to further study Caspr distribution in these areas (scale bar = 500 μm). **(B)** In MS-PP, the co-localization of Caspr with juxtaparanodal Kv1.2 was observed. In MS-P, a significant elongation of Caspr+ segments and disrupted localization of Kv1.2 was observed (scale bar = 10 μm). **(C)** The ratio between the measured length of Caspr+ segments vs. their diameter was significantly increased in MS-PP and MS-P, compared with non-neurological disease control (NNDC) samples. **(D)** Immunostaining of SMI-32, Caspr, and DAPI in serial section showed a re-distribution of Caspr along the whole internode in SMI-32 (+) degenerative axons within the lesion border (white arrowheads indicate diffuse expression of Caspr along the internode; scale bar = 10 μm; one-way ANOVA with *post hoc* Tukey's test, $^{****}P < 0.0001$, $n = 4$ for each patient samples). **(E)** Western blot for Caspr and **(F)** α-tubulin-loading control performed on brain white matter lysates of NNDC, Alzheimer's disease (AD), frontotemporal dementia (FTD), Huntington's disease (HD), and MS patients. **(G)** Densitometric quantification of full-length Caspr (FL-Caspr), **(H)** 64kDa degradation product of Caspr and **(I)** 45 kDa Caspr degradation product normalized by α-tubulin loading control (one-way ANOVA *post hoc* Tukey's test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 4$ for each patient samples). **(J)** Immunoprecipitation of Caspr and probed with anti-PrP^C. Western immunoblot for PrP^C from 5% input of pre-immunoprecipitation sample shown on the bottom **(K)** Densitometric quantification of Caspr bound PrP^C and **(L)** total di-PrP^C. **(M)** Western blot for Reelin. **(N)** Densitometric quantification of 140 kDa Reelin.

plasticity (Stephany et al., 2018). Furthermore, we have recently discovered that isolated cortical neurons from *ngr1*$^{-/-}$ mice exhibit potentiated anterograde vesicular axonal transport when compared to isolated cortical neurons from wild type littermates (Lee et al., 2019). Despite elevated neurotransmission observed in *ngr1*$^{-/-}$ mice, the velocities of compound action potentials (CAPs) are reduced in both spinal cord dorsal white matter tracts and optic nerves of these mice (Lee et al., 2017). Taken together, these data suggest a degree of complexity in electrophysiological mechanisms governed by NgR1 in white matter tracts of the CNS.

We have previously demonstrated NgR1 to be a key regulator of the distribution of an integral paranodal protein, Caspr, along with the intramembranous cleavage of the paranodal protein at the junction (Lee et al., 2017). This finding correlated with the altered ultrastructural organization at the paranode and internode of *ngr1*$^{-/-}$ mice and disrupted expression and localization of other key subdomain proteins and ion channels, resulting in delayed conduction velocity. Hence, through extensive ultrastructural molecular and electrophysiological studies of *ngr1*$^{-/-}$ mice, we identified an indirect role in the regulation of axo-glial units for NgR1 (Lee et al., 2017). It is plausible that NgR1 can also regulate the axonal localization of cellular prion protein (PrPc), thereby reducing its interaction with Caspr. Indeed, we found sequestered PrPc within the neuronal somata of the spinal cord gray matter leaving Caspr unbound at the axo-glial junction in *ngr1*$^{-/-}$ mice. Since PrPc has been reported to limit intramembranous Caspr proteolysis through the activity of Reelin at the Laminin-G-like domains (Devanathan et al., 2010), the reduced interaction of PrPc-Caspr may thereby lead to unopposed cleavage of Caspr by Reelin. Indeed, we detected increased proteolytic products of Caspr in the *ngr1*$^{-/-}$ spinal cords, while Reelin levels were

sustained. Intriguingly, despite the lack of the *ngr1* allele, Caspr expression was maintained in the context of cleavage (Lee et al., 2017). These data suggest that the *ngr1*$^{-/-}$ adult mouse CNS exhibits immature paranodal junctions and internodal myelin sheaths with constant myelin turnover. These molecular and ultrastructural findings were also electrophysiologically verified by delayed latency in CAP recordings of *ngr1*$^{-/-}$ when compared with wild-type mice. However, these changes in *ngr1*$^{-/-}$ mice did not compromise axonal integrity (Lee et al., 2017). The possibility of developmental myelination being sustained into adulthood in mice that lacking *ngr1* is therefore an open question.

The presence of Caspr at the paranodal junction is a fundamental factor regulating subdomains of the nodes (in coordination with Neurofascins, the nodal and paranodal cytoskeletal scaffolds, the nodal extracellular matrix, along with myelin membrane-bound lipids and glycolipids), that the segregation of sodium and potassium channels that is necessary for the propagation of action potentials at nodes of Ranvier (Bhat et al., 2001; Ohno et al., 2011; Gordon et al., 2014; Laquérriere et al., 2014). Interestingly, the expression of full-length Caspr was maintained in *ngr1*$^{-/-}$ mice during EAE indicating that, outside of its well-known role in axonal degeneration, NgR1 may also play a role in neuroinflammation-dependent axoglial dynamics (Lee et al., 2017). This was particularly highlighted when we investigated the chronic active lesions of progressive MS patients (**Figure 5**). The increased expression of NgR1 that we observed only in MS tissue (Lee et al., 2019) was associated with elevated Reelin-mediated cleavage of Caspr with significant ion channel re-distribution along the axons and potentiated axonal damage (**Figure 5**). A surprising finding in our study which investigated axo-glial dynamics of *ngr1*$^{-/-}$ mice (Lee et al., 2017), was that although there was increased cleavage of Caspr, no reduction of full-length Caspr was found in the spinal cords of *ngr1*$^{-/-}$ mice, implicating consistent expression and possibly a potential regulation of turnover of myelin by NgR1. We have recently reported that *ngr1*$^{-/-}$ mice also exhibit a sustained expansion of microglia without neuroinflammatory challenge and these cells exhibit increased levels of engulfed myelin proteins (Alrehaili et al., 2018). Furthermore, this observation is consistent with the different expression patterns of Nogo-A found along with the axo-glial units in the spinal cords of *ngr1*$^{-/-}$ mice, as mice deficient in Nogo-A exhibited faster remyelination upon lysolecithin-induced demyelination when compared to wild-type (Chong et al., 2012). Therefore, another open question is whether this endogenous activity, innate to *ngr1*$^{-/-}$ CNS tissues, is a consequence of potentiated clearance of unstructured myelin. This possibility warrants further investigation as it may provide a further understanding of myelin dynamics during disease and possibly also aging.

CONCLUSION

How NgR1 regulates the distribution of Caspr in a tightly orchestrated paranodal interaction with its glial membrane proteins that are integral to the synchrony of axonal myelin physiology is an important question. Future research should involve investigations into CNS remyelination in adult *ngr1*$^{-/-}$ mice where specific demyelinating lesions are observed during the repair. The cuprizone-mediated experimental demyelination model would be ideal to assess the initiation of paranode and internode formation in the adult CNS, without the influence of neuronal NgR utilizing Cre-deleted NgR1-floxed transgenic mice. Moreover, this model has the added advantage of having no invading autoreactive adaptive peripheral immune cells impacting CNS demyelination/remyelination (as can occur in models such as EAE) and axonopathy that can differentiate the role of NgR1 in myelin turnover during remyelination compared to the axonal degeneration occurring in MOG-induced models. Elucidating the precise coupling of NgR1-dependent neuronal activity with the molecular restructuring of the node of Ranvier and paranodal myelin is a critical line of investigation in neuroscience that will drive the development of future regenerative therapeutic interventions that target the Nogo-A/NgR1 cell signaling mechanism during neurodegenerative diseases governed by the sequelae of inflammation.

ETHICS STATEMENT

The AMREP Animal Ethics Committee (AEC nos. E/1532/2015/M and E/1602/2015/M) reviewed and approved the use of these animals for experimentation in this study, in accordance with the guidelines and regulations set out by the National Health and Medical Research Council of Australia. All animal experiments are governed by the Australian Code for the care and use of animals for scientific purposes (2013) and comply with the Victorian Cruelty to Animals Act 1986. All frozen human deep-cortical white matter tissues used for this study were acquired from the Victorian Brain Bank Network (VBBN) under the National Health and Medical Research Council guidelines and the Monash University Human Research Ethics Committee approval number CF13/1646-2013000831.

AUTHOR CONTRIBUTIONS

SP wrote the manuscript. PT, MK, MA and JL edited the manuscript and generated data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00227/full#supplementary-material.

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REVIEW
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Myelin Repair: From Animal Models to Humans

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It is widely thought that brain repair does not occur, but myelin regeneration provides clear evidence to the contrary. Spontaneous remyelination may occur after injury or in multiple sclerosis (MS). However, the efficiency of remyelination varies considerably between MS patients and between the lesions of each patient. Myelin repair is essential for optimal functional recovery, so a profound understanding of the cells and mechanisms involved in this process is required for the development of new therapeutic strategies. In this review, we describe how animal models and modern cell tracing and imaging methods have helped to identify the cell types involved in myelin regeneration. In addition to the oligodendrocyte progenitor cells identified in the 1990s as the principal source of remyelinating cells in the central nervous system (CNS), other cell populations, including subventricular zone-derived neural progenitors, Schwann cells, and even spared mature oligodendrocytes, have more recently emerged as potential contributors to CNS remyelination. We will also highlight the conditions known to limit endogenous repair, such as aging, chronic inflammation, and the production of extracellular matrix proteins, and the role of astrocytes and microglia in these processes. Finally, we will present the discrepancies between observations in humans and in rodents, discussing the relationship of findings in experimental models to myelin repair in humans. These considerations are particularly important from a therapeutic standpoint.

Keywords: myelin repair, oligodendrocyte, neural stem cells, subventricular zone, multiple sclerosis, therapeutic strategies

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INTRODUCTION

Myelin is an insulating sheath that surrounds the axons. It allows the propagation of saltatory impulses, resulting in an efficient acceleration of signal conduction along the axons, together with protection and metabolic support for neurons.

Many studies have suggested that there is a correlation between myelin content and cognitive function. According to the recently developed concept of "myelin plasticity," experience-dependent changes in myelin may modulate brain function (Purger et al., 2016) through effects on signal synchronization (Bells et al., 2019; Hasan et al., 2019).

Within the central nervous system (CNS), myelin is produced by oligodendrocytes (OLGs). These cells produce huge amounts of cytoplasmic membrane, which is then wrapped around the axons. The myelin membrane is unique in that 70% of its dry weight consists of lipids, including cholesterol and galactolipids in particular, and it contains a specific set of proteins including proteolipid protein (PLP) and myelin basic protein (MBP). The membrane loops are compacted to form myelin segments. During embryonic development, oligodendrocyte progenitor cells (OPCs) are specified from neuroepithelial neural stem cells in the spinal cord and the forebrain

(Rowitch and Kriegstein, 2010). Before birth, the OPCs proliferate and migrate throughout the CNS parenchyma, but myelination occurs principally during the postnatal period, when OPCs differentiate and become myelinating OLGs. The first OLGs expressing myelin proteins appear as early as mid-gestation in humans, but brain myelination is not complete until several weeks after birth in rodents and several years after birth in humans. Myelin is then continually remodeled throughout life, through several different processes: the addition of myelin segments, lengthening, retraction, and changes in thickness. Interestingly, a fraction of OPCs in the adult brain remain at the stage of immature progenitors and can respond to neuronal activity by proliferation and differentiation (Gibson et al., 2014), to adapt myelination to the needs of active networks. This adaptive myelination is required for the correct learning of new complex tasks, as shown by the impaired motor learning and fear memory of healthy adult mice in which the differentiation of OPCs is prevented (McKenzie et al., 2014; Xiao et al., 2016; Pan et al., 2020). Myelin is now considered to play a key role in the correct functioning of the brain.

Unfortunately, myelin may be injured or degraded and is affected by aging. OLGs produce large amounts of cell membrane, and their metabolic rates are, therefore, high. OLGs also have low reduced glutathione levels and a high iron content, rendering them susceptible to oxidative stress, excitotoxic damage, and inflammation (Juurlink et al., 1998; Benarroch, 2009). Deficiencies of glycolysis in OLGs were recently shown to trigger inflammasome activation, ultimately leading to demyelinaton (Zhang et al., 2020).

Consequently, many traumas, lesions, and infections trigger the death of OLGs, leading to demyelination, which contributes to functional disorders. Demyelination induces axonal defects, such as nodal complex alterations, conduction blocks, and/or irreversible axon losses that clearly disrupt long-range connectivity (Nave, 2010), as in multiple sclerosis (MS), an autoimmune disease targeting OLGs. Together, these disturbances cause motor, sensory, and cognitive dysfunctions.

REMYELINATION IS A MAJOR ISSUE FOR PREVENTING NEURODEGENERATION AND IRREVERSIBLE LOSSES OF FUNCTION

OLGs and axons are intricately connected, and this relationship is crucial for the preservation of axon integrity and correct signal conduction. The preservation of this "neuron–OLG" unit not only guarantees the maintenance of saltatory conduction, but also provides the neurons with neuroprotective trophic support. Indeed, the myelin sheath plays an important role in ensuring axon survival, by providing physical and metabolic support (Fünfschilling et al., 2012; Lee et al., 2012), and OLG dysfunction is sufficient to trigger neuronal death (Montag et al., 1994; Griffiths et al., 1998; Lappe-Siefke et al., 2003). In pathological conditions, denuded axons are exposed to a toxic inflammatory environment, in which early remyelination may limit excessive neuronal death by insulating the axons against the potentially hostile microenvironment. Several studies of the transplantation of myelin-forming cells have provided strong support for the concept that timely remyelination protects axons from degeneration and promotes functional recovery, in both MS (Irvine and Blakemore, 2008) and spinal cord injury models (Karimi-Abdolrezaee et al., 2006; Hawryluk et al., 2014; Nagoshi et al., 2018). Accelerating remyelination in experimental autoimmune encephalitis (EAE), a model of inflammatory demyelination, is also sufficient to preserve axonal integrity and neuronal function (Mei et al., 2016). During demyelination, the nodes of Ranvier are disorganized, altering nerve conduction. Following remyelination, the nodes are reorganized, allowing functional restoration, within a critical time window, consistent with the notion that node restoration is more beneficial if initiated before the occurrence of axonal damage (Saifetiarova et al., 2018). Nevertheless, the remyelination of damaged axons remains possible and may promote axonal recovery and, thus, neuronal survival (Schultz et al., 2017). Finally, remyelination index, which varies considerably between MS patients, leading to the classification of patients as "good" and "bad remyelinators," is highly correlated with functional outcome (Bodini et al., 2016).

Most neurons do not regenerate (with the exception of neurogenic niches in the dentate gyrus and subventricular zone), but spontaneous myelin repair is commonly observed. This process is highly efficient in rodent models, in which complete remyelination occurs within weeks or months following the experimental insult; by contrast, remyelination varies considerably between MS patients and between individual lesions in the same patient (Patrikios et al., 2006; Albert et al., 2007; Patani et al., 2007; Bodini et al., 2016). It is, therefore, crucial to understand all the elements at work in this regenerative process fully, to facilitate the identification of new targets and the development of therapeutic strategies.

CELL SOURCES FOR MYELIN REPAIR: WHAT WE HAVE LEARNED FROM ANIMAL MODELS

The complex nature of MS makes it difficult to mimic the disease faithfully in animal models, but these models have nevertheless been the source of most of our knowledge on the cell biology of myelin repair. The most widely used models of demyelination are as follows (Burrows et al., 2019): (1) focal demyelination of the white matter by the intracerebral injection of demyelinating agents, such as lysophosphatidylcholine (LPC, a membrane-dissolving agent) or ethidium bromide (a DNA-intercalating agent), which trigger rapid demyelination (over a period of a few hours for LPC, to a few days for ethidium bromide), followed by complete remyelination over a period of 2–3 weeks; (2) massive and widespread demyelination of the brain by the ingestion of cuprizone (a copper chelator) in feed, followed by complete remyelination in the acute (3–6 weeks of treatment) model, or incomplete remyelination in the chronic model (12 weeks of treatment); and (3) EAE, in which inflammation is the predominant feature observed after immunization. EAE can be triggered by two approaches: active immunization with

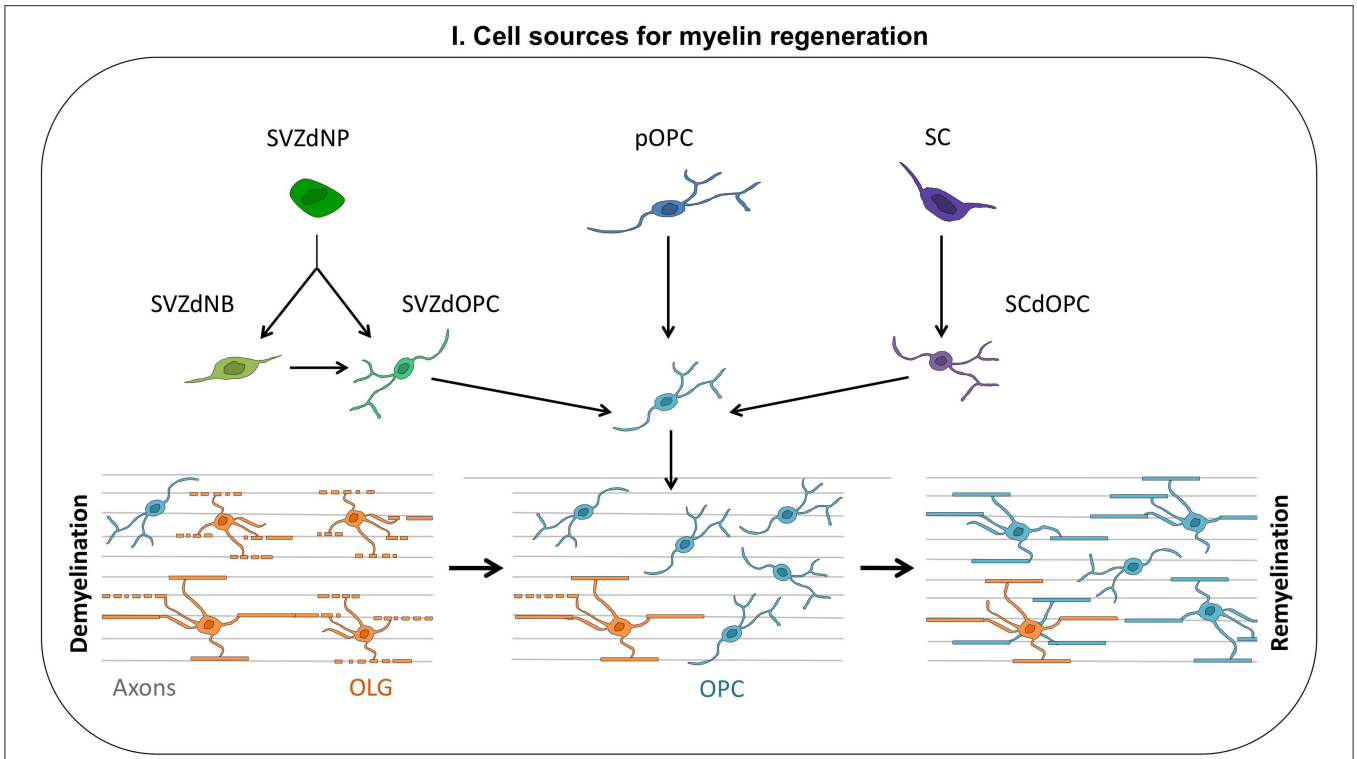

FIGURE 1 | Cell sources for myelin regeneration. Following a demyelination insult, oligodendrocyte progenitor cells (OPCs) are mobilized: they proliferate, migrate toward the injury, and finally differentiate into new myelinating oligodendrocytes (OLGs). These OPCs contributing to myelin repair may be derived from the subventricular zone (SVZdOPC) [directly or by reprogramming of neuroblasts (SVZdNB)], from embryo-derived OPCs (pOPC) or from Schwann cells (SCdOPC). Surviving OLG can also produce new myelin segments thus contributing to remyelination.

myelin peptides or passive induction *via* the adoptive transfer of activated myelin-specific Th1 or Th17 cells from immunized donors in naïve syngeneic recipients. EAE has proved very useful for studies of the pathogenesis of the disease and the role of immune cells, but the demyelination lesions generated are highly variable in size and unpredictable and occur at different stages of development. Furthermore, axonal integrity is compromised in this model, making it difficult to study remyelination. For these reasons, the LPC and cuprizone models (in which extensive demyelination is followed by robust remyelination) are preferred for studies of the cellular mechanisms of demyelination and remyelination. However, these models do not encompass the complexity of MS pathogenesis due to the absence of adaptive immune system involvement.

Progress in mouse genetic techniques opened up opportunities for lineage cell tracing, making it possible to identify the different cell types contributing to OLG replacement and remyelination (**Figure 1**). The data obtained in rodents concerning the cells involved in myelin regeneration are summarized below.

Oligodendrocyte Progenitor Cells

Most of the OPCs present in adult rodent brain are generated from the neuroepithelium during embryonic development; then they proliferate and disseminate throughout the brain parenchyma. OPCs are often characterized by NG2 and PDGFRa expression. They constitute the major population of dividing cells in the healthy adult brain. In physiological conditions, they have a very long cell cycle, with a prolonged G1 phase, and only a minority of these cells differentiate into OLGs (Dimou et al., 2008).

OPC Reactivity Following Demyelination

Early studies reported a rapid increase in OPC density following demyelination (Franklin et al., 1997; Levine and Reynolds, 1999; Reynolds et al., 2001; Chari and Blakemore, 2002), due to rapid recruitment of the pool of NG2-expressing cells through a shortening of their cell cycle (Simon et al., 2011) and the stimulation of short-distance migration toward the site of the lesion (Franklin and Blakemore, 1997). Indeed, in demyelinating conditions, OPCs are activated and revert to a more juvenile phenotype; they then produce the cytokine IL-1β and the chemokine CCL2, which enhance OPC mobilization and the repopulation of demyelinated areas (Moyon et al., 2015). OPC density gradually returns to normal levels with the reappearance of mature OLGs in the lesion. These observations suggested that the parenchymal OPC pool was responsible for spontaneous remyelination. This was definitively demonstrated by lineage cell tracing with PDGFRaCreERT2:RosaYFP and NG2CreERT2:TaumGFP mice, in which OPCs and their progeny

were labeled following tamoxifen injection. These studies provided direct evidence for the generation of remyelinating oligodendrocytes from OPCs (Zawadzka et al., 2010; Mei et al., 2016).

OPC Diversity and Remyelination

There is growing evidence to suggest that OPCs do not constitute a homogeneous cell population (for a review, see Werkman et al., 2020). However, it is difficult to differentiate between real cell diversity and different lineage stages within the same cell population, because only a few studies have shown phenotypic differences to be intrinsic and associated with functional specificity (Foerster et al., 2019).

The first evidence for OPC heterogeneity was provided by the difference in cell cycle kinetics between OPCs residing in the white and gray matter. The OPC cell cycle in the corpus callosum (CC) of young adult mice takes about 7 days, whereas that in the cortex takes between 21 and 50 days (Dimou et al., 2008; Rivers et al., 2008; Simon et al., 2011; Young et al., 2013). The OPCs in the white and gray matter also differ in terms of their differentiation potential. About 40% of the OPCs in the CC differentiate into OLGs, vs. only 11% in the cortex (Dimou et al., 2008). Grafting experiments have suggested that these differences are due to both the intrinsic properties of the OPCs and environmental cues (Viganò et al., 2013). Cultured rat gray matter OPCs have a less mature phenotype (in terms of morphology and gene expression), higher rates of proliferation, and slower differentiation than white matter OPCs. Furthermore, migration in response to cues secreted by astrocytes is weaker in white matter OPCs, which are more sensitive to the inhibition of proliferation and differentiation by TNFα and IFNγ (Lentferink et al., 2018). In the context of demyelination, these characteristics may confer advantages on advantages to gray matter OPCs.

The heterogeneity of OPCs is also revealed by their electrophysiological properties. OPCs are sensitive to neuronal activity due to the expression of ion channels and neurotransmitter receptors. However, not all OPCs are excitable, and white and gray matter OPCs have different electrophysiological signatures, often linked to their differentiation potential (Spitzer et al., 2019).

Recent studies have revealed not all OPCs contribute equally to myelin repair. During embryonic development, OPCs arise from different neuroepithelium domains. In response to demyelination in the adult brain, OPCs from dorsal origin are more strongly mobilized than ventral OPCs, but these cells are more sensitive to the age-related decline in differentiation potential (Crawford et al., 2016b). This is one of the rare examples of the functional diversity of OPCs. A subset of OPCs evenly distributed throughout the brain and characterized by the expression of the G protein-coupled receptor GPR17 has been identified as a reserve pool for repair purposes (Lecca et al., 2008; Viganò et al., 2016). In physiological conditions, GPR17 is required to initiate OPC differentiation, but must be downregulated to allow cells to undergo terminal maturation. Thus, in the absence of lesions, the subpopulation of GPR17$^+$ OPCs is quiescent progenitors that do not differentiate into OLGs (Fumagalli et al., 2017). GPR17 is activated by purines and leukotrienes, the levels of which increase after lesion formation. In MS models, a robust induction of GRP17 is observed in OPCs, together with a large increase in OPC proliferation in the CC (Coppolino et al., 2018; Nyamoya et al., 2019). However, although these activated progenitors produce mature OLGs in the cuprizone model, the process fails in EAE, suggesting that inflammation (in EAE) may lead to an overactivation of GPR17, thereby preventing the terminal differentiation of OPCs (Coppolino et al., 2018). GPR17 reactivity is not observed in the cortex of cuprizone-fed mice (Nyamoya et al., 2019). These results suggest that GPR17 may be a suitable molecular target for the promotion of endogenous myelin repair.

Another subpopulation of OPCs has been identified on the basis of levels of expression for ITPR2, an intracellular calcium channel. Motor learning strongly stimulates the production of ITPR2$^+$ OPCs, which then differentiate into OLGs, contributing to early learning by facilitating electrical transmission (Marques et al., 2016).

These differences probably account for remyelination occurring later in the cortex than in the CC: after acute cuprizone-induced demyelination, OPC repopulation and differentiation is much faster in the CC than in the cortex, resulting in later, incomplete remyelination in the cortex (Gudi et al., 2009; Baxi et al., 2017; Nyamoya et al., 2019). However, in a chronic model based on cuprizone treatment associated with rapamycin to inhibit OPC differentiation so as to improve reproduction of the characteristics of chronic MS, remyelination was faster and more robust in the cortex than in the CC (Bai et al., 2016), as observed in patients with chronic MS (Chang et al., 2012). In both cases (cuprizone model and patients), these differences in remyelination efficiency were associated with differential astrocyte reactivity in the cortex and CC (see below for the role of astrocytes in myelin repair).

Subventricular Zone-Derived Neural Progenitors

Evidence for Subventricular Zone-Derived Oligodendrogenesis

In the adult brain, the wall of the lateral ventricle is a stem cell niche with a unique cellular and extracellular organization (Mirzadeh et al., 2008; Tavazoie et al., 2008; Mercier, 2016). The neural stem cells present in the subventricular zone (SVZ) persist throughout life; they are quiescent but can be activated and re-enter the cell cycle when needed. They give rise to amplifying progenitors ("type C" cells), which, in turn, mostly produce neuroblasts that migrate along the rostral migratory stream to the olfactory bulb, where they differentiate into olfactory interneurons (Lois and Alvarez-BuyIIa, 1994; Doetsch et al., 1999). In parallel, a small fraction of C cells give rise to OPCs that invade periventricular areas (CC, striatum) and the cortex (Suzuki and Goldman, 2003; Menn et al., 2006). These low levels of oligodendrogenesis increase by a factor of four after LPC-induced demyelination (Menn et al., 2006), suggesting a role for SVZ-derived OPCs in myelin repair. Substantial levels of progenitor cell emigration from the SVZ to demyelinated

lesion sites are also consistently observed in MS models (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002; Magalon et al., 2007; Cayre et al., 2013). The migration of these cells from their niche to the lesion site is regulated by guidance cues, such as Slit1 (Deboux et al., 2020). Here again, genetic lineage tracing appears to be a useful tool for unequivocally demonstrating the contribution of SVZ-derived progenitors to myelin repair, particularly in periventricular areas, such as the rostrolateral CC (Xing et al., 2014; Brousse et al., 2015). It has also been suggested that SVZ progenitors are an important source of cells for repopulating the parenchymal OPC pool following demyelination-induced pOPC differentiation into myelinating OLGs (Serwanski et al., 2018).

Despite these demonstrations, the role of SVZ-derived progenitors in myelin repair has been called into question in a few studies. Guglielmetti et al. demonstrated, with MRI and bioluminescence imaging, an increase in olfactory bulb neurogenesis in cuprizone-fed mice, but they were unable to detect any migration of SVZ-derived progenitors to the demyelinated CC (Guglielmetti et al., 2014). Another study reported that SVZ progenitors responded rapidly to focal demyelination but failed to produce myelinating OLGs in the lesion (Kazanis et al., 2017). Given the multiple observations based on the genetic tracing and/or immunofluorescence studies of SVZ-derived progenitor cell migration to sites of injury and the differentiation of these cells into OLGs (Xing et al., 2014; Brousse et al., 2015; Samanta et al., 2015; Serwanski et al., 2018; Butti et al., 2019), it seems likely that these noninvasive imaging techniques do not detect mature cells efficiently and lack sensitivity for visualizing the migration of isolated cells.

Characterization of the SVZ Progenitors Mobilized

The stem cells in the SVZ are not homogeneous. They are organized into domains according to the combination of transcription factors that confer them functional specificities (Merkle et al., 2014; Bonaguidi et al., 2016). Thus, particular subpopulations of SVZ progenitors may be mobilized after demyelination. A subset of progenitors expressing GLI1, a Sonic Hedgehog effector, has been shown to be particularly responsive after the formation of lesions; surprisingly, the mobilization of these cells is further increased by GLI1 inhibition (Samanta et al., 2015).

Tracing experiments with NestinCreERT2 mice cannot determine the type of progenitors mobilized after demyelination. Neuroblasts have been reported to exit the SVZ and rostral migratory stream following experimentally induced demyelination (Picard-Riera et al., 2002; Cayre et al., 2013), suggesting a possible role in the repair process. Indeed, grafting experiments showed that neuroblasts transplanted into the CC of shiverer mice lacking MBP massively differentiate into myelinating OLGs (Cayre et al., 2006). The upregulation of Chordin in the SVZ of demyelinated mice is partly responsible for this change in fate from neuroblast to OLG (Jablonska et al., 2010). This lineage plasticity was further documented in recent studies showing spontaneous neuroblast conversion into OLGs in cuprizone-fed mice (El Waly et al., 2018). These studies demonstrate that both SVZ-derived OPCs and SVZ-derived neuroblasts contribute to OLG replacement after demyelinating insults. The respective roles of each of these cell populations in the contribution of the SVZ to myelin repair remain unknown.

Bioinformatics and *in silico* genomic analyses have identified a catalog of small molecules as potential regulators of SVZ microdomain-specific lineages (Azim et al., 2017). For example, GSK3β and PI3K/Akt inhibitors have been shown to promote oligodendrogenesis from SVZ neural stem cells and to promote remyelination in response to focal demyelination (Azim et al., 2017). This study provides proof of concept that the pharmacological stimulation of SVZ neural stem cells to produce new OLGs is a potentially valuable strategy for myelin repair.

Alternative Roles of SVZ-Derived Progenitors in Myelin Repair

Martino's laboratory investigated the role of SVZ-derived progenitors in myelin repair, using nestin-thymidine kinase-transgenic mice to kill neural progenitors in a specific manner in cuprizone-fed mice (Butti et al., 2019). They concluded that SVZ-derived progenitors were dispensable for remyelination but provided partial protection against greater axonal loss (Butti et al., 2019). Along the same lines, our laboratory showed that some SVZ-derived progenitors mobilized to the demyelinated CC remain undifferentiated and produce factors capable of modulating microglial activation, thereby playing a protective role (Brousse et al., 2020, https://doi.org/10.1101/2020.06.18.158782).

Thus, endogenous SVZ progenitors may promote myelin repair *via* two different mechanisms: OLG replacement and immunomodulation/neuroprotection.

Schwann Cells

Schwann cells are responsible for peripheral nervous system (PNS) myelination. They are derived from the neural crest during embryonic development. Schwann cells are remarkably plastic and respond to lesions by dedifferentiation and re-entry into the cell cycle, facilitating rapid PNS myelin repair. Early observations described an invasion of the CNS by Schwann cells following LPC-induced demyelination of the spinal cord (Blakemore et al., 1977) and in myelin-deficient mutants (Duncan and Hoffman, 1997). It was originally assumed that Schwann cells invaded the CNS following a breach of the glia limitans, but another mode of Schwann cell contribution to CNS remyelination is now recognized (Blakemore, 2005). Indeed, fate mapping strategies demonstrated that OPCs could differentiate into Schwann cells following CNS demyelination (Zawadzka et al., 2010). After spinal cord injury, extensive Schwann cell-mediated remyelination occurs, much of which is driven by OPC-derived Schwann cells (Assinck et al., 2017). The bone morphogenic protein (BMP)/Wnt pathway partly drives decisions concerning the fate of adult OPCs (differentiation into OLGs or Schwann cells): BMP4 upregulation during demyelination drives OPCs to differentiate into Schwann cells, whereas reactive astrocytes within nonvascular areas inhibit BMP/Wnt, thereby favoring OLG differentiation (Ulanska-Poutanen et al., 2018).

Interestingly, recent studies have shown that the specific deletion of Fbxw7 (a E3 ubiquitin ligase component) in Schwann

cells is sufficient to induce the production of thicker myelin sheaths by these cells, together with a myelination of multiple axons similar to that observed with OLGs (Harty et al., 2019). These recent findings raise questions about the relationship between PNS and CNS remyelination and suggest that the demarcation between the biology of Schwann cells and that of OLGs may be less marked than previously appreciated. However, it remains to be demonstrated that Schwann cells can provide appropriate metabolic support to CNS axons and restore effective signal conduction (Chen et al., 2021).

Mature Oligodendrocytes

Following demyelination, some mature oligodendrocytes are spared and survive. A possible role for these cells in myelin repair was considered but quickly ruled out when it was shown that they do not re-enter the cell cycle and proliferate after experimentally induced demyelination and that they do not migrate to the lesion or extend processes to the lesion site (Keirstead and Blakemore, 1997, 1999; Crawford et al., 2016a). OLGs were, thus, considered to be highly differentiated and specialized cells with a complex morphology and no postlesional plasticity. However, recent studies have suggested that these spared OLGs are not passive witnesses of demyelination and can instead play a significant role in the repair process. OLGs located at the border of the lesion produce heparan sulfates, which, in turn, enhance remyelination *via* Sonic Hedgehog signaling (Macchi et al., 2020). Using 3D electron microscopy in large-animal models (cats and nonhuman primates), Duncan and coworkers provided the first evidence of direct remyelination by mature OLGs (Duncan et al., 2018). The development of *in vivo* two-photon video microscopy with longitudinal follow-up over several weeks subsequently made it possible to demonstrate unequivocally that surviving OLGs were able to generate new myelin sheaths after cuprizone-induced demyelination (Bacmeister et al., 2020). However, the laboratory of Bergles failed to detect such OLG plasticity with similar techniques (Orthmann-Murphy et al., 2020), suggesting that it probably makes only a minor contribution to myelin repair, in particular conditions, such as during motor learning tasks.

In conclusion, four main sources of OLG-forming cells may contribute to remyelination following demyelination: OPCs, SVZ-derived progenitors, Schwann cells, and surviving mature OLGs. OPCs, and OLGs are present throughout the central nervous system and can therefore participate in myelin repair at any lesion site. In this respect, they occupy a privileged position. SVZ-derived progenitors have a strong migratory potential and are equipped to respond to inflammatory cues, but their contribution to myelin repair is unlikely to extend very far from periventricular structures. Remyelination quality may also depend on the source of remyelinating cells. For instance, the myelin sheaths formed by SVZ-derived OLGs appear to be thicker than those formed by OLGs generated from parenchymal OPCs (Xing et al., 2014). Interestingly, surviving mature OLGs produce fewer new myelin sheaths than newly formed OLGs, but they better preserve the pattern of myelination in the cortex, with potential implications for functional recovery (Bacmeister et al., 2020). Indeed, the optimal processing capabilities of cortical circuits may be dependent on specific and stable cortical neuron myelination. Finally, remyelination by Schwann cells leads to myelin sheaths of a different molecular composition, which are not affected in MS patients. This is an interesting and potentially advantageous feature. Conversely, peripheral myelin is less compacted, which may affect conduction efficiency in the CNS. Furthermore, given the 1:1 ratio between Schwann cells and myelin segments, extensive CNS remyelination would require an extremely large number of Schwann cells and little is currently known about the mechanisms driving the differentiation of OPCs into Schwann cells.

WHAT CURBS SPONTANEOUS MYELIN REPAIR?

Successful remyelination implies progenitor cell proliferation, migration to the lesion site, and differentiation into OLGs. The newly formed OLGs must then engage in dialog with axons, which they must ensheath to form compacted functional myelin sheaths. A glitch in any one of these steps may lead to remyelination failure. We will now consider the conditions that have been shown to inhibit spontaneous myelin repair.

Aging

Like postlesional regeneration and most plasticity events, the potential for remyelination declines with age. Remyelination is still observed in old rodents, but it is much slower than in younger animals (Shields et al., 1999; Sim et al., 2002). Interestingly, in MS patients, the transition from relapsing–remitting to progressive MS occurs at about the same age, regardless of age at disease onset (Confavreux and Vukusic, 2006b; Tutuncu et al., 2013), suggesting that remyelination failure and disease progression are tightly linked to aging. Aging affects OPC recruitment and differentiation in a cell-autonomous or non-cell-autonomous fashion *via* the alteration of other cell types.

OPCs from aged mice transplanted into neonatal brain recover the proliferation and differentiation rates of newborn OPCs (Segel et al., 2019), suggesting that environmental cues play a crucial role in the age-related decrease in myelin formation. The mechanical properties of the microenvironment may be involved: a recent study revealed that tissue stiffness increases with age, impairing OPC proliferation and differentiation *via* the mechanoresponsive ion channel Piezzo1 (Segel et al., 2019).

The expression profile of growth factors involved in OPC recruitment (PDGFRa, FGF2) and in OPC differentiation (IGF1, TGFβ1) following demyelination is altered in old mice, in which the upregulation of these factors is both weaker and delayed (Hinks and Franklin, 2000).

Consistent with cell-autonomous effects, the epigenetic control of OPC differentiation into myelinating OLGs is disrupted with aging (Shen et al., 2008), and the OPCs of aged mice fail to respond to growth factors and differentiation signals (Neumann et al., 2019). Furthermore, single-cell RNA sequencing-based comparisons of OPCs obtained from young and old mice have revealed mitochondrial dysfunction and a greater activity of the inflammasome and pathways associated

with nutrient signaling in aged OPCs (Neumann et al., 2019). These findings were recently confirmed by a proteomic analysis revealing that the levels of proteins associated with oxidative phosphorylation and inflammatory responses increase with age, whereas those of proteins associated with cholesterol biosynthesis and the cell cycle decrease (de la Fuente et al., 2020). Several studies have suggested that, unlike neurogenesis, SVZ-derived oligodendrogenesis does not decline with age (Capilla-Gonzalez et al., 2013; Weissleder et al., 2016). The contribution of SVZ progenitors to myelin repair can also be enhanced by stimulation of the EGF pathway (Aguirre and Gallo, 2007; Cantarella et al., 2008) and enrichment of the environment (Magalon et al., 2007). The SVZ may therefore be an interesting target reservoir for treatments designed to promote myelin repair in elderly patients.

Finally, aging also affects microglial cells and macrophages, which play a crucial role in remyelination, by removing myelin debris that inhibits remyelination (for a review, see Pinto and Fernandes, 2020). Aged microglial cells become dystrophic, and their processes become less motile (Wong, 2013; Hefendehl et al., 2014; Rawji et al., 2018). These cells become more immunogenic, produce inflammatory cytokines and reactive oxygen species, and thus, have a deleterious phenotype (Hammond et al., 2019). In the context of demyelination, aged microglial cells fail to take up myelin debris efficiently by phagocytosis (Ritzel et al., 2015; Rawji et al., 2018).

Interestingly, the process can be reversed, as old mice exposed to a youthful systemic environment *via* heterochronic parabiosis recover a remyelination potential similar to that of young mice (Ruckh et al., 2012). The clearance of myelin debris and remyelination can also be restored in old mice by systemic injections of niacin, which acts by upregulating CD36 (Rawji et al., 2020). Fasting and treatment with metformin (a fasting mimetic drug) also lead to a recovery of OPC responsiveness to differentiation signals in old mice (Neumann et al., 2019).

Inflammation

CNS injury triggers a cascade of cellular and molecular events leading to inflammation. In MS, breakdown of the brain–blood barrier allows autoreactive T lymphocytes and macrophages to infiltrate the brain, increasing local levels of proinflammatory cytokines. Glial cells also make an active contribution to these environmental changes. Inflammation may itself cause demyelination, as in MS, in which leptomeningeal immune cell infiltration and compartmentalized inflammation within the subarachnoid space are tightly associated with the development of cortical lesions (Choi et al., 2012; Magliozzi et al., 2018).

Inflammatory mediators may have a negative or positive effect on progenitor cell-mediated remyelination. Acute inflammation is required for the correct remyelination of demyelinated lesions (Prineas et al., 1989; Foote and Blakemore, 2005; McMurran et al., 2016). In the cuprizone model, a lack of TNFα or MHC-2 leads to low levels of OPC proliferation and remyelination failure (Arnett et al., 2001, 2003). The delayed but prolonged expression of cytokines such as IL1β, Il6, and TNFα is associated with delayed remyelination in old rats (Zhao et al., 2006). Overall, these data suggest that early acute inflammation is required for the correct recruitment of OPCs to the lesion site, but that chronic inflammation may impede remyelination (**Figure 2**).

Inflammation can inhibit remyelination in several ways: (1) neural progenitors contributing to remyelination may be directly attacked by inflammatory cues or immune cells (Imitola et al., 2003, 2004). Activated T lymphocytes induce the progressive collapse of the process and the apoptotic death of neural progenitors and OPCs *via* the secretion of semaphorin (Giraudon et al., 2004); (2) inflammation affects axonal integrity, in turn altering axon–OPC communication during remyelination; and (3) the extensive remodeling of the extracellular matrix (ECM) associated with sustained inflammation renders the microenvironment nonpermissive for remyelination, mostly by preventing OLG differentiation. These inhibitory signals, secreted into the ECM, are generated principally by microglial cells and astrocytes activated upon injury. They have multiple complex functions in pathological conditions. Both microglia and astrocytes can present different phenotypes, behaving like Dr. Jekyll and Mr. Hyde (**Figure 2**).

Deleterious Effects of Astrocytes on Remyelination

A transcriptomic analysis of astrocytes activated by different types of insults (ischemia, LPS injection) revealed that two types of reactive astrocytes could be distinguished: A1 astrocytes with detrimental effects on cell survival and regeneration and A2 astrocytes presenting protective effects (Zamanian et al., 2012). Astrocyte reactivity upon demyelination is greater in white matter than in gray matter, probably due to the differential induction of various factors. Astrocyte reactivity is regulated by proinflammatory cytokines and myelin debris. Myelin debris are more abundant in white matter, contributing to higher levels of astrocyte activation. In addition, microglial reactivity occurs later and is weaker in the gray matter, resulting in lower levels of cytokine production (Gudi et al., 2009; Buschmann et al., 2012; Werkman et al., 2020). The activation state of astrocytes determines their permissive or inhibitory influence on remyelination.

Astrogliosis is one of the hallmarks of MS. Reactive astrocytes have been shown to increase the recruitment of OPCs to the lesion *via* the secretion of chemokines, such as CXCL1, 8, and 10 (Omari et al., 2005). They also promote the recruitment of microglial cells *via* CXCL10, thereby regulating myelin debris clearance (Skripuletz et al., 2013). In this way, astrocytes contribute to the repair process. Conversely, they also promote peripheral immune cell recruitment, thereby enhancing demyelination (Brambilla et al., 2014). Astrocytes can also regulate the myelin-specific autoreactive response of effector T cells *via* interleukin secretion (Correale and Farez, 2015). In EAE, astrocytes display impaired glutamate transporter expression, leading to deficiencies of glutamate uptake and excitotoxicity (Ohgoh et al., 2002).

Finally, astrocytes make a major contribution to deleterious ECM modifications through their production of hyaluronan, chondroitin sulfate proteoglycan (CSPG), fibronectin, and BMP, all of which inhibit OPC maturation and remyelination (Back et al., 2005; Pendleton et al., 2013; Harlow and Macklin, 2014). Fibronectin accumulates in demyelinated lesions and prevents

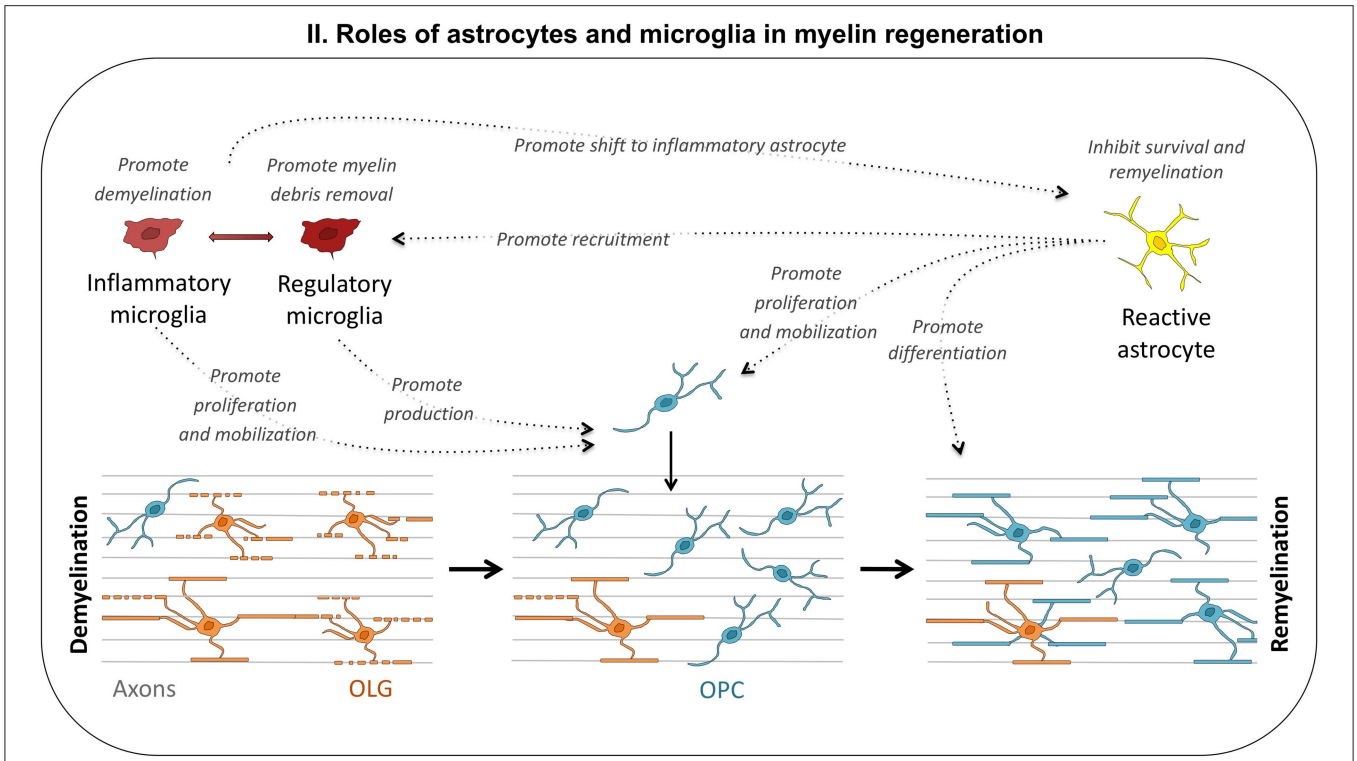

FIGURE 2 | Roles of astrocytes and microglia in myelin regeneration. Depending on environmental cues, microglial cells may adopt different phenotypes, from proinflammatory to regulatory. Inflammatory microglia promotes demyelination and astrocyte reactivity, but may also be useful to the repair process, by stimulating OPC proliferation and mobilization. Regulatory microglia promotes remyelination by enhancing debris removal and OPC production. Reactive astrocytes also play multiple roles: they inhibit remyelination through inflammatory cytokine production and ECM modifications, promote myelin debris removal by recruiting microglia, and stimulate oligodendrogenesis.

OPC differentiation in the EAE model, but not in toxin-induced demyelination, suggesting that fibronectin aggregation is mediated by inflammation. In MS patients, fibronectin aggregates are found in chronic lesions but not in remyelinated lesions (Stoffels et al., 2013). Fibronectin may therefore contribute to remyelination failure.

In addition, these changes to ECM composition increase tissue stiffness, which has been shown to downregulate OPC proliferation and differentiation (Jagielska et al., 2012, 2017; Urbanski et al., 2016; Segel et al., 2019). Tissue stiffness is higher in chronic lesions than in actively remyelinating lesions in MS patients; similarly, tissue stiffness decreases slightly in the acute cuprizone model, but increases in the chronic model with long-term administration (Urbanski et al., 2019).

Roles of Microglia in Remyelination

Microglial cells are the resident immune cells of the CNS, and as such, they are the sensors of pathological events. They are, thus, among the first cells to be activated upon injury. Activated microglia proliferate and migrate to the lesion site, where they accumulate, with both beneficial and detrimental functions. Microglial ablation during the symptomatic phase of EAE has been shown to reduce CNS inflammation and promote recovery (Nissen et al., 2018), but impeding microglial function may also limit remyelination (Stangel et al., 2017; Lloyd and Miron, 2019). Indeed, microglia are crucial for the elimination of myelin debris by phagocytosis, a prerequisite for remyelination (Napoli and Neumann, 2010; Voß et al., 2012; Lampron et al., 2015).

Activated microglia adopt different phenotypes according to the combination of cytokines and factors they express. Traditionally, microglia have been categorized into M1 (proinflammatory) and M2 (immunomodulatory), although this classification now appears too simplistic. Indeed, the development of single-cell RNA sequencing analyses has led to the detection of microglial heterogeneity in both physiological and pathological conditions, suggesting that microglial activation is a dynamic response involving transcriptionally and spatially different subpopulations (Keren-Shaul et al., 2017; Hammond et al., 2019; Plemel et al., 2020). In the LPC demyelinating model, different phenotypes predominate at different stages of demyelination/remyelination, with an initial proinflammatory signature at the time of OPC recruitment, followed by a shift to a more regenerative state during remyelination (Miron et al., 2013). Inflammatory microglial necroptosis occurs, shutting down proinflammatory signals, and is followed by the repopulation of the lesion with regulatory microglia, a process required for efficient remyelination (Lloyd et al., 2019). Regulatory microglia can also promote SVZ-derived

oligodendrogenesis and remyelination *via* Wnt7a production (Mecha et al., 2020).

Inflammatory microglia have several deleterious effects, including shifting astrocytes from a protective to an inflammatory phenotype *via* the secretion of extracellular vesicles and cytokines, such as IL1a, TNFα, and C1q. The astrocytes lose their ability to promote neuronal survival, become neurotoxic, block OLG differentiation, and enhance OLG death (Liddelow et al., 2017; Lombardi et al., 2019). Furthermore, like astrocytes, activated microglia secrete chemokines of the CXC and CC family, contributing to the intracerebral recruitment of T cells and antigen-presenting cells, such as macrophages and dendritic cells (O'Loughlin et al., 2018).

As mentioned above, microglia are sensitive to aging, becoming increasingly inflammatory and less effective at the phagocytosis of myelin debris, thereby contributing to remyelination failure.

REMYELINATION IN HUMANS: OBSERVATIONS FROM MS PATIENTS

MS is an autoimmune disease leading to myelin destruction and neuronal dysfunction, finally triggering neurodegeneration. The pathological hallmark of MS is the occurrence of focal demyelinated lesions (or plaques) disseminated throughout the CNS, causing diverse functional deficits according to their location. These plaques can be classified into several categories on the basis of their inflammatory properties and the presence or absence of ongoing demyelination (Kuhlmann et al., 2017). Active lesions are characterized by the presence of macrophages/microglia throughout the lesion. In mixed active/inactive lesions, these immune cells are present only at the border of the lesion, and chronic lesions are hypocellular, with an almost complete absence of macrophages/microglia. The first two categories can be subdivided into demyelinated lesions (ongoing myelin destruction visible as myelin degradation products in the cytoplasm of macrophages/microglia) and postdemyelinating lesions.

Evidence of Heterogeneous and Variable Myelin Repair in MS Patients

Observations of postmortem tissues from MS patients long ago raised the possibility of spontaneous myelin repair in humans (Prineas and Connell, 1979; Prineas et al., 1984). Remyelinated areas have thinner myelin sheaths, characterized by myelin of a paler color, and are known as "shadow plaques." Lesions more frequently display partial remyelination, usually starting from the periphery; such partial remyelination is observed in all lesion types, but varies considerably between lesions and between patients (Patrikios et al., 2006; Patani et al., 2007). Extensive sampling of brain tissues from MS patients has suggested that remyelination may be more extensive than previously thought, with a mean of 47% remyelination in the white matter. Within the white matter, 22% of lesions were found to be shadow plaques, 73% were partially remyelinated, and only 5% were completely demyelinated (Patani et al., 2007).

Independently of chronicity and location, some MS patients are "good remyelinators" and others are "bad remyelinators" (Patrikios et al., 2006; Bodini et al., 2016). Little is known about the factors underlying this variability in myelin repair. The regenerative process is probably influenced by both genetic and environmental factors, although no specific genes or factors involved in this process have yet been clearly identified. Aging obviously plays a role in decreasing the efficiency of repair in chronic MS, probably through mechanisms reminiscent of those observed in animal models (see *Aging* section), but it cannot provide a complete explanation. Both disease duration and the location of the lesions affect remyelination potential. Many studies have suggested that remyelination is more prominent at the beginning of the disease than in chronic MS, in which the extent of remyelination is often limited (Prineas et al., 1993; Goldschmidt et al., 2009; Chen et al., 2013; Frischer et al., 2015). Depletion of the OPC pool through repeated demyelination is another possible explanation, although sustained demyelination rather than repeated insults is required to deplete OPCs and impair remyelination in rodent models (Penderis et al., 2003).

A recent study suggested that there may be multiple reasons for remyelination failure in MS patients, according to lesion stage: in a subset of active lesions, a lack of myelin sheath formation was observed despite the presence of mature OLGs, whereas in inactive lesions, OLG loss and a hostile microenvironment were identified as the major causes of remyelination failure (Heß et al., 2020).

Subcortical lesions display more extensive remyelination than periventricular and cerebellar lesions, which are often poorly remyelinated (Goldschmidt et al., 2009). Lesions in cortical areas are consistently more extensively remyelinated than white matter lesions (Albert et al., 2007; Strijbis et al., 2017), as clearly shown by the analysis of leukocortical regions, which embrace both white and gray matter (Chang et al., 2012). Cortical lesions are observed in all forms of MS, but are most prominent in long-term progressive MS and are more strongly associated with functional disability than white matter lesions. There is evidence to suggest that environmental cues, produced by astrocytes and microglia in particular, may underlie these differences in remyelination potential (Chang et al., 2012). Indeed, cortical lesions often lack the pathological hallmarks of MS white matter lesions, instead displaying only low levels of inflammation and gliosis (Peterson et al., 2001; Bø et al., 2003).

Finally, the sex of the patient may affect disease susceptibility, progression, and regeneration. Indeed, MS affects three times more women than men, but appears to follow a less aggressive course in women (Confavreux and Vukusic, 2006a). The rate of relapses decreases strongly during the last trimester of pregnancy, when estrogen and progesterone levels are at their highest, whereas there is a rebound after delivery, coinciding with the decrease in hormone levels (Confavreux et al., 1998). *In vitro* experiments and preclinical studies also support the hypothesis that sex hormones may improve remyelination (for a review, see El-Etr et al., 2011).

It therefore appears likely that several mechanisms and factors contribute to impaired remyelination, ranging from genetic background to neuropathological subtype.

Cell Sources for Myelin Repair in MS Patients

The identification of cells contributing to myelin repair is of course more difficult and less reliable in humans than in animal models. However, a number of observations suggest that several cell types may be involved. NG2-positive OPCs are present in MS lesions and can produce new OLGs characterized by short internodes (Chang et al., 2002, 2012). However, in chronic white matter lesions, OPC differentiation into OLGs like that observed in early active lesions does not occur (Kuhlmann et al., 2008), suggesting that inhibitory factors block differentiation. In the spinal cord of MS patients, myelin sheaths labeled with P0, a major constituent of peripheral nervous system myelin, can be detected, indicating the contribution of Schwann cells to CNS remyelination (Itoyama et al., 1983, 1985). Finally, SVZ activation is observed in MS patients, with increases in oligodendrogenesis and the ectopic migration of progenitors that are thought to be involved in remyelination (Nait-Oumesmar et al., 2007; Wu et al., 2009). Based on these observations, it seems likely that myelin repair in the human brain can, as in rodents, proceed from different cell sources, including OPCs, Schwann cells, and SVZ-derived progenitors.

Recent studies using ^{14}C levels to date the birth of cells concluded that, in healthy adult human white matter, only a very small number of new OLGs are generated (Yeung et al., 2019). More surprisingly, newborn OLGs were almost undetectable in the remyelinated lesions of MS patients, except in very aggressive forms of the disease (Yeung et al., 2019). The absence of new OLGs in shadow plaques led the authors to conclude that myelin repair in the human brain does not stem from OPCs, instead originating from surviving OLGs that form new myelin sheaths. This contrasts sharply with observations and demonstrations in animal models, in which remyelination from mature OLGs is restricted to very particular conditions (Duncan et al., 2018; Bacmeister et al., 2020). This finding also calls into question the use of rodent models in the design of therapeutic strategies and suggests that the use of larger animal models, such as cats and nonhuman primates, should be considered (Duncan et al., 2018). Indeed, no single animal model can recapitulate the entire spectrum of heterogeneity for human MS lesions and repair mechanisms, and the best option would be to use several models. A role for mature OLGs in remyelination would have important consequences for the design of new therapeutic strategies: in this case, efforts should be made to promote OLG survival and plasticity.

However, many questions remain unresolved due to several obstacles, making it impossible to draw definitive conclusions from these studies. First, it is difficult to date the beginning of the disease and lesion initiation in humans. Second, if histology is not coupled with longitudinal *in vivo* imaging, it is not possible to conclude with certainty that remyelination has occurred. The histological hallmark of remyelination is the observation of shadow plaques, visible because the regenerated myelin sheath is thinner than the original one; however, this assumption is currently questioned (Neumann et al., 2020). In particular, in areas in which the axon diameter is small, as in the CC, the difference is minimal, and remyelinated areas may be very similar to healthy areas, leading to an underestimation of remyelination rates. Conversely, a shadow plaque may not necessarily be associated with remyelination: paler coloration may result from partial demyelination or a decrease in axonal density. It is not, therefore, yet possible to rule out a contribution of OPCs to myelin repair in the human brain.

POST-LESIONAL PLASTICITY AND MYELIN REPAIR: TRANSLATION INTO THERAPY

MS is primarily an immune-mediated disease, and until very recently, treatments were designed to target immune cells and inflammation. These immunomodulation therapies proved efficient for limiting and reducing lesion formation and relapse rate but failed to prevent the progression of the disease toward neuron loss and irreversible disability.

As mentioned in the *Remyelination Is a Major Issue for Preventing Neurodegeneration and Irreversible Losses of Function* section, many studies have provided support for the idea that targeting remyelination is a sound strategy for promoting functional recovery. Here, we focus on strategies promoting myelin repair from endogenous cell sources; we do not, therefore, consider cell transplantation approaches. Bearing in mind the various factors shown to be involved in the production of new OLGs and in remyelination failure, several strategies for enhancing spontaneous repair may be considered.

Promoting OPC Recruitment and/or Myelin Formation

Boosting OPC proliferation and differentiation appears to be a straightforward strategy for promoting remyelination. However, the reality is more complex. Lesions at different stages coexist in MS patients, and neuropathological studies indicate that there is a high degree of heterogeneity between lesions (Lucchinetti et al., 1999, 2000), with some lacking OPCs (Boyd et al., 2013) and others full of OPCs with blocked differentiation programs (Wolswijk, 1998; Kuhlmann et al., 2008). It is therefore difficult to determine the most appropriate time window for the use of proliferating or differentiating agents. Indeed, promoting OPC proliferation (potentially inhibiting cell differentiation) would be at best useless and possibly even detrimental for lesions containing progenitors unable to differentiate into myelinating OLGs; conversely, promoting OLG differentiation in lesions with only a few rare OPCs would be highly inefficient.

Two approaches currently exist for the development of new treatments targeting OPCs and OLGs (**Table 1**). The first is based on our current knowledge of factors or receptors governing oligodendrogenesis and myelination. The thyroid hormone and retinoic X receptor gamma (RXRγ) have excited considerable interest in this context, due to their promyelinating effects (Harsan et al., 2008; Huang et al., 2011; de la Fuente et al., 2015). The second approach is blind to mechanisms of action and based on the *in vitro*

TABLE 1 | Compounds and therapies promoting remyelination.

			Preclinical studies		Clinical trial		
	Compounds/ therapy	**Mode of action**	**Experimental models**	**Effects observed**	**Trial**	**Results**	**References**
Pro-myelinating compounds	Retinoic acid, bexarotene	RXR agonist; promotes the development of regulatory Tcells and suppresses the development of T helper 17 cells.	Ethidium bromide in rats; LPC in mice	Promote OPC differentiation and remyelination	Phase 2 "CCMR one"	Slightly improved lesion remyelination (MRI), reduced visual evoked potential latency but side effects (hypothyroidism, hypertriglyceridemia)	Huang et al., 2011; Chandraratna et al., 2016
	Thyroïd hormone, Sobetirome	Thyroïd hormone agonist	Cuprizone in mice; EAE in mice	Protects against demyelination and axonal degeneration, improves remyelination and clinical outcome.	Phase 1	Short-term safety	Harsan et al., 2008; Wooliscroft et al., 2020; Chaudhary et al., 2021
	Clemastin	H1 receptor but act as anti-M1 mAchR	LPC in mice, Cuprizone in mice	Promotes OPC differentiation and remyelination	Phase 2 "ReBUILD" and "ReCOVER"	Slightly reduced evoked potential latency but no clinical improvement	Mei et al., 2014; Li et al., 2015; Green et al., 2017
	Benzatropin	Anticholinergic (M1/M3 receptor antagonist)	Cuprizone and EAE in mice	Enhances remyelination and decreases disease severity	No		Deshmukh et al., 2013
	Miconazole	Antifungal drug acting *via* MAPK and *via* cholesterol biosynthesis	LPC and EAE in mice	Promotes OPC differentiation and remyelination	No		Najm et al., 2015
	Clobetazol	Immunosuppressor, acts *via* glucocorticoid receptor signaling	LPC and EAE in mice	Promotes OPC differentiation and remyelination; immunosuppression	No		Najm et al., 2015
	Bazedoxifene	Selective estrogen receptor modulator, but acts *via* cholesterol biosynthesis	LPC in mice	Promotes OPC differentiation and remyelination	Phase2	Ongoing	Rankin et al., 2019
	Olesoxime	Mitochondria, microtubule	Cuprisone and LPC in mice	Promotes OPC differentiation and accelerates remyelination	Phase 1		Magalon et al., 2012, 2016
	Biotin	Co-factor for enzymze involved in fatty acid synthesis and energy production.	Rat OPC primary culture; biotidinase KO mice	Promotes myelin synthesis and protects against axonal degeneration	Phase 3	Failed to improve disability in patients with progressive MS	Pindolia et al., 2012; Sedel et al., 2016; Tourbah et al., 2016; Cree et al., 2020; Cui et al., 2020
	Neuronal activity	Local translation of MBP	LPC in mice	Promotes OPC differentiation and functional improvement	Transorbital electrical stimulation	Ongoing	Ortiz et al., 2019
Suppression of inhibitory signals	Temelimab	Monoclonal antibody GNbAC1 against the envelop of human endogenous retrovirus prevents TLR4 activation	Human primary OPC culture	Env-mediated stimulation of TLR4 on OPC induces inflammatory cytokines and prevent myelin protein expression	Phase 2	Decreased cortical atrophy,slight effect on remyelination	Kremer et al., 2013; Derfuss et al., 2015
	Opicinumab	Anti-LINGO-1, inhibits RhoA activation	EAE in rats and mice	Increased axonal integrity and remyelination, improved clinical score	phase 2 "Affinity" "Synergy" and "Renew"	Reduced evoked potential latency in acute optic neuritis but failure to improve physical and cognitive function in RRMS patients	Mi et al., 2007; Klistorner et al., 2018; Cadavid et al., 2019
	Metformin	Anti-diabetic, rejuvenating	Ethidium bromide in rats	Reverses age-related changes in OPCs, improves remyelination in aged animals	Phase 1	Ongoing	Neumann et al., 2019

(Continued)

TABLE 1 | Continued

	Compounds/ therapy	Mode of action	Preclinical studies: Experimental models	Preclinical studies: Effects observed	Clinical trial: Trial	Clinical trial: Results	References
Suppression of inhibitory signals	DHA and EPA	Polyunsaturated fatty acids. Switch microglia phenotype	Cuprizone, culture	Enhance myelin debris phagocytosis, reduce demyelination, improve cognitive function			Chen et al., 2014
	Endocannabinoid 2-AG	Activates CB1, CB2, and TRVP1 receptors	TMEV-IDD viral murine model	Enhances the clearance of myelin debris, promotes OPC differentiation			Mecha et al., 2019
	Niacin (vitamin B3)	Regulates CD36 expression	LPC, culture	Increases myelin debris phagocytosis by macrophages and microglia and improves remyelination			Rawji et al., 2020
	rHIgM22	IgM antibody binds CNS myelin	TMEV-IDD viral murine model, cuprizone, culture	Stimulates myelin debris phagocytosis by microglial cells, promotes remyelination	Phase 1	Well tolerated, positive trend on clinically stable MS patients	Warrington et al., 2007; Eisen et al., 2017; Mullin et al., 2017; Zorina et al., 2018
	Dietary restriction	Anti-inflammatory, rejuvenating	EAE and cuprizone in mice	Reduces pro-inflammatory cytokines, promotes OPC regeneration and remyelination, reduces clinical severity	Special diets	Ongoing	Choi et al., 2016; Neumann et al., 2019

screening of compounds from libraries. This approach was made possible by the recent development of high-throughput platforms assessing myelination (Mei et al., 2014; Lariosa-Willingham and Leonoudakis, 2018). Several promising small molecules have been identified, including miconazole, clobetazole, clemastine, and benzatropine, highlighting new pathways regulating OLG differentiation (the MAP kinase, glucocorticoid receptor, and muscarinic acetylcholine receptor pathways) (Deshmukh et al., 2013; Najm et al., 2015).

Bazedoxifene (BZA), a selective estrogen receptor modulator, has been identified as a promyelinating agent, with further studies validating its remyelinating effect *in vivo* after demyelinating insults (Rankin et al., 2019). This molecule is now being evaluated in a phase 2 clinical trial on MS patients. One recent study revealed a common mechanism of action for these diverse compounds, independent of their canonical pathways: they all interfere with the cholesterol biosynthesis pathway, leading to the accumulation of 8,9-unsaturated sterols, which stimulate the differentiation of OPCs into myelinating OLGs (Hubler et al., 2018; Rankin et al., 2019).

A small cholesterol-like compound, olesoxime, has been shown to promote oligodendrocyte maturation, remyelination, and functional recovery in rodent models of demyelination, *via* binding to mitochondria and the modulation of ROS levels (Magalon et al., 2012, 2016). Great hopes were raised by biotin, an essential cofactor for fatty-acid synthesis and energy production that was found to have beneficial effects against progressive MS in a pilot study (Sedel et al., 2016; Tourbah et al., 2016); unfortunately, it provided no improvement in clinical outcome in a phase 3 clinical trial.

Recent studies in animal models have highlighted the crucial role of neuronal activity in OPC proliferation and *de novo* myelination (for a review, see Sampaio-Baptista and Johansen-Berg, 2017). Based on these observations, two clinical trials were launched on electrical stimulation of the optic nerve in patients suffering from acute optic neuritis.

Finally, given the minimal *de novo* generation of OLGs from OPCs in the brains of adult humans, including MS patients (Yeung et al., 2014, 2019), efforts should also be made to identify factors capable of boosting mature OLG survival and plasticity during acute demyelination. Creatine was found to be effective for this purpose in the lysolecithin model, in which it protected OLGs against caspase-dependent apoptosis by enhancing mitochondrial function (Chamberlain et al., 2017).

If OLGs are found to play a major role in remyelination in the human brain, they should, indeed, be considered as new targets for drug development.

Removing Inhibitory Signals and Reversing the Effects of Aging

Blocking or removing signals that inhibit OLG differentiation and myelination should render the microenvironment more permissive for regeneration.

The Notch, Wnt, and LINGO-1 signaling pathways have been identified as major inhibitors of CNS remyelination (Wang et al., 1998; Mi et al., 2005; Zhang et al., 2009). Preclinical studies provided strong evidence that LINGO-1 inhibition enhances repair in demyelinating disease (Bourikas et al., 2010; Mi et al.,

2013; Sun et al., 2015). Clinical trials with LINGO-1 antagonists have provided encouraging preliminary results, at least for optic neuritis.

The CSPG and hyaluronan produced by reactive astrocytes exert potent inhibitory signals on OLG development (Back et al., 2005; Lau et al., 2012, 2013; Pu et al., 2018). Most drugs known to induce OPC differentiation *in vitro* are ineffective in the presence of CSPG (Keough et al., 2016). MS lesions are enriched in CSPG, indicating that they may be good targets for neutralization to improve remyelination. In preclinical studies, CSPG biosynthesis inhibitors were shown to be efficient for rescuing OPC differentiation *in vitro* and accelerating remyelination in mice (Lau et al., 2012; Keough et al., 2016). A better knowledge of CSPG and the signaling cascades operating in OPCs will be required for the design of future therapeutic strategies. Similarly, low molecular weight hyaluronans impair OLG differentiation and remyelination, and their concentrations are high in chronic MS lesions (Back et al., 2005; Sloane et al., 2010). The inhibition of low molecular weight hyaluronans may, therefore, also be therapeutically relevant. Finally, the recent discovery of OPC mechanosensitivity and of the impact of tissue stiffness on OLG maturation and myelination opens up new perspectives for treatment (Makhija et al., 2020), although we will need to understand much more about the mechanobiology of OPCs before translation into clinical practice.

Myelin debris is a potent inhibitor of remyelination. The transmembrane protein EphrinB3 has been shown to be an important mediator of this inhibition of OLG maturation; the masking of EphrinB3 epitopes promotes remyelination in a focal demyelination rat model (Syed et al., 2016). This discovery has been patented and may lead to the future development of new therapeutic neutralizing antibodies. Improving myelin clearance is a major challenge. No drug is currently available for specifically preventing the aging-induced decrease in microglial phagocytosis, although several candidate molecules have been identified (Pinto and Fernandes, 2020). These molecules include two polyunsaturated fatty acids (DHA and EPA) (Chen et al., 2014), the endocannabinoid 2-AG (Mecha et al., 2019), and an experimental recombinant human antibody rhIGM22 (Zorina et al., 2018). Vitamin B_3 (niacin) also seems to be a promising compound for safely enhancing myelin phagocytosis by microglia (Rawji et al., 2020). TREM2 is another potentially interesting candidate target. It is expressed by microglia and is involved in the proliferation and phagocytic activity of these cells. One very recent study of a TREM2 agonistic antibody in the cuprizone model reported enhanced myelin debris uptake and degradation, together with improved remyelination (Cignarella et al., 2020). However, as microglia also take up synapses by phagocytosis, a fine balance must be achieved to promote the phagocytosis of myelin debris specifically, without eliminating synapses.

Another approach could involve targeting lipid metabolism, as the uptake of too much cholesterol-rich myelin debris converts microglia into cells with a proinflammatory profile. Molecules triggering the upregulation of ATP-binding cassette (ABC) transporters promote lipid efflux from human macrophages and are, thus, potentially good candidates (Pinto and Fernandes, 2020).

Finally, approaches fighting cellular aging may represent an interesting strategy. Preclinical studies have shown that OPCs can be rejuvenated by alternate-day fasting or by treatment with the fasting mimetic metformin (Neumann et al., 2019). Interestingly, in EAE mice, a fasting mimicking diet strongly reduces clinical severity and inflammation and promotes axonal remyelination (Choi et al., 2016). Several clinical trials targeting dietary interventions have been completed, but they provided insufficient evidence of efficacy for translation into clinical practice. However, most of these trials included limited numbers of patients and short treatment durations (Evans et al., 2019). Other clinical trials are still underway, testing various specific regimens, such as a ketogenic diet and intermittent fasting. These approaches, with few deleterious side effects, may be an interesting complement to drug administration.

CONCLUSION

In recent years, the discovery of several compounds that effectively promote remyelination and provide neuroprotection in animal models has raised hopes for the development of new treatments for MS patients, particularly for preventing or treating the progressive form of the disease, for which very few options are currently available. However, many obstacles will need to be overcome before this goal can be achieved.

First, it should be remembered that no animal model fully reproduces all the characteristics of MS in humans, and young rodents are used, at ages at which regenerative potential is optimal. Drugs found to be effective in such experimental designs may be less effective in less favorable conditions. The use of larger animal models in preclinical studies may be required to overcome this problem.

It is reasonable to assume that the combination of immunomodulatory treatments with compounds alleviating inhibitory signals for remyelination, together with the use of other treatments stimulating OLG differentiation, would have the greatest effect. However, the design of such combinatorial therapies is complicated and they are difficult to test in clinical trials. For instance, it is not immediately obvious whether the various drugs should be administered simultaneously or in a particular time window. The negative effects of permanently stimulating OPC differentiation should also be taken into account, because such treatment may ultimately lead to the depletion of the OPC pool. Will all patients benefit from the same protocol, or do we need to characterize the clinical and neuropathological specificities of each patient more precisely to propose adapted therapy? If we are to achieve this ambitious objective, we will first need to identify valuable biological markers and to develop imaging techniques of better predictive value. Indeed, demyelination/remyelination

follow-up in patients included in clinical trials requires improvement, to ensure that treatment efficacy is properly evaluated. MRI techniques are still being developed, to improve signal specificity, but reliable blood biomarkers are still lacking, and neurophysiological measurements will also be required, to estimate functional recovery.

AUTHOR CONTRIBUTIONS

MC wrote the original draft and MF generated the figures. All authors contributed to the writing of this article, approved the submitted version, involved in developing the plan for the article, and in reviewing and editing the manuscript.

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