MYELIN-MEDIATED INHIBITION OF AXONAL REGENERATION: PAST, PRESENT, AND FUTURE

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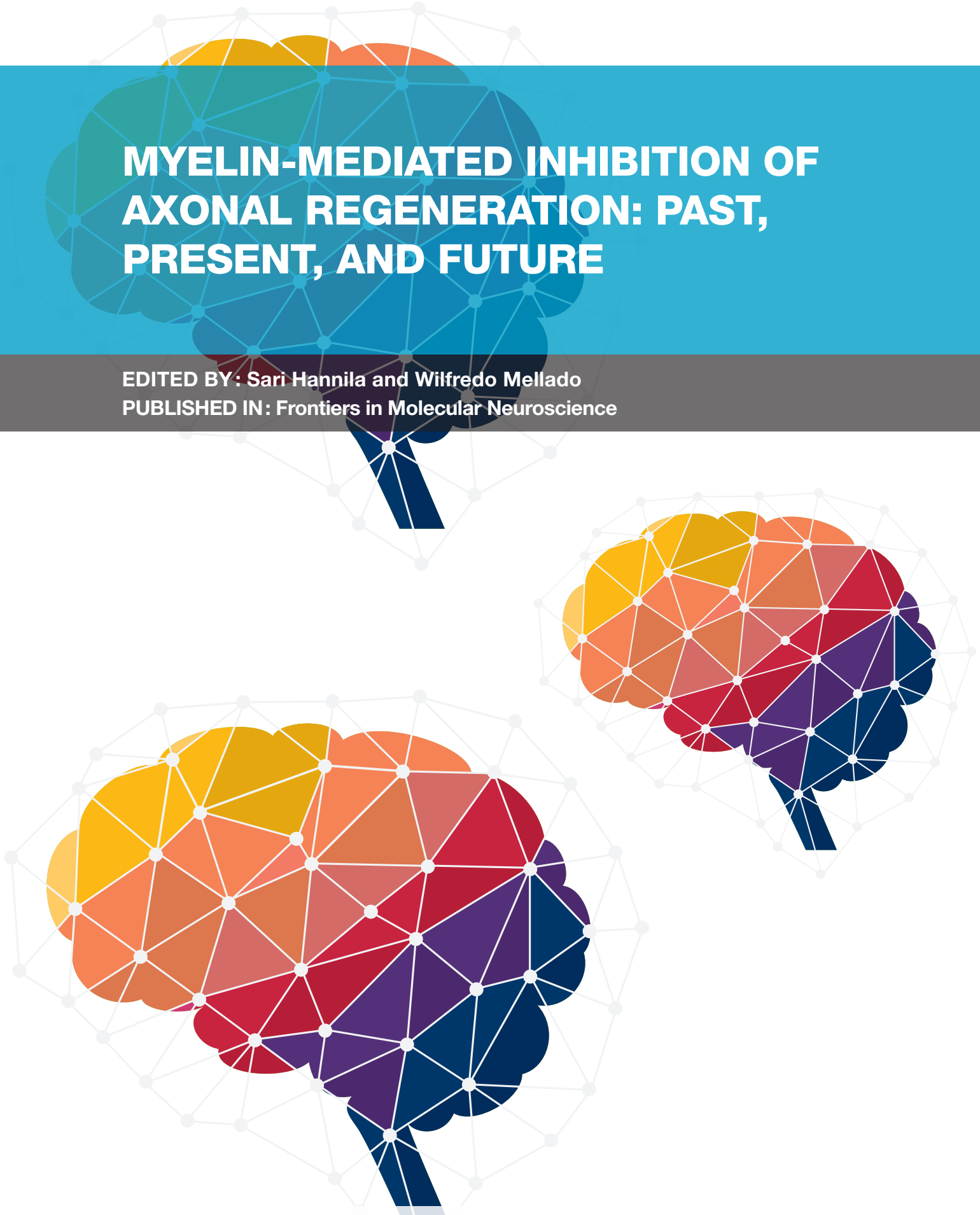

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MYELIN-MEDIATED INHIBITION OF AXONAL REGENERATION: PAST, PRESENT, AND FUTURE

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In memory

Marie T. Filbin
1955-2014

Marie at a lab lunch around 2002, New York City. Photo by Wilfredo Mellado

Pioneering studies conducted in the 1980's laid the foundation for the hypothesis that axonal regeneration is limited by CNS myelin, and the identification of myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte myelin glycoprotein (OMgp) as inhibitors of neurite outgrowth firmly established myelin as a key factor in regenerative failure. Mechanistically, it has been shown that MAG, Nogo, and OMgp mediate inhibition by binding to either Nogo receptor (NgR) or paired immunoglobulin receptor B (PirB), and initiating a signaling cascade that culminates in the activation of RhoA.

Since the discovery of these proteins, there has been tremendous interest in identifying compounds and molecular mechanisms that are capable of overcoming myelin-mediated inhibition. Many studies have focused on pharmacological antagonism of receptors and signaling intermediates, while others have sought to identify and enhance endogenous pro-regenerative pathways. The most notable example of the latter is the conditioning lesion effect, which led to the discovery of cyclic AMP's ability to overcome inhibition by MAG and myelin. Many of the agents tested in these studies have been shown to promote axonal regeneration in vivo, and this research topic allows researchers to share information about new treatments that have been developed in both academia and industry.

As we look toward the future, it is becoming increasingly clear that reversal of myelin-mediated inhibition alone will not be sufficient to produce functional recovery from spinal cord injury, and that other factors, such as astroglial scarring, the expression of chondroitin sulfate proteoglycans, neuronal cell death, and lack of neurotrophic support, must also be taken into consideration. Combinatorial approaches therefore hold a great deal of promise, and we hope to initiate a dialogue on how stem cell transplantation, chondroitinase ABC, gene therapy, growth-promoting agents, and other methods can be combined to optimize functional recovery.

We introduce this topic in honor of the life and work of Dr. Marie T. Filbin (1955-2014). Through these articles, we highlight past achievements in the field, novel findings, unanswered questions and innovative ideas that we hope will lead to new advances in axonal regeneration.

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EDITORIAL
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Editorial: Myelin-Mediated Inhibition of Axonal Regeneration: Past, Present, and Future

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Keywords: myelin inhibitors, myelin associated glycoprotein, Nogo, OMgp, neuronal regeneration

Editorial on the Research Topic

Myelin-Mediated Inhibition of Axonal Regeneration: Past, Present, and Future

Impact: what every scientist seeks in their career. It is a sign of widespread influence, a reflection of the field's continued interest, and validation of our ideas. The impact of a scientist's work is often expressed using simple numbers found in citation reports, journal impact factors, and h-indices, but perhaps the truest measure is the respect of their peers. By this metric, the impact of Dr. Marie T. Filbin's life and career was profound, as evidenced by the many articles published by friends and colleagues in remembrance of Marie since her passing (Ashe and Roskams, 2014; Maddox, 2014; Melendez-Vasquez, 2014; Roskams et al., 2014; Stephenson, 2014). For those of us who had the privilege of working in her laboratory, our fond personal memories are intertwined with a deep desire to preserve and promote her scientific legacy, and this was the driving force behind this Frontiers Research Topic. There were few things that Marie enjoyed more than talking about science, so we could think of no better way to honor her memory than to invite our colleagues to share perspectives on the current state and future direction of the field she helped create.

The hypothesis that central nervous system (CNS) myelin inhibits axonal regeneration was first proposed in the early 1980s (Berry, 1982), and Marie played a central role in identifying myelin-associated glycoprotein (MAG) as the first myelin-associated inhibitor (McKerracher et al., 1994; Mukhopadhyay et al., 1994). This was soon followed by the discovery of other myelin proteins that inhibit axonal growth, such as Nogo-A (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000), oligodendrocyte-myelin glycoprotein (Kottis et al., 2002; Wang et al., 2002a), and ephrin-B3 (Benson et al., 2005), as well as the receptors and co-factors that mediate their effects, including Nogo receptor (Fournier et al., 2001; Domeniconi et al., 2002; Oertle et al., 2003), paired immunoglobulin receptor B (Atwal et al., 2008), the p75 neurotrophin receptor (Wang et al., 2002b), and low-density lipoprotein receptor-related protein 1 (Stiles et al., 2013). Since their discovery, these proteins and receptors have been widely investigated as potential targets for promoting axonal regeneration after spinal cord injury.

This e-book begins with several reviews that expand on the theme of myelin and its function in the injured CNS. While myelin-associated inhibitors are typically viewed in a negative light, these proteins do have normal physiologic roles that have been discussed by Baldwin and Giger. The next chapter is a historical and personal overview of the myelin-associated inhibitor field by McKerracher and Rosen. It is followed by a review from Soheila Karimi-Abdolrezaee's lab that describes the pathophysiologic events that affect oligodendrocytes after CNS injury, as well as cellular approaches currently used to promote remyelination (Alizadeh et al.). This topic is examined from a neuronal perspective by Kaplan et al. in their discussion of how neuron-intrinsic factors contribute to both axonal regeneration and inhibitory factor neutralization in the extracellular environment (Kaplan et al.), which serves as a fitting counterpart to the review by

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Rao and Pearse that comprehensively describes how specific axon-glia signaling pathways influence both myelination and axonal regeneration.

In 1999, the Filbin lab made another seminal contribution to the axonal regeneration field when they reported that elevation of cyclic AMP (cAMP) in postnatal rat neurons was sufficient to overcome inhibition by MAG and CNS myelin (Cai et al., 1999). They subsequently showed that the increase in cAMP enhanced protein kinase A activity and downstream activation of transcription factors such as cAMP-responsive element binding protein (CREB; Gao et al., 2004). To identify which genes were upregulated in response to cAMP, Jason Carmel performed a microarray analysis of cAMP-treated neurons plated on myelin substrates, and together with Wise Young and Ronald Hart, he describes his findings in this collection of reviews (Carmel et al.). Several genes identified in this screen—arginase I, interleukin 6, secretory leukocyte protease inhibitor, and metallothionein I/II—were shown to overcome MAG inhibition in their own right, and their effects and mechanisms are discussed by Siddiq and Hannila.

Interestingly, analysis of the promoter regions of some of these genes revealed that they did not contain cAMP response elements, which suggests that other transcription factors are involved in reversing the effects of myelin-associated inhibitors. Using embryonic mouse neurons grown in the presence of MAG, it was recently demonstrated that another transcription factor, activator protein 1, functions synergistically with CREB to induce arginase I expression (Ma et al., 2014). An earlier study identified several compounds that allowed cerebellar neurons to overcome myelin inhibition, but surprisingly, they did not elevate cAMP (Usher et al., 2010). This led to the hypothesis that non-cAMP-regulated genes also play a substantial role in blocking myelin-mediated inhibition, and it raises the interesting question of how to identify and manipulate regeneration-associated genes to enhance axonal regeneration, a topic that is discussed by Ma and Willis.

Fittingly, we end with a contribution from Marie's laboratory on the subject that first brought her to prominence: MAG biochemistry and function. As a member of the Siglec family, it is well known that MAG can bind complex gangliosides such as GT1b and GD1a (Kelm et al., 1994; Vinson et al., 2001), but the role of sialic acid-binding in MAG-mediated inhibition of neurite outgrowth remains contentious. Najat Al-Bashir's review presents a new working model describing how sialic acid binding at Arg 118 is required to mediate inhibition by soluble but not membrane-bound forms of MAG (Al-Bashir et al.). While this story would appear to bring Marie's career full circle, it is in fact a reminder of all that remains unknown in this field and the work that still lies ahead. New discoveries regarding the effects of MAG, Nogo, and other myelin-associated inhibitors will undoubtedly provoke debate, and that is something Marie would have welcomed.

She also would have been deeply grateful to all of the distinguished scientists who contributed to this Research Topic, and we extend our deepest thanks to each of them for their time and efforts. We also would like to thank the reviewers for their input and the staff of *Frontiers in Molecular Neuroscience* for their support and guidance throughout this process, one that has been personally and scientifically rewarding for both of us.

AUTHOR CONTRIBUTIONS

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

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MINI REVIEW
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Insights into the physiological role of CNS regeneration inhibitors

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The growth inhibitory nature of injured adult mammalian central nervous system (CNS) tissue constitutes a major barrier to robust axonal outgrowth and functional recovery following trauma or disease. Prototypic CNS regeneration inhibitors are broadly expressed in the healthy and injured brain and spinal cord and include myelin-associated glycoprotein (MAG), the reticulon family member NogoA, oligodendrocyte myelin glycoprotein (OMgp), and chondroitin sulfate proteoglycans (CSPGs). These structurally diverse molecules strongly inhibit neurite outgrowth *in vitro*, and have been most extensively studied in the context of nervous system injury *in vivo*. The physiological role of CNS regeneration inhibitors in the naïve, or uninjured, CNS remains less well understood, but has received growing attention in recent years and is the focus of this review. CNS regeneration inhibitors regulate myelin development and axon stability, consolidate neuronal structure shaped by experience, and limit activity-dependent modification of synaptic strength. Altered function of CNS regeneration inhibitors is associated with neuropsychiatric disorders, suggesting crucial roles in brain development and health.

Keywords: NogoA, MAG, OMgp, CSPGs, regeneration, synaptic plasticity

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Neural Network Plasticity—a Delicate Balancing Act Orchestrated by Many Players

Proper function of the adult mammalian CNS requires precise assembly, refinement, and maintenance of an elaborate network of neuronal connections. During development, axon guidance molecules facilitate the formation of intricate neuronal networks. After this initial assembly, many circuits are refined in an activity-dependent manner during a short time period of heightened plasticity, called the critical period (CP). At the end of the CP, the fine tuning of networks is complete and they acquire their mature shape. Synaptic contacts in the mature brain are stable over long time periods (Holtmaat and Svoboda, 2009), however, limited structural remodeling does occur and is thought to form the cellular basis of learning and the acquisition of new memories. Some degree of circuit plasticity is vital for proper brain function, and when improperly regulated can result in defects in learning and memory or cause nervous system diseases such as autism or schizophrenia (Mironova and Giger, 2013; Zagrebelsky and Korte, 2014).

To ensure rapid and accurate propagation of electrical impulses among different neural ensembles, most long axons are enwrapped with myelin sheaths. In addition to increasing the speed of impulse propagation, myelin provides metabolic support for axons (Franklin et al., 2012; Saab et al., 2013) and has neuroprotective properties (Franklin et al., 2012). Recent evidence shows that neuronal activity-regulated mechanisms exist that influence the extent of CNS myelination

(Wake et al., 2011; Lundgaard et al., 2013; Gibson et al., 2014; Hines et al., 2015; Mensch et al., 2015), suggesting that adaptive myelination represents an as of yet underexplored form of activity-dependent nervous system plasticity. Indeed, perturbation of experience-dependent oligodendrocyte (OL) maturation may lead to neurological disorder (Long and Corfas, 2014).

Here we focus on the physiological function of CNS regeneration inhibitors in the naïve mammalian CNS. Vital roles for these molecules in the uninjured CNS raise important considerations for manipulating their function during therapeutic approaches directed toward augmenting neural plasticity and enabling nervous system repair.

Synopsis of CNS Regeneration Inhibitors

MAG

The neurite outgrowth inhibitory properties of MAG were discovered independently by the laboratories of Marie Filbin (Mukhopadhyay et al., 1994) and Peter Braun (McKerracher et al., 1994) more than 20 years ago. MAG is a type-1 transmembrane protein and a prominent member of the family of sialic acid-binding Ig superfamily (siglec) proteins. MAG is expressed by myelinating glia, Schwann cells in the periphery and oligodendrocytes (OL) in the CNS. MAG is abundant in the CNS and is enriched in Schmidt-Lanterman incisures and the periaxonal membrane of myelin sheath, allowing for complexes with receptors to form on the axonal surface (Trapp et al., 1989). The direct apposition of MAG and the axon membrane led to the early hypothesis that MAG plays an important role in regulating axon-myelin interactions and myelin development. *In vivo* studies with *Mag* knockout mice revealed surprisingly normal myelin development, yet closer examination uncovered a delay in OL differentiation and transient hypomyelination of the optic nerve in these mutants (Li et al., 1994; Montag et al., 1994; Pernet et al., 2008). At the ultrastructural level, peripheral and central nervous system myelin sheaths in *Mag* null mice display numerous subtle structural abnormalities, including aberrant myelin outfolds and uncompacted myelin wraps (Pernet et al., 2008). Additionally, loss of MAG delays node of Ranvier formation and alters distribution of nodal proteins, including paranodin and sodium channels (Marcus et al., 2002). Aging studies in *Mag* knockout mice revealed increased axonal "drop out" and axonal atrophy, indicating that MAG plays a crucial role in maintenance and long-term stability of the axon-glial unit (Fruttiger et al., 1995).

Our understanding of the molecular mechanisms employed by MAG to exert its different functions is still incomplete. Several receptors for MAG have been identified, including the complex brain gangliosides GD1a and GT1b (Yang et al., 1996), the Nogo receptor family members NgR1 (Domeniconi et al., 2002; Liu et al., 2002) and NgR2 (Venkatesh et al., 2005), paired Ig-like receptor B (PirB) (Atwal et al., 2008), β1-integrin (Goh et al., 2008), and low density lipoprotein receptor-related protein 1 (LRP1). Except for the interaction with LRP1, MAG binds to its neuronal surface receptors in a sialic acid-dependent manner (Robak et al., 2009; Stiles et al., 2013). Myelination and nodal defects in *B4galnt1* null mice, which lack major brain gangliosides (including GD1a and GT1b), display striking similarities to *Mag* null mice, suggesting that gangliosides may be the primary receptors responsible for MAG-mediated axon protection (Schnaar and Lopez, 2009). Whether NgR1, NgR2, PirB, β1-integrin, or LRP1 play a role in MAG mediated axon protection *in vivo* has not yet been addressed conclusively and will likely require the generation of compound mutant mice to deal with potential redundant functions among different MAG receptors. MAG modulates the axonal cytoskeleton, affecting axon caliber, neurofilament spacing (Yin et al., 1998), post-translational modification of microtubules (Hsieh et al., 1994; Nguyen et al., 2009), phosphorylation of MAP2 (Dashiell et al., 2002) and activation of the RhoA/ROCK signaling pathway (Mimura et al., 2006). In future studies it will be important to determine whether the molecular mechanisms of MAG-mediated axon protection and neurite outgrowth inhibition can be dissociated, and if so, whether this can be exploited therapeutically to selectivity promote axon protection while at the same time eliminating neuronal growth inhibitory constraints imposed by MAG.

NogoA

NogoA is a membrane-associated protein that belongs to the reticulon family (GrandPre et al., 2000). Originally identified as a neurite growth inhibitory "activity" enriched in a spinal cord white matter fraction (Caroni and Schwab, 1988; Caroni et al., 1988), three laboratories described the molecular identity of Nogo-A more than 15 years ago (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). NogoA harbors at least two distinct growth inhibitory motifs, Nogo-66 (Fournier et al., 2001) and NogoΔ20 (Oertle et al., 2003). In the injured spinal cord, acute antibody blockade of Nogo-A promotes axon sprouting and is associated with improved behavioral outcomes (Schnell and Schwab, 1990; Merkler et al., 2001; Liebscher et al., 2005). NogoA is expressed by many cell types, though its expression is highest in OLs and principal neurons in brain regions with a heightened degree of network plasticity, including the hippocampus and neocortex (Huber et al., 2002; Zhang et al., 2014). In the OL lineage, NogoA appears to be an important regulator of myelin development. Treating oligodendrocyte precursor cells (OPCs) with a Nogo-A function-blocking antibody impairs differentiation of OPCs into mature OLs *in vitro* (Huang et al., 2012). Additionally, *NogoA* knockout mice show reduced OPC differentiation *in vivo* (Chong et al., 2012). Similar to *Mag* null mice, the optic nerves in *NogoA* null mice are hypomeylinated during development, but not in adulthood (Pernet et al., 2008). NogoA also participates in contact-mediated competitive interactions between OLs to regulate the myelogenic potential (Chong et al., 2012). Cocultures of WT and *NogoA* null OPCs revealed that the spacing of myelin internodes formed by WT OLs depends on NogoA expression in neighboring OLs. This regulation is likely accomplished by NogoΔ20, since bead-bound NogoΔ20 significantly inhibits the number of myelin internodes formed per OL in culture. *In vivo*, *NogoA* null mice show expansive and aberrant myelination, including hypermyelination of the superficial layers in the neocortex (Chong et al., 2012).

OMgp

OMgp is a 110-kDa leucine-rich repeat protein linked to the cell membrane by a glycosylphosphatidylinositol (GPI)-anchor. OMgp is expressed by OLs and neurons in the CNS (Vourc'h et al., 2003) and is also found in astrocytes (Zhang et al., 2014). Two independent studies identified OMgp as a potent growth inhibitory molecule enriched in CNS myelin (Kottis et al., 2002; Wang et al., 2002). Compared to MAG and NogoA, significantly less is known about the physiological role of OMgp. However, there may be some degree of overlap, as OMgp, similar to NogoA and MAG, associates with NgR1 and PirB. Analogous to NogoA, antibody blockade of OMgp leads to impaired differentiation of OPCs into mature OLs *in vitro* (Huang et al., 2012). *OMgp* null mice display defects of the nodal and paranodal architecture (Nie et al., 2006) and hypomyelination of the spinal cord that correlates with slower propagation of ascending and descending electrical impulses (Lee et al., 2011). OMgp was shown to be enriched near nodes of Ranvier, where it reportedly blocks axon collateral sprouting from non-myelinated segments (Huang et al., 2005). However, a subsequent study, using the same anti-OMgp antiserum in WT and *OMgp* knockout tissue, showed that *OMgp* is not enriched at nodes (Chang et al., 2010). Additional studies are needed to describe the physiological properties of OMgp *in vivo* and to define the extent to which this underexplored molecule contributes to aspects of axon-myelin communication.

CSPGs

Another prominent group of CNS regeneration inhibitors, chondroitin sulfate proteoglycans (CSPGs), are extracellular matrix (ECM) proteoglycans that consist of a protein core with covalently attached glycosaminoglycan (GAG) side chains (Properzi et al., 2003; Silver and Silver, 2014). CSPGs are secreted by astrocytes, neurons, and oligodendrocytes (Ogawa et al., 2001), and they are strongly enriched at the glial scar after CNS injury where they inhibit regenerative growth and restrict plasticity (Bradbury et al., 2002; Morgenstern et al., 2002; Silver and Miller, 2004). CSPGs are a major component of the brain ECM with developmentally regulated expression, therefore they are thought to play a role in neural development, axon guidance, and synaptic plasticity (Kwok et al., 2012). During development, immature OLs express brevican precisely when they extend processes to ensheathe axons (Ogawa et al., 2001), and loss of brevican perturbs ECM distribution near nodes of Ranvier in adulthood (Bekku et al., 2009). As discussed below, CSPGs play important roles in visual system development and plasticity (Pizzorusso et al., 2002), and also in the formation, refinement, and modification of synaptic structures in other brain regions (Orlando et al., 2012) and the protection of memories from erasure (Gogolla et al., 2009).

CNS Regeneration Inhibitors Consolidate Neuronal Architecture at the End of Critical Periods

Proper formation, maintenance, and activity-dependent modification of synaptic contacts may be achieved through dynamic regulation of molecules that promote structural plasticity and also molecules that stabilize existing structures (Mironova and Giger, 2013; de Wit and Ghosh, 2014; Zagrebelsky and Korte, 2014). Strong evidence that CNS regeneration inhibitors may be involved in limiting neuronal plasticity stems from work in the rodent visual system. In the juvenile brain, normal development of the primary visual cortex involves a CP of heightened plasticity. During the CP, visual experience drives refinement of visual neuronal architecture, including the formation of ocular dominance (OD) columns. Once the CP is closed, mature networks are maintained and OD plasticity is more restricted (Levelt and Hubener, 2012). Though most extensively studied in the visual cortex, activity-dependent refinement of neural circuits is not unique to the visual system. CPs exist in many other brain regions and are important for the acquisition of language and certain forms of higher cognitive processing. Elegant studies by Pizzorusso et al. showed that enzymatic digestion of CSPG glycosaminoglycan chains by local infusion of chondroitinaseABC, a bacterial enzyme that digests the GAG side chains on CSPGs, greatly augments OD plasticity in the binocular zone of the adult visual cortex (Pizzorusso et al., 2002). McGee and colleagues found that in *NgR1* mutant mice, there is no temporal limit to the CP, and robust OD plasticity persists throughout adulthood (McGee et al., 2005). In a similar vein, OD plasticity is extended in *Nogo* (McGee et al., 2005) and *PirB* (Syken et al., 2006) mutant mice, indicating that CNS regeneration inhibitors and their receptors function as negative regulators of experience-driven neuronal plasticity at the end of the CP in the visual system. Thus, one important physiological function of CNS regeneration inhibitors is to consolidate neuronal architecture established at the end of CPs, thereby stabilizing microcircuits that are highly tuned and difficult to assemble.

Neuronal Expression of Inhibitory Ligands and their Receptors

CNS regeneration inhibitors are expressed by glia and neurons. Nogo-A, OMgp and several CSPGs are expressed by neurons and found along axons and dendrites (Mironova and Giger, 2013). Interestingly, Nogo-A and OMgp are present in presynaptic and post-synaptic density fractions isolated from the rodent hippocampus (Lee et al., 2008; Raiker et al., 2010). In a similar vein, NgR1 a receptor for Nogo-66, OMgp and CSPGs, is also found in synaptic density fractions, as is PirB (Fournier et al., 2001; Wang et al., 2002; Atwal et al., 2008; Filbin, 2008). PirB is a member of the leukocyte immunoglobulin-like receptor family (LILRB3), and compared to NgR1 it is much less prominently expressed in the CNS (Raiker et al., 2010; Zhang et al., 2014). The NogoΔ20 domain of NogoA does not interact with PirB or members of the Nogo receptor family. A recent study identified sphingosine 1 phosphate receptor 2 (S1PR2) as a novel receptor for NogoΔ20 (Kempf et al., 2014). S1PR2 is expressed in hippocampal neurons, and as discussed below, it is necessary for NogoΔ20-elicited changes in synaptic function (Nie et al., 2006). CSPGs are secreted by astrocytes and neurons, and they are found at synaptic sites and enriched in perineuronal nets (PNNs) (Orlando et al., 2012; Miao et al., 2014). A number of neuronal

surface receptors bind CSPGs and inhibit neurite outgrowth *in vitro*, including NgR1, NgR3, leukocyte common antigen-related protein (LAR), and its homolog RPTPσ (Shen et al., 2009; Fisher et al., 2011; Dickendesher et al., 2012). NgR1, LAR, and RPTPσ are present both pre and post-synaptically (Mironova and Giger, 2013; Takahashi and Craig, 2013). Challenges for future studies, therefore, will be to dissect which CSPGs exert their functions through which receptor complexes and to define their functional relationship to different classes of ligands already known to operate in a LAR family or Nogo receptor family dependent manner.

CNS Regeneration Inhibitors Can Influence Synaptic Structure and Density

Dendritic spines are specialized post-synaptic compartments that receive excitatory inputs from presynaptic axon terminals. Dendritic spines display various morphologies, ranging from immature thin or filopodia-like protrusions to mature mushroom-shaped structures. Spine morphology is thought to reflect the maturity and strength of excitatory synaptic connections (Sala and Segal, 2014). In the hippocampus of *NgR1* knockout mice, CA1 dendritic spines have a less mature distribution profile than wild-type littermate controls, suggesting that NgR1 is required for the proper development or maintenance of mature spines (Lee et al., 2008). Studies with Nogo receptor compound mutant mice revealed that loss of all three NgR family members (NgR1, NgR2, and NgR3) increases synaptic density in the juvenile hippocampus, suggesting that NgRs function as negative regulators of synaptogenesis. In primary hippocampal neurons, loss of any one NgR family member increases dendritic spine density. *In vivo*, however, an increase in spine density is only observed in *NgR* triple knockout mice, suggesting some degree of functional redundancy among these related molecules (Wills et al., 2012). In a recent study NgR1 was reported to be a key molecule in limiting dendritic spine turnover in the somatosensory cortex of adult mice (Akbik et al., 2013). However, this finding was challenged by a subsequent study that found no role for NgR1 in restricting dendritic spine turnover using the same transgenic mouse model (Park et al., 2014). It is unclear which NgR ligands are responsible for regulation of synaptic density and dendritic spine morphology, though CSPGs, NogoA, and OMgp are present in synaptic density fractions and therefore are likely candidates (Raiker et al., 2010; Takahashi and Craig, 2013). Similar to NgR1, NogoA promotes spine maturation in hippocampal pyramidal neurons. In hippocampal slice cultures, antibody blockade of NogoA shifts dendritic spine morphology toward a more immature phenotype but does not affect spine density (Zagrebelsky et al., 2010). *In vivo,* administration of NogoA function-blocking antibodies into the motor cortex of adult male rats leads to a net increase in dendritic spine density due to an increase in spine formation (Zemmar et al., 2014).

The correlation of ECM maturation with reduced spine dynamics at the closure of the CP suggests that CSPGs, since they are ECM components, may play an active role in restricting spine formation and maturation. Indeed, treatment of hippocampal slices with ChaseABC to digest perisynaptic CSPGs increases dendritic spine density and formation of spinehead protrusions through a mechanism that requires β1-integrin (Orlando et al., 2012). In neuron-glia co-cultures, enzymatic digestion of brevican and neurocan GAG chains promotes formation of synaptic puncta (Pyka et al., 2011), suggesting that CSPGs restrict synaptogenesis. CSPGs may restrict synapse formation through a mechanism involving RPTPσ or NgRs since mice lacking these receptors display increased dendritic spine density and length *in vivo* (Horn et al., 2012; Wills et al., 2012).

Regulation of Synaptic Function

In the mature brain, alterations in neuronal structure are thought to reflect prolonged changes in neuronal activity. Long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission are opposing forms of activity-dependent synaptic strength modifications, and are thought to underlie aspects of learning and memory formation (Siegelbaum and Kandel, 1991). Neurotrophic factors such as fibroblast growth factors (FGFs) promote neuronal growth and plasticity and may antagonize the growth inhibitory effects of CNS regeneration inhibitors. In acutely isolated hippocampal slices of WT mice, exogenously applied FGF2 does not alter LTP at CA3-CA1 synapses. In $NgR1^{-/-}$ slices, LTP at CA3-CA1 synapses is indistinguishable from WT controls. However, exogenous application of FGF2 greatly increases LTP in $NgR1^{-/-}$ slices. Pharmacological studies show that elevated LTP in $NgR1^{-/-}$ slices requires FGFR kinase activity (Lee et al., 2008). In juvenile hippocampal slices of $NgR1^{-/-}$ mice, NMDAR dependent LTD at CA3-CA1 synapses was absent, suggesting that synaptic depression requires NgR1 signaling (Lee et al., 2008). Moreover, treatment of acute hippocampal slices with soluble Nogo66 or OMgp suppresses hippocampal LTP in an *NgR1*-dependent manner (Raiker et al., 2010). These results demonstrate that CNS regeneration inhibitors and their receptors can regulate the strength of synaptic transmission, and may accomplish this, in part, by antagonizing growth promoting signaling pathways.

NogoA strongly attenuates LTP at hippocampal CA3-CA1 synapses, since it is observed that antibody blockade of the NogoΔ20 region leads to increased LTP (Delekate et al., 2011). Counter-intuitively, treatment of hippocampal slices with soluble NogoΔ20 leads to an increase in LTP. This NogoΔ20-mediated increase in LTP may be caused by rapid endocytosis of NogoA (Joset et al., 2010), resulting in an opposite effect since internalization of the receptor complex attenuates NogoΔ20 signaling. Surprisingly, recordings from acute hippocampal slices of *NogoA* knockout mice show normal LTP and normal LTD at CA3-CA1 synapses, suggesting that related mechanisms exist that compensate for chronic loss of NogoA (Delekate et al., 2011). Inhibition of the NogoΔ20 receptor S1PR2 enhances LTP in the hippocampus and motor cortex of WT mice, but not in *NogoA* knock-out mice (Kempf et al., 2014), indicating that NogoA may restrict activity-dependent plasticity through S1PR2. Collectively, these studies establish the NogoΔ20 region of NogoA as a negative regulator of activity-dependent synaptic plasticity.

CSPGs are known to influence activity-dependent synaptic strength, but the mechanisms are less clear. Mice lacking *RPTPσ* display altered basal synaptic transmission, including greater paired-pulse facilitation along with increased frequency

of miniature excitatory post-synaptic currents (mEPSCs) (Horn et al., 2012). Additionally, *RPTPσ* null mice have reduced LTP, yet enhanced novel object recognition memory (Horn et al., 2012). These alterations in synaptic function may not be specific to CSPGs, since RPTPσ, similar to NgR1 and NgR3, also interacts with heparan sulfate proteoglycans (HSPGs) (Coles et al., 2011; Dickendesher et al., 2012). The cross-talk between CSPG and HSPG family members at the synapse, and in neural network plasticity in general, is an exciting but underexplored issue.

Signaling Pathways

Collectively, the above studies suggest that an intricate cross-talk occurs between growth promoting and growth inhibitory signaling pathways to achieve proper regulation of synaptic plasticity and stability. Cross-talk between CNS regeneration inhibitors and growth factors that increase plasticity may occur at multiple levels. This is well illustrated by examining how CNS regeneration inhibitors antagonize brain-derived neurotrophic factor (BDNF) signaling. At the cell surface, BDNF binds TrkB to activate several growth promoting pathways, including mTOR complex 1 (mTORC1) and MAP kinase/ERK. However, CSPG binding to RPTPσ attenuates activity of TrkB (Kurihara and Yamashita, 2012). At the level of cell signaling and protein translation, BNDF-mediated activation of mTORC1 leads to increased local protein synthesis at dendritic spines and promotes synaptic plasticity (Tang et al., 2002; Leal et al., 2014). In primary cortical neurons, pretreatment with crude CNS myelin or recombinant Nogo66 attenuates the BDNF-mediated increase in phosphorylation of p70S6K, a downstream target of mTORC1 (Raiker et al., 2010). This suggests that NogoA and NgR1 may restrict synaptic plasticity through negative regulation of mTORC1 signaling, and perhaps the (local) translation of synaptic proteins. In synaptic density fractions isolated from the hippocampus of *NgR1* mutant mice, levels of phosphorylated ERK are significantly increased, suggesting that NgR1 negatively regulates ERK signaling (Lee et al., 2008). Cross-talk might also occur at the transcriptional level through regulation of cAMP response element-binding protein (CREB). Elevating cAMP levels overcomes myelin-mediated inhibition of neurite outgrowth in a CREB dependent manner (Gao et al., 2004), while NogoΔ20 decreases activation of CREB (Joset et al., 2010).

Role of CNS Inhibitory Molecules in CNS Disorders

Many human neuropsychiatric disorders are associated with defects in synaptic structure or function and may be caused by a shift in excitatory/inhibitory balance (Pittenger, 2013). Given that CNS myelin inhibitors play important roles in regulating these processes, altered expression or function may contribute to developmental brain disorders. In the aging brain, hippocampal expression of several CNS regeneration inhibitors increases, and correlates with, deficits in spatial learning and memory (Vanguilder et al., 2012). *NgR1* null mice show impaired memory function, including impaired fear extinction and consolidation (Park et al., 2014) as well as slow acquisition of a spatial memory task (van Gaalen et al., 2012). Rats with reduced expression of NogoA display defects in cognition and social behavior associated with schizophrenia (Tews et al., 2013). Interestingly, mutations in both human *NgR1* and *NogoA* have been associated with schizophrenia (Sinibaldi et al., 2004; Willi and Schwab, 2013).

Concluding Remarks

CNS regeneration inhibitors play important physiological roles in the uninjured brain and spinal cord. The myelin-associated inhibitors MAG, NogoA, and OMgp regulate myelin formation and axon-myelin interactions. NogoA, OMgp, and CSPGs regulate synapse formation and maturation, and they influence activity-dependent synaptic strength. Growing evidence suggests that some CNS regeneration inhibitors participate in an intricate cross-talk with growth promoting molecules at the level of several key signaling molecules. This finely tuned balance of excitation and inhibition in the developing and mature CNS may be necessary for proper formation and function of neural networks.

The physiological role of these molecules raises important considerations for therapeutic strategies designed to promote neural regeneration following injury. The acquisition of a large number of ligand-receptor systems that restrict neural network plasticity may have been a prerequisite that enabled the evolution of larger and more powerful neural networks. Larger and more complex brains may be more vulnerable to aberrant changes in synaptic connectivity and therefore, once fully developed, need to be consolidated and protected by molecules that constrain excessive network rearrangement. Following injury to the adult CNS, molecules that restrict aberrant growth and plasticity may be detrimental since they limit attempts to modify or rebuild nearby networks to compensate for lost neural circuits. Therefore, a deeper understanding of the physiological roles played by CNS regeneration inhibitors is of great interest both clinically and biologically.

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MAG, myelin and overcoming growth inhibition in the CNS

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While neurons in the central nervous system (CNS) have the capacity to regenerate their axons after injury, they fail to do so, in part because regeneration is limited by growth inhibitory proteins present in CNS myelin. Myelin-associated glycoprotein (MAG) was the first myelin-derived growth inhibitory protein identified, and its inhibitory activity was initially elucidated in 1994 independently by the Filbin lab and the McKerracher lab using cell-based and biochemical techniques, respectively. Since that time we have gained a wealth of knowledge concerning the numerous growth inhibitory proteins that are present in myelin, and we also have dissected many of the neuronal signaling pathways that act as stop signs for axon regeneration. Here we give an overview of the early research efforts that led to the identification of myelin-derived growth inhibitory proteins, and the importance of this family of proteins for understanding neurotrauma and CNS diseases. We further provide an update on how this knowledge has been translated towards current clinical studies in regenerative medicine.

Keywords: myelin, myelin-associated glycoprotein, axon regeneration, myelin-derived inhibitors, neurotrauma, Rho, Nogo

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Introduction

Ramon y Cajal (1991), the Spanish neuroscientist who won the Nobel Prize in Medicine in 1906, first described in detail the frustrated growth response of axons injured in the spinal cord. Cajal observed in 1905 that cut axons first form growth cones similar to those seen in development of the nervous system, but then retract into round, static bulbs. Until the early 1980s it was assumed that there was a lack of an intrinsic ability of central nervous system (CNS) neurons to regenerate. Albert Aguayo first showed that CNS axons can regenerate their injured axons when provided with a peripheral nerve graft (Richardson et al., 1980; David and Aguayo, 1981). Later, Aguayo showed that regenerated fibers could form synapses and functional connections, even in adult animals (Keirstead et al., 1985). All of which raised the question: If CNS neurons were able to regenerate, what was it that was stopping them from doing so after an injury?

Advances in our understanding of the control of axonal regeneration have followed from the discovery of growth inhibitory molecules in the CNS, which can signal to block axonal extension. In its most basic form, CNS injury causes the release of fragmented, disrupted myelin, and this myelin debris inhibits neurite outgrowth. However, the discovery of this growth inhibitory activity was not confirmed until the individual myelin proteins responsible were identified (reviewed by Filbin, 2003). Following the description of these inhibitory proteins, the identification of their specific neuronal receptor molecules further increased support for the rather surprising finding that there existed an endogenous neuronal growth inhibition in the CNS.

Myelin-Derived Inhibitors of Axon Growth

The inhibitors of CNS regeneration can be classified into three main categories: (1) Myelin-associated inhibitors; (2) Inhibitors associated with the glial scar that forms after injury; and (3) Inhibitors of the "guidance type". We focus here on the myelin-derived inhibitors (see **Figure 1**). They are important because myelin is disrupted in both traumatic injury (Richardson et al., 1982) and neurological diseases including multiple sclerosis (Simons et al., 2014) and neurodegenerative disorders (Bartzokis, 2011).

MAG and Inhibition of Neurite Outgrowth

The first myelin-associated CNS growth inhibitory protein to be identified was myelin-associated glycoprotein (MAG), and this activity was discovered independently by Marie Filbin and collaborators at Hunter College NY (Mukhopadhyay et al., 1994), and my group and collaborators at McGill University (McKerracher et al., 1994). The two studies used different approaches to discern that MAG has growth inhibitory activity, a surprising finding at the time because MAG is also expressed in peripheral nerve myelin, peripheral nerves regenerate naturally after injury, and it had previously been reported that MAG promoted neurite outgrowth (Johnson et al., 1989; Turnley and Bartlett, 1998).

My group at McGill was trying to understand why CNS nerves do not regenerate, and we had characterized changes in axonal transport and gene expression that were associated with CNS injury (Fournier and McKerracher, 1995, 1997). To understand how the environment of the CNS might inhibit regeneration, we set up a rapid neurite outgrowth assay using NG108 cells as a screening tool. We teamed up with two other McGill researchers, biochemist Peter Braun and neuroscientist Sam David, as we set out to purify the inhibitory proteins from myelin. We isolated myelin from calf brain and screened fractionated myelin proteins, seeking those proteins that would block neurite extension. We found several fractions that blocked neurite outgrowth, and the major peak of activity co-localized with the fractions where MAG eluted from the columns (McKerracher et al., 1994). Using purified recombinant MAG, we confirmed that MAG completely blocked neurite growth (McKerracher et al., 1994), and that growth cones retracted after making even a single filopodial contact (Shibata et al., 1998).

Simultaneously, and in complementary studies, Marie Filbin and collaborators Patrick Doherty and Frank Walsh at Guy's Hospital in London discovered the growth inhibitory activity of MAG (Mukhopadhyay et al., 1994). Using a different approach, Filbin and colleagues were trying to understand how neurons interact with MAG by plating primary neurons on MAG substrates. To better simulate the presentation of MAG to primary neurons, they co-cultured neurons plated onto Chinese hamster ovary (CHO) cells expressing MAG on their surface. Surprisingly, they found that neurite outgrowth from cerebellar neurons was inhibited by MAG, an unexpected finding because it was initially believed that MAG promoted neurite outgrowth. When they tested dorsal root ganglion (DRG) neurons, they found that embryonic DRG neurons grew long neurites on the MAG-CHO cells, whereas MAG inhibited neurite outgrowth from neurons isolated from 7 day-old rats (Mukhopadhyay et al., 1994). These were the first studies to show a developmentally regulated switch in the neuronal response to myelin-derived growth inhibitory proteins, with MAG promoting the growth of embryonic neurons, while blocking axonal extension from postnatal neurons. Extending this line of studies, Filbin's group went on to show that the developmental switch in growth responsiveness to myelin-MAG was regulated by the endogenous levels of cyclic AMP (cAMP; Cai et al., 2001). They identified a developmental decrease in neuronal cAMP levels and downstream PKA activity that was highly correlated with the change in neuronal responsiveness to MAG as it became an inhibitor of neurite outgrowth.

The finding that MAG was a growth inhibitory protein was both surprising and highly controversial at the time. Part of the reason for this was that Martin Schwab and collaborators were engaged in a search for a much larger, 250 kd growth inhibitory protein in the CNS (Caroni and Schwab, 1988b; Cloned 6 years after the identification of MAG, this was the myelin-derived inhibitor now known as Nogo). Immediately after the two papers reporting MAG as an inhibitor of regeneration (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Melitta Schachner's group, in collaboration with Martin Schwab, studied the myelin purified from MAG knockout mice, and determined that growth inhibitory activity remained (Bartsch et al., 1995). This paper showed that other inhibitors of neurite outgrowth were present in the myelin of MAG knockout mice, but it unleashed a fire-storm of controversy about the importance of MAG in growth inhibition, a controversy clearly explained in a classic paper published by Filbin (1996) entitled "The muddle with MAG". Over the next decade Marie Filbin's group carefully characterized the neuronal response to MAG and clarified the importance and role of MAG in growth inhibition in the CNS. My group went on to try to understand the neuronal signaling response to growth inhibitory proteins, focusing on Rho (Lehmann et al., 1999). Thus it is mainly the large body of work contributed by Filbin that has carefully characterized the neuronal response to MAG (for review, see Chaudhry and Filbin, 2007).

MAG in Myelin and Released from Myelin

MAG is a minor constituent of myelin, and it is localized in mature, compact myelin only in the innermost membrane in contact with the axon (Trapp, 1990). It is present in both CNS and peripheral nervous system (PNS) myelin and is thought to be important for the long-term stabilization of myelinated axons (Fruttiger et al., 1995). Myelination is a relatively late event in neurodevelopment and therefore axonal growth cones would likely never come in contact with myelin, so it is not likely to play a role in stabilizing neuronal networks, as proposed for the other growth inhibitory proteins. Only after myelin is damaged and axons are attempting to regenerate would growth cones encounter MAG (Tang et al., 2001). In the PNS, where MAG

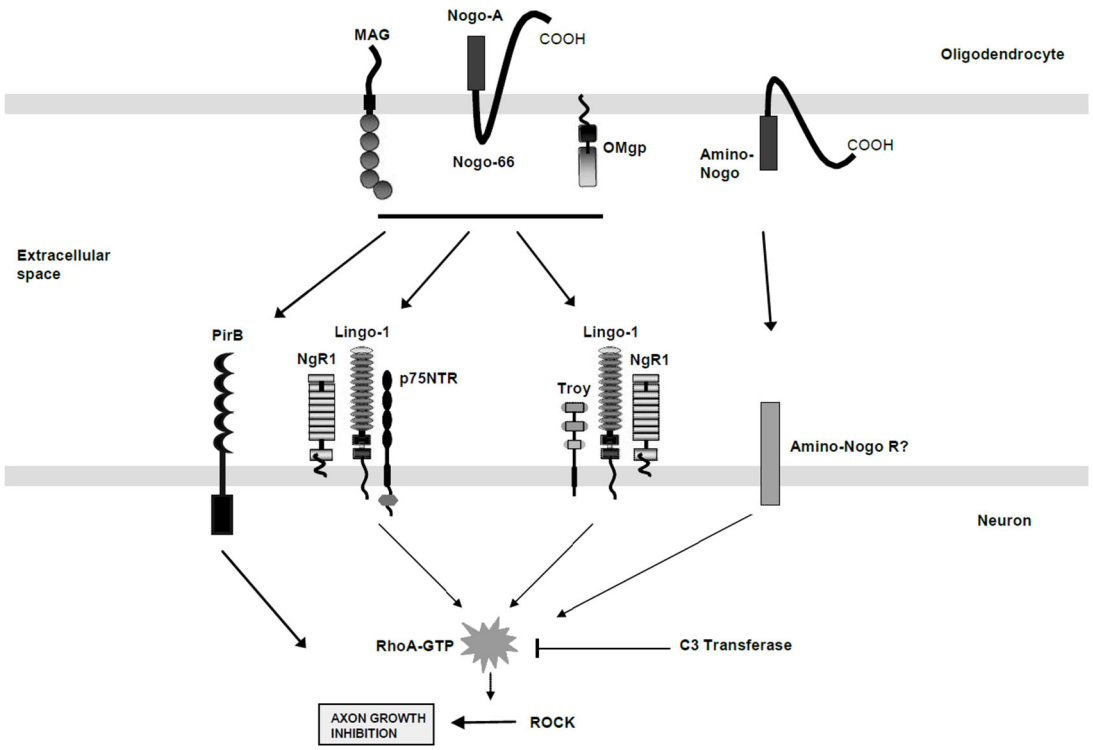

FIGURE 1 | A schematic diagram of the protein components involved in the myelin protein-derived inhibition of neurite growth. The oligodendrocyte derived proteins released from the cell membrane following injury include MAG, Nogo-A (Nogo-66), Oligodendrocyte myelin glycoprotein (OMgp) and Amino-Nogo. The multicomponent cell surface receptor complexes that are localized to the neuronal cell membrane include the Nogo Receptor 1 (NgR1), Lingo-1, p75NTR, and TROY, all of which signal to change the activation of Rho (active Rho is indicated by RhoA-GTP). The PirB receptor also binds the myelin-derived inhibitory molecules but its absolute signaling pathway remains unclear. The C3 transferase enzyme, isolated from Clostridial species, is known to block these Rho activation pathways and to allow for neurite outgrowth in the face of myelin-derived protein inhibition. (Adapted by permission from Macmillan Publishers Ltd: J. Cereb. Blood Flow Metab.; Chaudhry and Filbin; 27(6):1096–107, Copyright 2007).

may also be exposed after nerve injury, the high concentration of laminin is sufficient to override the growth inhibitory property of MAG (David et al., 1995). By contrast, the CNS does not contain much laminin or other favorable neuronal growth substrates.

Filbin made a major discovery about MAG and myelin-derived growth inhibition that, to date, has been underappreciated. She discovered that MAG becomes a much more potent component of CNS growth inhibitory activity after its release from damaged myelin in a diffusible form (Tang et al., 1997, 2001). Being a myelin biochemist, Filbin knew the historical literature on MAG, and the finding more than 10 years earlier that a soluble form of MAG exists, called dMAG (Sato et al., 1984). Filbin showed that the dMAG protein contains the entire extracellular domain of MAG, and it has a potent growth inhibitory activity. The Filbin group made use of MAG knockout mice to show that damaged white matter from normal but not MAG knockout mice inhibited neurite growth, and that immunodepletion of MAG from the soluble fraction removed the growth inhibition (Tang et al., 1997, 2001). These results show that MAG is the major inhibitor of axon growth that is released from damaged myelin and is present in the soluble fraction that can diffuse in the CNS and affect neurons that are not in direct contact with myelin debris. These findings have a major implication for other CNS diseases where myelin disruption occurs, such as multiple sclerosis, and could explain why, in the early phase of multiple sclerosis, neurons can degenerate without obvious evidence of frank myelin disruption, as soluble, released MAG fragments could be acting prior to the bulk collapse of myelin (Bjartmar and Trapp, 2001). Importantly, studies that leveraged knockout mice lines targeting myelin-associated inhibitor genes, either individually, or in combination, have left a certain amount of debate as to the precise role for each of these proteins (Ji et al., 2008; Cafferty et al., 2010; Lee et al., 2010). Much of the perceived difficulty relating to these mouse models likely can be ascribed to differences in the constructs targeting specific genes, the impact of the absence of these proteins throughout development, as well as the background of the mice on which they were generated. If nothing else, these studies have effectively shown that there is no simple answer to the role of myelin inhibitory proteins in the regeneration of the CNS for either localized regeneration or synaptic plasticity or the hope for growth of long axon tracts.

Nogo

Despite the importance of MAG and other growth inhibitory proteins such as other myelin-derived proteins, chemorepulsive guidance molecules and proteoglycans present in the CNS, a great deal of recent research activity has focused on Nogo. Nogo's extensive history in the regeneration literature is, in part, due to the assertion that it represents the majority of the growth inhibitory activity first detected by Martin Schwab. In this study he showed that neurons in culture could grow into peripheral nerves but not into explanted optic nerves (Schwab and Thoenen, 1985). These early findings led Schwab to hypothesize that a growth inhibitory activity exists in the CNS, and that a lack of axon regeneration was not simply due to a lack of appropriate growth factors.

The Schwab group created a monoclonal antibody, called IN-1, raised against an inhibitory fraction of myelin, and showed that it was able to attenuate the inhibitory properties of CNS myelin *in vitro* (Caroni and Schwab, 1988a). In adult rats, injection of the IN-1 antibody directly into the spinal cord promoted regeneration of axons in the corticospinal tract, and these axons grew past the lesion into the distal spinal cord (Schnell and Schwab, 1990). At a Neuroscience meeting in 1999, the Schwab group presented some peptide sequencing data from a high molecular weight protein, thought to be the elusive high molecular weight growth inhibitory protein. The cloning and sequencing of Nogo followed soon after (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000). This Nogo protein was one of three isoforms (Nogo-A, Nogo-B and Nogo-C) produced from the Nogo gene by alternative splicing. Nogo-A is expressed in adult CNS neurons and oligodendrocytes but not in Schwann cells (GrandPré et al., 2000). Two inhibitory domains of Nogo have been identified: a 66 amino acid loop (Nogo-66) common to all three isoforms of Nogo and a unique amino-terminal region (amino-Nogo) specific to Nogo-A (Prinjha et al., 2000; Oertle et al., 2003). Although Nogo-A has been well characterized as a myelin-associated inhibitor for axonal regrowth in the injured CNS, the normal physiological function of Nogo-A in oligodendrocytes has yet to be fully elucidated. In Nogo knockout mice, delays in oligodendrocyte differentiation, myelin sheath formation and axonal caliber growth within the first postnatal month are observed, and the combined deletion of Nogo and MAG leads to transient hypomyelination (Pernet et al., 2008).

OMgp

Oligodendrocyte myelin glycoprotein (OMgp) is a glycosylphosphatidylinositol (GPI)-anchored CNS myelin protein that is yet another myelin-derived protein that can inhibit neurite outgrowth (Kottis et al., 2002; Wang et al., 2002b). Intriguingly, OMgp is expressed in both neurons and oligodendrocytes in the CNS (Habib et al., 1998), and the expression of OMgp correlates with the onset of myelination (Mikol et al., 1990). OMgp plays a role in mediating the oligodendrocyte-oligodendrocyte and oligodendrocyte-axonal membrane interactions at the nodes of Ranvier (Mikol et al., 1990). Later evidence found a role for OMgp in oligodendroglial-like cells in preventing collateral sprouting and determining the spacing of the nodes of Ranvier (Huang et al., 2005).

Neuronal Receptors for Growth Inhibitory Proteins

The identification of extracellular fragments of myelin-derived proteins as inhibitors of axonal regeneration in the CNS supported the notion of the existence of cell surface receptor molecules that would be involved in transmitting the growth inhibitory signal. The receptors for the various myelin-derived growth inhibitory proteins have taken significantly longer to identify, and new receptors/components are still being added to the list. One difficulty with identifying potential receptors is that it appears that MAG, Nogo and OMgp signal through receptor complexes on the neuronal membrane that may have a different array of constituents dependent upon the specific type of neuron being examined. Importantly, however, it appeared that MAG, Nogo and OMgp all signaled their inhibitory commands through a receptor complex containing, at a minimum, the Nogo-66 receptor NgR1 (Fournier et al., 2001; Domeniconi et al., 2002; Wang et al., 2002b). But given that NgR1 is a GPI-linked cell surface protein, additional partners are needed to transmit an intracellular signal inhibiting axonal outgrowth.

Continuing research into the nature and constituents of the neuronal receptor for myelin-derived growth inhibitors has now led to the identification of multiple proteins as potential participants. Work performed in the laboratory of Zhigang He identified the neurotrophin receptor molecule p75NTR as a component of a complex involving NgR1 (Wang et al., 2002a). Yet, other studies failed to identify an activation of downstream inhibitory Rho signaling if only NgR1 and p75NTR were expressed, which ultimately led to the identification of another component of this inhibitory signaling receptor complex, LINGO-1 (Mi et al., 2004). As the search for additional components continued, it was abundantly clear that multiple populations of CNS neurons expressed either very little or no detectable p75NTR. Research to address this question led to the identification of the TNF receptor family member TROY as yet an additional participant in an inhibitory signal transducing receptor complex (Park et al., 2005; Shao et al., 2005). Importantly, numerous studies have made it clear that all of these proteins are involved in signaling their inhibitory message to Rho. More recently, an additional transmembrane receptor protein has been identified as a high affinity binding site for myelin-derived inhibitory proteins, the paired immunoglobulin receptor B protein (PirB). Originally identified in the nervous system as an important modulator of visual cortical developmental plasticity (Syken et al., 2006), collaborative work between the Shatz and Tessier-Lavigne labs showed that all three myelin-derived inhibitory molecules bind to PirB with high affinity and it signals to inhibit neurite outgrowth (Atwal et al., 2008). The linkage between these inhibitory receptor complexes and CNS plasticity is one that continues to be explored, especially in the context of managing repair in the CNS.

Translation to Clinical Studies

Advances in our understanding of the role of growth inhibitory protein in blocking axon regeneration and functional repair in the CNS have led to the development of viable drug candidates to treat neurotrauma. Clinical studies with MAG as drug target to promote nerve repair have been carried out by GlaxoSmithKline. They have studied the use of anti-MAG antibodies for potential efficacy in treating stroke. A humanized monoclonal antibody to MAG, GSK249320, blocks growth inhibition by MAG. This antibody was tested in 42 squirrel monkeys +24–72 h after stroke (Barbay et al., 2015). Trained on a pellet retrieval task, the monkeys had 30 mg/kg anti-MAG administered intravenously once weekly for 7 weeks. Treated animals showed a more rapid recovery of dexterity, with increased performance as early as 3 days after treatment. It is likely too early to be a result of axon regeneration, suggesting a neuroprotective effect (Barbay et al., 2015). Nogo receptor signaling to Rho plays an important role in neuroprotection (Dubreuil et al., 2003). Therefore blocking MAG inhibitory activity though binding the Nogo receptor likely accounts for the early recovery by reducing cell death. Further, mutant mice lacking NgR also recover from stroke better than controls (Lee et al., 2004).

Overall, MAG and other targets in the growth inhibitory signaling pathway show promise for translation to clinical study for treatment of various disorders, from multiple sclerosis and Alzheimer's Disease to spinal cord injury (for review, see Hawryluk et al., 2008; Schwab, 2010; Schmandke et al., 2014).

Summary

Today it is known that there is a plethora of growth inhibitory proteins expressed in the CNS, from the myelin-derived growth inhibitory proteins to chemorepulsive factors that are important in axonal guidance in development. The role of many of these proteins is still being elucidated, and it is now clear that growing axons respond to both "stop" and "go" signals, and that the preponderance of stop signals in the CNS plays a key role in preventing repair after traumatic injury. These findings have led to a new generation of approaches to overcoming growth inhibition in the CNS to promote regeneration and functional repair after injury. Marie Filbin's pioneering work on MAG has both withstood the test of time and led the way for clinical advances to treat many different neurological disorders.

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Conflict of Interest Statement: Dr. Lisa McKerracher is the Founder and CEO of BioAxone BioSciences Inc. and Dr. Kenneth Rosen is an employee of BioAxone. BioAxone BioSciences is a company that is developing compounds to treat spinal cord injury.

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REVIEW
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Myelin damage and repair in pathologic CNS: challenges and prospects

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Injury to the central nervous system (CNS) results in oligodendrocyte cell death and progressive demyelination. Demyelinated axons undergo considerable physiological changes and molecular reorganizations that collectively result in axonal dysfunction, degeneration and loss of sensory and motor functions. Endogenous adult oligodendrocyte precursor cells and neural stem/progenitor cells contribute to the replacement of oligodendrocytes, however, the extent and quality of endogenous remyelination is suboptimal. Emerging evidence indicates that optimal remyelination is restricted by multiple factors including (i) low levels of factors that promote oligodendrogenesis; (ii) cell death among newly generated oligodendrocytes, (iii) inhibitory factors in the post-injury milieu that impede remyelination, and (iv) deficient expression of key growth factors essential for proper re-construction of a highly organized myelin sheath. Considering these challenges, over the past several years, a number of cell-based strategies have been developed to optimize remyelination therapeutically. Outcomes of these basic and preclinical discoveries are promising and signify the importance of remyelination as a mechanism for improving functions in CNS injuries. In this review, we provide an overview on: (1) the precise organization of myelinated axons and the reciprocal axo-myelin interactions that warrant properly balanced physiological activities within the CNS; (2) underlying cause of demyelination and the structural and functional consequences of demyelination in axons following injury and disease; (3) the endogenous mechanisms of oligodendrocyte replacement; (4) the modulatory role of reactive astrocytes and inflammatory cells in remyelination; and (5) the current status of cell-based therapies for promoting remyelination. Careful elucidation of the cellular and molecular mechanisms of demyelination in the pathologic CNS is a key to better understanding the impact of remyelination for CNS repair.

Keywords: demyelination, spinal cord injury, cell therapy, oligodendrocytes, remyelination, neural stem cells, oligodendrocyte precursor cells, astrocytes

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Introduction

Myelin is a cholesterol rich extension of oligodendrocytes and Schwann cells (SCs) plasma membrane, which serves as a specialized insulation sheath for axons in the nervous system. Myelin facilitates axon signal conduction through enabling "saltatory conduction" (see review by Miron and Franklin, 2014). However, the importance of myelin in the central nervous system

(CNS) is beyond its role in rapid signal conduction along axons as its disturbance also cause other severe functional and neurobehavioral disabilities (as reviewed by Love, 2006). Myelin is important for axon maintenance and function (Nave and Trapp, 2008). Perturbations of myelin structure and function or "demyelination" is associated with a long list of CNS pathologies from congenital and autoimmune disorders to metabolic disturbances (Love, 2006). Progressive demyelination also results in axonal degeneration due to the disruption of axo-oligodendrocyte signaling. A healthy cross talk between axons and oligodendrocytes is required to maintain proper metabolic function of axons, trophic support, cytoskeletal arrangement, ion channel organization, and axonal transport (Edgar et al., 2004; Devaux and Scherer, 2005; Kassmann and Nave, 2008; Nave and Trapp, 2008; Bruce et al., 2010; Nave, 2010; Fünfschilling et al., 2012). Axons become dependent on myelinating glia as myelin appears during the development. This concept was demonstrated in PLP/DM20 deficient mice where the absence of these essential myelin proteolipids resulted in axonal swellings only in myelinated axons with no evidence of axonal pathology in normal unmyelinated fibers (Griffiths et al., 1998). Moreover; mice lacking *Cnp1*, which encodes 2′,3′-cyclic nucleotide phosphodiesterase in oligodendrocytes, show no structural abnormality in myelin but develop axonal swelling and degeneration (Lappe-Siefke et al., 2003). These studies suggest that myelinated axons receive signals from oligodendrocytes that trigger their dependency to myelin. Interestingly, such a dependency have not yet been observe in non-myelinated axons (Griffiths et al., 1998; Lappe-Siefke et al., 2003). Survival of oligodendrocytes is also dependent on axons. Following injury, oligodendrocytes distal to the site of an axonal injury degenerate due to lack of trophic support from the injured axon (Lappe-Siefke et al., 2003). Considering the reciprocal axo-oligodendrocytes signaling, replacement of oligodendrocytes and renewal of myelin sheath around surviving demyelinated axons following injury is a vital repair strategy for CNS regeneration and functional recovery.

Oligodendrocyte precursor cells (OPCs) and neural stem/progenitor cells (NPCs) are two endogenous cell populations, capable of replacing lost oligodendrocytes and remyelinating spared axons following injury (Beattie et al., 1997; Eftekharpour et al., 2008; Meletis et al., 2008; Barnabe-Heider et al., 2010). Despite the spontaneous response and activation of both OPCs and NPCs upon injury, adequacy, and quality of remyelination is challenged due to multiple factors including modifications in the extracellular matrix, astrogliosis, and downregulation of essential trophic and growth factors (Karimi-Abdolrezaee et al., 2006; Meletis et al., 2008; Barnabe-Heider et al., 2010; Karimi-Abdolrezaee et al., 2012; Lau et al., 2012; Gauthier et al., 2013; Lukovic et al., 2015). These injury-induced events either limit oligodendrocyte differentiation or impede the process of axonal ensheathment and remyelination. Over the last decade, cellular and pharmacological repair strategies have been developed to induce remyelination by recruiting endogenous precursor cells or through stem cell therapies (Karimi-Abdolrezaee et al., 2006; Eftekharpour et al., 2007, 2008; Joubert et al., 2010; Kotter et al., 2011; Karimi-Abdolrezaee and Eftekharpour, 2012; Rodgers et al., 2013; Plemel et al., 2014). In this review, (1) we will provide an overview on the precise molecular and ion channel organization of myelinated axons and the reciprocal axo-myelin interactions that warrant properly balanced physiological activities within the CNS, (2) we will dissect the underlying cause of demyelination and the structural and functional consequences of demyelination in axons by focusing on spinal cord injury (SCI) and multiple sclerosis (MS) models, (3) we will discuss the role of activated glia in demyelination and remyelination following demyelination, and (4) we will review the current status of cell-based therapeutic interventions that are designed to promote oligodendrocyte differentiation and facilitate remyelination. Understanding the functional ramification of demyelination and remyelination and the cellular and molecular basis of these events will aid in developing targeted therapies to more effectively promote myelin repair and prevent disease progression in demyelinating conditions.

Normal Molecular Organization of Myelin and Nodes of Ranvier

Myelin is a modified plasma membrane of oligodendrocytes in the CNS, which enwraps a segment of axon in a spiral fashion (Barres et al., 1993). Myelination affects function and molecular organization of axons allowing faster signal propagation with reduced energy consumption (Frankenhaeuser and Schneider, 1951; Bishop and Levick, 1956; Homma et al., 1983; Saab et al., 2013; Stiefel et al., 2013). Several proteins in myelin have been identified to play essential roles in axonal maintenance and function. Proteolipid protein (PLP) and its spliced derivative, DM20, are essential for proper axonal function (Griffiths et al., 1998; Lappe-Siefke et al., 2003). Loss of either PLP or DM20 will affect myelin periodicity and cause axonal swelling (Griffiths et al., 1998; Lappe-Siefke et al., 2003). Swollen axons will gradually become dysfunctional and degenerate causing functional deficits at later stages (Griffiths et al., 1998). Myelin basic protein (MBP) is another structural protein that plays a vital role in myelin compaction and thickening in the CNS (Condorelli et al., 2003; Eftekharpour et al., 2007). *Shiverer* mice that lack MBP demonstrate dysmyelinated axons associated with axonal dysfunction and motor impairments (Loers et al., 2004; Sinha et al., 2006). Interestingly, *Shiverer* mice do not develop axonal swelling and show minimal axonal degeneration compared to PLP/DM20 deficient mice even up to 2–3 months following birth (Griffiths et al., 1998; Loers et al., 2004). Myelin associated glycoprotein (MAG) is essential for the initiation of myelination (Biffiger et al., 2000). Mice with double knockout of MAG and Fyn (a downstream signaling molecule in MAG/Fyn pathway) demonstrate severe optic nerve hypomyelination despite the unaffected presence of oligodendrocytes (Biffiger et al., 2000). MAG is also known to be essential for survival and integrity of myelinated axons (Yin et al., 1998; Pan et al., 2005; Nguyen et al., 2009), however, such a role has not been established for Fyn (Biffiger et al., 2000). CNPase (2,3-cyclic nucleotide

3-phosphodiesterase) is an enzyme that is synthesized in myelinating mature oligodendrocytes and can be found in non-compact regions of the myelin sheath (Nagy et al., 1997). Lack of CNPase has not been shown to affect myelination but myelinated axons will eventually become swollen and degenerate (Lappe-Siefke et al., 2003; Rocco et al., 2004). This evidence demonstrates the importance of the various myelin compartments/proteins for the proper functioning of axons and oligodendrocytes. However, further investigations are required to elucidate the role of each myelin protein in this complex relationship.

Myelinated axons show a high degree of structural organization. A myelinated axon can be separated into distinct domains including node of Ranvier, paranode, juxtaparanode, and internode (Eftekharpour et al., 2008; Ohno et al., 2014; Plemel et al., 2014) (**Figure 1A**). Node of Ranvier is the gap between two adjacent myelin sheaths and contains high concentrations of voltage-dependent Na^+ channels on the axonal membrane (Amor et al., 2014). Electrical impulse cannot flow through the high resistance myelin sheath, but instead flows through the node of Ranvier and depolarizes the axonal membrane at each node resulting in saltatory conduction (Ohno et al., 2011).

In myelinated axons, node of Ranvier was characterized by the localization of voltage-gated sodium (Na_v) and KCNQ K^+ channels (Chiu and Ritchie, 1980; Rasband et al., 1998). Node of Ranvier also contains a collection of adhesion molecules, adaptor proteins, and cytoskeletal structures including, βIV-spectrin, ankyrin G, neuron-glia-related cell adhesion molecule (NrCAM) and a 186 kDa isoform of neurofascin (NF186) (Davis et al., 1996; Salzer, 2003; Amor et al., 2014) (**Figure 1A**). Among these molecules, βIV-spectrin and ankyrin G play a major role in stabilizing the Na_v channels at nodal region (Lai and Jan, 2006). During the development of axons, $Na_v1.2$ channels are initially expressed along pre-myelinated axons with the capability to generate an action potential (Caldwell et al., 2000; Rasband and Shrager, 2000). As myelination ensues, Na_v 1.6 channels begin to cluster at mature nodes of Ranvier (Boiko et al., 2001; Kaplan et al., 2001). $Na_v1.2$ and $Na_v1.6$ channels are both rapidly activating and inactivating channels but $Na_v1.6$ is known to produce a larger persistent current (Caldwell et al., 2000; Rush et al., 2005). Glial cells play an essential role in the formation of normal nodes of Ranvier with their typical nodal Na_v and paranodal K_v channel distribution. As it has been reviewed by Schafer and Rasband (2006), there are similarities in the contribution of glial cells in node formation between the CNS and PNS. In both systems glial cell adhesion molecules (CAMs) in close association with axonal CAMs and cytoskeletal domain, form a structural framework that clusters ion channels with specific formation in nodal and paranodal areas (Schafer and Rasband, 2006) (see **Figure 1A**).

Paranode is the adjacent segment to the node of Ranvier where myelin loops provide an anchor by tethering the myelin to the axonal membrane (Poliak et al., 1999). Evidence has established a determining role for paranodal axo-oligodendrocyte junction in precise localization of ion channels into specialized domains of myelinated axons (Barres et al., 1992a,b; Davis et al., 1996; Kiernan et al., 1996; Kaplan et al., 1997). Paranodal junctions are critical in preventing lateral diffusion of ion channels along the axons to ensure proper segregation of Na^+ and K^+ channels at discrete domains on axonal membrane (Gard et al., 1995; Peles and Salzer, 2000). The paranodal region was characterized by the presence of Contactin and Contactin-Associated Protein (Caspr) that form a complex in the "septate-like junctions" between myelin loops and axolemma (Einheber et al., 1997). Caspr is critical for the establishment of axo-glia junction in myelinated fibers through its interactions with contactin and NF-155 (Lyons and Talbot, 2008). Caspr deficiency results in disruption of the paranodal region and aberrant distribution of ion channels along the axons (Kiernan et al., 1996) (see **Figure 1A**).

The juxtaparanode contains delayed-rectifier *voltage-gated* Kv^+ channels and Na^+/K^+ ATPase channels that allow for rapid exchange of axoplasmic Na^+ for extracellular K^+ and restoration of the resting membrane potential (Poliak et al., 2003; Traka et al., 2003). As ion channel clustering evolves, shaker type Kv1.1 and Kv1.2 channels begin to localize in juxtaparanodal region of the myelinated axons (Wang et al., 1993; Rasband et al., 1998). These channels are associated with the Caspr2/TAG-1 adhesion complex (Poliak et al., 1999, 2003; Traka et al., 2002, 2003; Horresh et al., 2008) (**Figure 1A**). Upon receiving of an action potential, Na_v channels open, allowing an influx of Na^+ into the axon causing depolarization. After each depolarization, Na^+/K^+ ATPase pumps, located at the juxtaparanodal and internodal regions, exchange axonal Na^+ for extracellular K^+ (Meta et al., 1991). This process is an energy dependent mechanism and is essential for rapid and repetitive axonal firing. Similarities exist between the molecular organization of nodes and the axon initial segment; however, while myelin is crucial for the proper molecular organization of nodes, the axon initial segment appears to be intrinsically organized by the neuron (Dzhashiashvili et al., 2007; Yang et al., 2007). Evidence from our group and others have shown that demyelination due to injury and disease results in disruption of the precise nodal organization causing axonal dysfunction (Davis et al., 1996; Kaplan et al., 1997; Nashmi et al., 2000; Karimi-Abdolrezaee et al., 2004; Eftekharpour et al., 2005, 2007; Sinha et al., 2006). Additionally, myelination provides extrinsic trophic signals, which influence the normal maturation, maintenance, and long-term survival of axons (White et al., 2009; Mar and Noetzel, 2010; Castelvetri et al., 2011; Lassmann et al., 2012; Mekhail et al., 2012; Teixeira et al., 2014). Structural and functional importance of nodal organization will be discussed in subsequent sections.

Demyelination and Its Pathophysiological Consequences

Demyelination is damage or loss of the myelin sheath around axons. It is mainly a consequence of oligodendroglia cell death that can occur through multiple mechanisms depending on the type of disease or injury, including genetic defects, infectious agents, autoimmune reactions, trauma, and some by unknown mechanisms (Zimmerman, 1956; Popescu and Lucchinetti, 2012; Kutzelnigg and Lassmann, 2014). Several genetic disorders exist that can cause defects in myelin through improper myelination

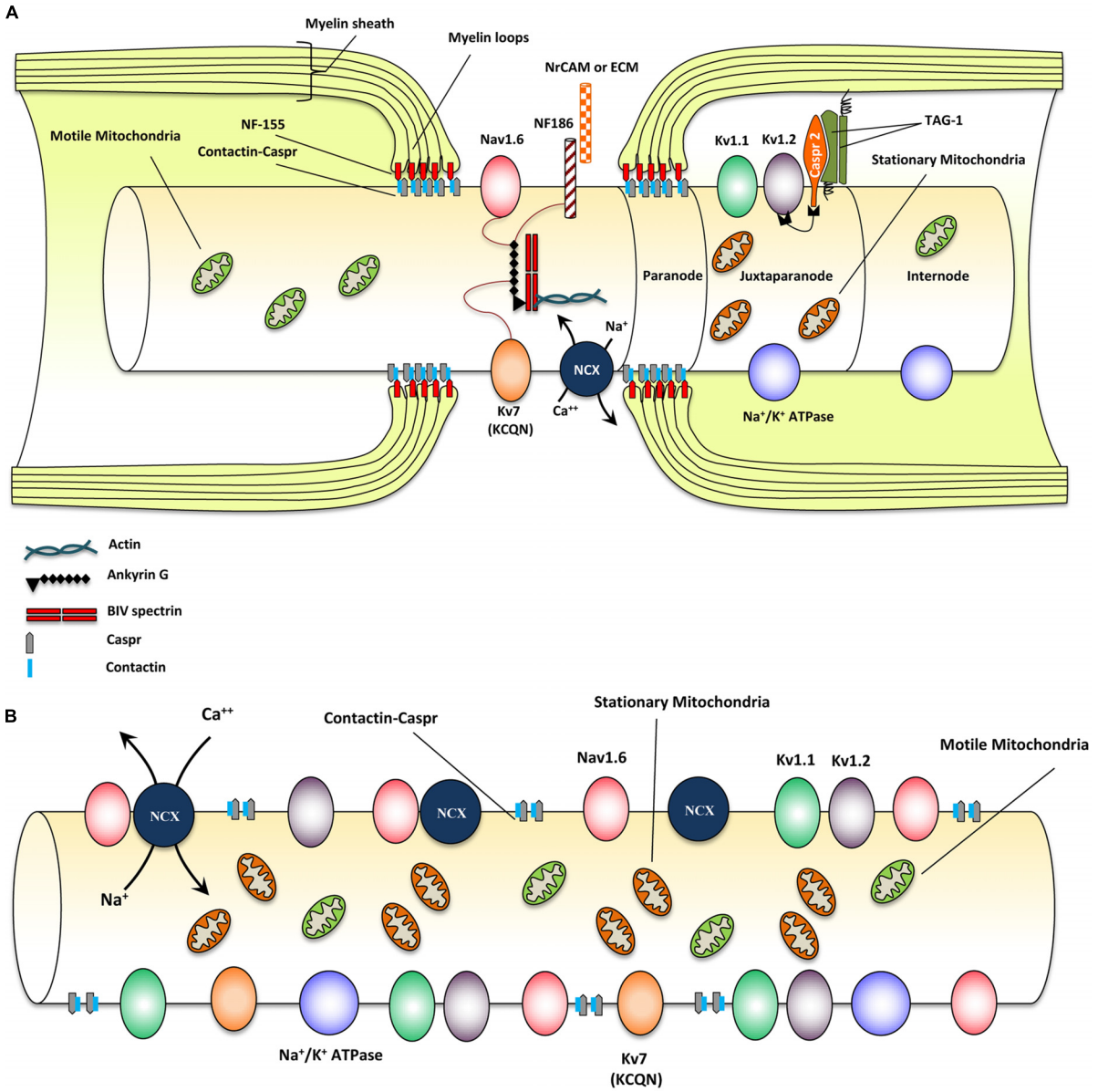

FIGURE 1 | Structural and molecular organization of myelinated axons in normal and demyelinating conditions. (A) Schematic diagram shows structure and molecular configuration of a myelinated axon at the node of Ranvier, paranodal and juxtaparanodal regions. Nav 1.6 and Kv7 (KCQN) are located in the nodal region and are essential for formation and propagation of action potential. Na^+/Ca^{2+} exchanger (NCX) is also located in nodal area and exchanges intracellular sodium with extracellular Ca^{2+} in an ATP dependent manner. Ion channels are precisely localized to specific domains of axons through their contact with adhesion molecules such as neurofascin (NF)-186. These adhesion molecules aid in stabilizing ion channels by connecting them with extracellular matrix (ECM) and glial cell processes surrounding the nodal region. Paranodal junctions are the region where myelin loops are tethered to axonal membranes. Contactin and contactin associated protein (Caspr) play key roles in formation of paranodal loops through their interaction with neurofascin (NF)-155 and other adhesion molecules from myelinating glia. Juxtaparanode contains voltage gated Kv^+ channels (Kv1.1 and 1.2) that are essential for restoring resting membrane potential. Kv channels allow for potassium to exit the axons quickly following depolarization. Caspr2/TAG-1 adhesion complex stabilizes these Kv1.1 and Kv1.2 channels in axonal membrane. Stationary mitochondria (brown) are mainly located in juxtaparanodal and internodal regions where Na/K ATPases are abundant to provide energy for ion homeostasis. There is another mitochondrial population called motile mitochondria (green) which can translocate in both retrograde and anterograde directions along the axon. These mitochondria are being produced in the cell body and can stop in stationary sites. They are important for the turnover and redistribution of mitochondria along the axons and during changes in energy demand. **(B)** Following demyelination, due to the disruption of paranodal myelin loops, all ion channels, pumps and exchangers become dispersed along the axon and sodium influx increases through Nav1.6 channels. Expression of Nav1.6, Kv1.1, and Kv1.2 channels increases significantly following demyelination. Sodium overload causes axonal calcium to reach toxic levels as it is being exchanged with sodium through NCXs by an energy dependent process. Following demyelination, speed of mitochondrial transportation and size of stationary mitochondria significantly increase to compensate for the increased energy demand. Despite robust increase in mitochondrial content, demyelinated axons are unable to maintain a balance between their energy production and expenditure that results in axonal degeneration eventually.

and myelin maintenance or progressive demyelination over time. Charcot-Marie-Tooth disease (CMT), Alexander disease, and Krabbe disease are examples of the many known genetic diseases characterized by axonal demyelination or dysmyelination (Ida et al., 1990; Satoh et al., 1997; Rocco et al., 2004; da Silva Pereira et al., 2013; Perveen et al., 2015).

Multiple Sclerosis is a classic example of autoimmune demyelination in the CNS (Bitsch et al., 2000; Kuhlmann et al., 2008). The early stages of MS involve relapsing-remitting where patient experience demyelination associated with loss of function (i.e., vision and gait), which is usually regained following remyelination. In the progressive stages of MS, irreversible functional deficit occurs which has been associated with progressive loss of axons and neurons (Kurtzke et al., 1977; Flachenecker and Hartung, 1996). Degeneration of chronically demyelinated axons is now considered to be a major contributor to the permanent neurological disability that MS patients eventually endure (Saito et al., 1990; Bjartmar and Trapp, 2001, 2003; Stys, 2004; Su et al., 2009; Dziedzic et al., 2010; Cambron et al., 2012).

Demyelination can also occur through traumatic injury. In the chronically injured spinal cord, there is varying degree of demyelination and dysmyelination in the subpial rim surrounding the lesion site (Nashmi and Fehlings, 2001). Following SCI, some axons and oligodendrocytes are initially lost through necrosis due to mechanical injury. As injury evolves, progressive loss of oligodendrocytes occurs through apoptosis and autophagy that results in demyelination of injured spared axons (Abe et al., 1999; Casha et al., 2001; McTigue and Tripathi, 2008; Kanno et al., 2009; Plemel et al., 2014). Remyelination occurs spontaneously by both OPCs and NPCs following injury even in the chronically injured spinal cord (Beattie et al., 1997; Salgado-Ceballos et al., 1998; Hesp et al., 2015). However, this remyelination attempt is often limited and inadequate due to changes to the post-injury environment (Barnabe-Heider et al., 2010; Karimi-Abdolrezaee et al., 2010, 2012; Xing et al., 2014; Hesp et al., 2015). Therapeutic strategies aimed at promoting remyelination have demonstrated the potential to promote axonal sparing and limit progressive axonal dieback in chronic SCI (Karimi-Abdolrezaee et al., 2010).

Animal models of demyelinating disease such as MS provide invaluable tools to study myelin–axon interactions and understand the pathological effects of demyelination on axonal integrity and function. Here, we will primarily focus on the effects of demyelination on axons in models of MS and SCI, however, many of the details provided here also correlate with other findings in the literature in other demyelinating conditions.

Changes to Ion Channels Following Demyelination

Loss of myelin sheath causes aberrant distribution of ion channels, where Na_v channels diffuse away from the nodes and redistribute across the axonal surface (Waxman et al., 2004). Additionally, there appears to be an overall increase in the expression of Na_v channels in chronically demyelinated axons (Bostock and Sears, 1978; Foster et al., 1980; Waxman et al., 2004). Following experimental autoimmune encephalomyelitis (EAE), $Na_v1.2$, and $Na_v1.6$ channels are up-regulated in demyelinated axons (Craner et al., 2003, 2004a) (see **Figure 1B**). Sodium channel redistribution causes an overall increase in Na^+ influx during impulse conduction and increased demand for ATP during repolarization (Waxman et al., 2004). Furthermore, increased sodium influx has been associated with axonal degeneration through a Ca^{2+}-mediated effect by causing the reversal of the Na^+/Ca^{2+} exchanger (NCX) (Stys et al., 1992; **Figure 1**). Inhibition of sodium channels and NCX has been shown to prevent axonal degeneration (Rosenberg et al., 1999; Bechtold et al., 2004; Hains et al., 2004). Increased axonal Ca^{2+} can activate proteolytic enzymes and eventually lead to degeneration of chronically demyelinated axons (Stys et al., 1992). Ca^{2+} influx is not normally toxic in axons, however, increased energy demand and thus lack of ATP in axons following demyelination causes failure in the energy-dependent Ca^{2+} buffering system to efficiently remove excess Ca^{2+}. This results in a rise in the Ca^{2+} concentration to toxic levels (Stys and Lopachin, 1998; Trapp and Stys, 2009; Plemel et al., 2014). Lack of ATP in chronically demyelinated axons is thought to render axons vulnerable to cellular death over time (Stys, 1998).

Axonal degeneration caused by sodium influx is thought to be mainly mediated through $Na_v1.6$ channels. $Na_v1.6$ channels produce a persistent current, which is much larger than that of $Na_v1.2$ (Smith et al., 1998). $Na_v1.6$ has been shown to be co-localized with NCX following demyelination of axons in EAE model (Craner et al., 2004a) and in postmortem cervical spinal cord and optic nerve tissue of acute MS patients (Craner et al., 2004b). $Na_v1.6$ is co-localized in 60% of axons, which express β-amyloid precursor protein (APP), a marker for axonal injury, whereas it is only expressed in 20% of axons, which are β-APP negative (Craner et al., 2004b). Importantly, in chronic lesions of MS, $Na_v1.6$ is expressed in patches in only a third of axons (Black et al., 2007). Evidence shows that demyelinated axons are more susceptible to axonal injury than dysmyelinated axons, which may be explained by the altered expression of $Na_v1.6$. Dysmyelination in $MBP^{-/-}$ *Shiverer* mice have altered expression of Na_v channels in their axons with $Na_v1.2$ being retained into adulthood and $Na_v1.6$ not being expressed (Boiko et al., 2001). Similarly, $PLP^{-/-}$ mice also show a loss of $Na_v1.6$ channel clustering and an increased expression of $Na_v1.2$ (Rasband et al., 2003). Because $Na_v1.2$ channels are expressed in pre-myelinated axons and can produce signals, it is believed that newly produced $Na_v1.2$ channels support conduction in demyelinated axons (Craner et al., 2004b).

Juxtaparanodal voltage-gated Kv^+ channels are also influenced by demyelination after SCI or genetic dysmyelination (Nashmi et al., 2000; Karimi-Abdolrezaee et al., 2004; Eftekharpour et al., 2005, 2007). We have shown that spinal cord axons in dysmyelinated *Shiverer* mice exhibit a dispersed distribution of Kv^+ channel subunits Kv1.1 and Kv1.2 associated with the loss of the characteristics of juxtaparanodal and paranodal structures (Eftekharpour et al., 2007) (**Figure 1B**). Our investigations on dysmyelinated *Shiverer* mice and Long

Evans Shaker (LES) rats elucidated the role of K^+ channels in axonal function (Eftekharpour et al., 2005; Sinha et al., 2006). These studies revealed aberrant localization and increased expression of both Kv1.1 and Kv1.2 channel subunits along axolemma of dysmyelinated axons while these channels were confined to juxtaparanodal regions of the wild-type axons. Using sucrose gap recording of spinal cord monophasic compound action potentials (CAP), we demonstrated that *Shiverer* spinal cord axons have significantly lower CAP amplitude and area compared to wild-type counterparts (Sinha et al., 2006). Kv^+ channel blockage by specific (DTX-I, DTX-K) and non-specific (4-AP) blockers improved axonal conductance; however, this effect was shown to be more dependent on a combination of subunits as a specific blocker of Kv1.1 failed to improve axonal conduction significantly (Sinha et al., 2006). Interestingly, Kv^+ channels are also important for remyelination (Bacia et al., 2004). Administration 4-aminopyridine (4-AP), a broad-spectrum K^+ channel antagonist that blocks fast K^+ channels, to a mouse model of cuprizone-induced demyelination, resulted in impaired oligodendrocyte regeneration and remyelination (Bacia et al., 2004). These studies collectively demonstrate the pivotal role of axo-myelin interactions in ion channels distributions and functions and more importantly on axonal physiology.

Effect of Demyelination on Axonal Transport and Metabolism

Axonal transport shuttles critical cell body-derived components back and forth between the soma and axon and across synapses in neurons (Millecamps and Julien, 2013). Dysfunction of axonal transport causes neuronal homeostasis imbalance and as a result makes axons more susceptible to axonal degeneration. Axonal transport disturbances are thought to precede the initiation of neurodegeneration in diseases including, hereditary spastic paraplegias (HSPs), AD and Huntington's disease (Gunawardena et al., 2003; Ebbing et al., 2008; Millecamps and Julien, 2013). Accumulation of APP is known as; an early marker of injury in MS patients and is believed to accumulate due to lack of axonal transport following injury (Ferguson et al., 1997; Smith et al., 2003).

Oligodendrocytes and their myelin sheath are critical in regulating slow and fast anterograde transport rates (Kirkpatrick et al., 2001; Edgar et al., 2004). Reduced fast axonal transport can cause degeneration in distal parts of the axons as observed in X-linked spastic paraplegia type 2, which is caused by a mutation of the PLP1 gene, a major protein of the myelin sheath. Absence of PLP causes swelling of axons and deficits in retrograde and anterograde transport (Griffiths et al., 1998; Edgar et al., 2004). In both humans and mice, absence of PLP causes selective axonal degeneration of long tracts including the fasciculus gracilis and distal corticospinal tracts (Garbem et al., 2002). Conversely, *Shiverer* mice, lacking MBP, demonstrate a significant increase in slow axonal transport associated with increased density and instability of microtubules in axons (Kirkpatrick et al., 2001).

Demyelination-induced defects in axonal transport has been also detected in MS models (Lin et al., 2014; Sorbara et al., 2014). Studies in an EAE model of optic neuritis in rats suggest that the extent of disruption in axonal transport appear to be correlated with the severity of inflammation, demyelination, and axonal injury (Lin et al., 2014). Live imaging of individual axonal organelles in the spinal cord of mice with acute EAE revealed that the anterograde and retrograde transport of mitochondria and peroxisomes were markedly reduced in spinal axons, which passed through the lesion (Sorbara et al., 2014). In chronic model of MS, when axonal transport is restricted, there is reduced density in distal organelles which can ultimately lead to "starvation" of distal axonal arbors and axonal degeneration (Sorbara et al., 2014). Transport deficits were shown to occur prior to any marked alteration of microtubule tracks (Sorbara et al., 2014). Dysfunction of axonal transport recovers within days following insult. However, in chronic MS lesions, transport deficits were apparent resulting in lack of distal organelle supply (Sorbara et al., 2014). Reduced axonal transport has been attributed to reactive oxygen and nitrogen species released by immune cells, which alters the attachment of motor/cargo complexes to microtubules (Sorbara et al., 2014). In chronic MS models, anterograde transport from the soma to the synapses appears to be considerably affected resulting in reduced organelle transport from cell body to the axonal terminal at synapses (Sorbara et al., 2014). Hence, interventions to restore transport tracks and axonal transport seem to be vital strategies to slow down the progression of axonal degeneration and reverse these degenerative effects (d'Ydewalle et al., 2011; Denton et al., 2014; Sorbara et al., 2014).

The majority of white matter tract axons were entirely enwrapped by myelin; therefore, it is likely that axons cannot obtain proper nutrient support from their external environment on their own and require metabolic support from glial cells (Saab et al., 2013). It is currently unknown whether glucose transporters are present at the node of Ranvier (Saab et al., 2013). In axons, the highest energy requirement occurs at the Na^+/K^+ ATPase pump, which is located along the internodes (Meta et al., 1991). It is proposed that axons receive their metabolic support mainly through the "pan-glial" network of oligodendrocytes and astrocytes (Saab et al., 2013). Astrocytes were further connected to the blood–brain-barrier and to the nutritive support by brain capillaries (Karimi-Abdolrezaee and Billakanti, 2012). Oligodendrocytes, expressing connexin (Cx) 47, were coupled to astrocytes, expressing Cx30, through gap-junctions (Tress et al., 2012). Double KO mice of Cx47 and Cx30 results in axonal loss, and death in mice (Tress et al., 2012). This evidence suggests that myelination has a dual role in supporting the metabolic activity of neurons by saving energy of axons through saltatory conduction and providing nutrients to neurons.

Ensheathment of axons by oligodendrocytes are shown to drastically diminish the ATP consumption of neurons by reducing the energy required by axons to transmit signals over long distances through saltatory conduction (Barron et al., 2004). However, is it important to take into consideration that the metabolic cost of myelin synthesis and maintenance might be higher than the saved energy (Harris and Attwell, 2012). Nonetheless, myelin does save the amount of energy required in neurons by decreasing the energy expenditure required to

maintain its resting membrane potential and to propagate signals (Saab et al., 2013). Following demyelination, there is an overall increased demand for ATP (Andrews et al., 2006). The energy required to maintain the intra-axonal ionic balance and the resting membrane potential by the Na^+/K^+ ATPase pump were increased, due to redistribution and an overall increase in the number of Na^+ channels (Craner et al., 2004a; Black et al., 2007). *Shiverer* mice exhibit a significant change in the density and activity of mitochondria in their axons in comparison to wild type animals (Andrews et al., 2006). Similarly, unmyelinated segments of retinal ganglion cell axons in the lamina cribrosa also show increased metabolic activity in comparison to myelinated segments (Bristow et al., 2002; Barron et al., 2004). Increased metabolism requirements for demyelinated axons may make these axons more susceptible to death through disease mechanisms such as inflammation (Millecamps and Julien, 2013).

Mitochondria are the major source of axonal ATP and play a critical role in apoptosis, reactive oxygen species generation and calcium buffering (Sheng and Cai, 2012). Two separate populations of mitochondria exist in myelinated axons, stationary and motile mitochondria. The majority of mitochondria are located throughout the axons in stationary sites where multiple mitochondria reside (Ohno et al., 2011; Saxton and Hollenbeck, 2012). Stationary mitochondrial are predominantly located in juxtaparanodal/internodal axoplasm, containing multiple morphologies of varying length, whereas those located in the nodes/paranodal axoplasm appear uniformly short or absent altogether (Ohno et al., 2011). Energy consumption in axons is highest at the juxtaparanodal and internodal regions where Na^+/K^+ ATPases are abundantly present (Meta et al., 1991). These stationary mitochondria do not translocate and usually vary in length but typically contain the same diameter throughout the population (Ohno et al., 2011). A separate population of relatively small but motile mitochondria also exist which translocate in both anterograde and retrograde directions (Detmer and Chan, 2007). These motile mitochondria are produced in the cell body, and can stop within stationary sites. They are essential for the turnover and redistribution of mitochondria and have been shown to fuse with or bud from stationary mitochondria (Detmer and Chan, 2007; Berman et al., 2009; Sheng and Cai, 2012). The rate of transport and docking of these motile mitochondria can be influenced by axonal metabolic demand, such as increases in axonal firing (Ohno et al., 2011).

Recent evidence has shed some light onto the changes that occur to mitochondria in the acute stages of demyelination. Following demyelination there is an overall increased demand for ATP mainly due to changes in ionic homeostasis (Barron et al., 2004). Moreover, the size of stationary sites and the speed of mitochondrial transport is increased in demyelinated axons (Kiryu-Seo et al., 2010). *In vitro* studies on myelinated rat dorsal root ganglion (DRG) axons showed 2.2-fold increase in the size of stationary mitochondria sites and 47% increase in the velocity of motile mitochondria following demyelination (Kiryu-Seo et al., 2010). This response is shown to be an axonal response to the increased ATP demand of demyelinated axons mediated, at least partially, through activating transcription factor 3 (Kiryu-Seo et al., 2010). Increased volume of mitochondria at these stationary sites are shown to be a protective response by demyelinated axons mediated through syntaphilin, a protein, which tethers mitochondria to microtubules at stationary sites (Ohno et al., 2014). Chronically demyelinated axons exhibit increased expression of syntaphilin (Mahad et al., 2009). Demyelinated axons deficient in syntaphilin degenerate at a significantly greater rate than wild type axons associated with smaller increases in stationary mitochondrial volume indicating the importance of mitochondrial migration to these stationary sites (Ohno et al., 2014). In summary, increasing mitochondrial stationary site size is important in protecting neurons from degeneration following CNS demyelination (Kiryu-Seo et al., 2010; Ohno et al., 2014).

Despite this protective response from axons, mitochondrial function appears to be limited in chronically demyelinated lesions of MS (Sheng and Cai, 2012). Following demyelination, changes to the energy balance in axons and dysfunctions of axonal mitochondria contribute to degeneration of chronically demyelinated axons (Sheng and Cai, 2012). There appears to be an overall decrease in the ability of neurons to produce ATP through their mitochondria (Dutta et al., 2006; Mahad et al., 2009; Campbell et al., 2011). In postmortem MS tissues, there was a decreased expression of mitochondrial electron transport chain genes which was associated with decreased ability of mitochondria to exchange electrons in respiratory chain complex I, III (Dutta et al., 2006), and IV (Mahad et al., 2009). This decrease in respiration was later shown to be mediated through deletion of mitochondrial DNA in axons (Campbell et al., 2011). These data suggest that mitochondria in the chronically demyelinated axons have a reduced ability to produce ATP, which can contribute to the axonal degeneration over time.

Endogenous Oligodendrocyte Replacement and Remyelination Following SCI

It is well-known that spontaneous remyelination occurs naturally after CNS injury (Gensert and Goldman, 1997; Salgado-Ceballos et al., 1998). However, the extent and quality of remyelination is limited following injury resulting in limited reorganization of nodes of Ranvier and continued axonal dysfunction (Nashmi et al., 2000; Karimi-Abdolrezaee et al., 2004, 2006). In rat compressive chronic SCI, we found considerable number of chronically injured axons in the rim of white matter that exhibited aberrant distribution of Kv1.1, Kv1.2, and Caspr along the paranodal and juxtaparanodal regions, an established characteristic of axonal demyelination or dysmyelination (Nashmi et al., 2000; Karimi-Abdolrezaee et al., 2004). Additionally, electron micrographs of the injured white matter showed that the spontaneous remyelination after SCI is suboptimal and incomplete as the newly formed myelin around the injured axons is thinner than normally myelinated axons (Nashmi and Fehlings, 2001; Karimi-Abdolrezaee et al., 2006). Considerable evidence over the past years has uncovered that

failure of the injured and diseased spinal cord for adequate remyelination is attributed to multiple factors that include (1) the limited replacement of myelinating oligodendrocytes by spinal cord progenitor cells (Mothe and Tator, 2005; Meletis et al., 2008; Barnabe-Heider et al., 2010; Karimi-Abdolrezaee et al., 2012; Gauthier et al., 2013), (2) insufficient levels of key growth factors for oligodendrocyte maturation and myelination (Kakinuma et al., 2004; Almad et al., 2011; Gauthier et al., 2013), (3) inadequate clearance of myelin debris that interferes with the process of axonal ensheathment and remyelination (Naumann et al., 2003; Miron et al., 2011; Lampron et al., 2015), (4) inhibitory factors mainly driven by activated glia that inhibit migration and maturation of OPCs, differentiation of NPCs to oligodendrocytes, and axonal ensheathment (Larsen et al., 2003; Kuhlmann et al., 2008; Karimi-Abdolrezaee et al., 2010; Lukovic et al., 2015). In the following sections, we will discuss endogenous mechanisms of remyelination and the role of injury microenvironment in modulating the replacement of oligodendrocytes and axonal remyelination.

Replacement of New Myelinating Cells Following SCI

Spinal cord injury results in loss of oligodendrocyte population acutely due to necrosis caused by the primary tissue damage (Almad et al., 2011). However, oligodendrocyte cell loss continues progressively through apoptosis-mediated cell death at subacute and chronic stages of SCI (Casha et al., 2001; Grossman et al., 2001; Almad et al., 2011). Evidence shows that oligodendrocytes are highly susceptible to cell death even after moderate contusive injury resulting in the loss of over 90% of the oligodendrocytes at the lesion epicenter by seven days after injury (McTigue et al., 2001). Interestingly, apoptotic oligodendrocyte death is also observed chronically along the long fiber tracts as a consequence of axonal degeneration and loss of trophic support from axons (Crowe et al., 1997). Similar process has been observed chronically in primate models of contusive SCI (Crowe et al., 1997) and in human SCI (Guest et al., 2005). Multiple secondary injury mechanisms contribute to oligodendrocyte loss in SCI including digestion by proteolytic enzymes released from damaged cells and toxic blood components (Juliet et al., 2009), ischemic damage, and oxidative stress (Thorburne and Juurlink, 1996; McAdoo et al., 1999; Jana and Pahan, 2007), glutamate and ATP mediated excitotoxicity (Wang et al., 2004; Xu et al., 2004; Gudz et al., 2006; Matute et al., 2007), pro-inflammatory cytokines released from infiltrated neutrophils and lymphocytes (Antel et al., 1994; Popovich et al., 1997; Takahashi et al., 2003; Demjen et al., 2004; Pineau and Lacroix, 2007; Donnelly and Popovich, 2008; Kanno et al., 2009) and autophagy (Kanno et al., 2009).

Despite extensive cell death, new oligodendrocytes form and remyelination occurs spontaneously following SCI and demyelinating CNS diseases (Chari, 2007; Zawadzka et al., 2010). Mature oligodendrocytes are post-mitotic and unable to contribute to cell renewal (Keirstead and Blakemore, 1997). However, the spinal cord harbors a population of adult OPCs that contribute to oligodendrocyte replacement following injury (McTigue et al., 2001; Zawadzka et al., 2010). These OPCs can be identified by the expression of platelet derived growth factor receptor α (PDGFR-α) and NG2 proteoglycan (Levine and Nishiyama, 1996; Keirstead et al., 1998; McTigue et al., 2001; Schonberg et al., 2007). Recent findings have shown that resident adult spinal cord OPCs become activated, and change their gene transcription pattern resembling immature OPCs (Moyon et al., 2015). OPCs differentiate into myelinating oligodendrocytes and remyelinate spared axons following demyelination (Gensert and Goldman, 1997; McTigue et al., 2001; Hesp et al., 2015). In addition to OPCs, the spinal cord also contains a population of endogenous NPCs, which is known to contribute to oligodendrocyte replacement following injury (Horky et al., 2006; Meletis et al., 2008; Barnabe-Heider et al., 2010; Karimi-Abdolrezaee et al., 2012). These NPCs exist in the ependymal layer of the intact spinal cord (Weiss et al., 1996; Barnabe-Heider et al., 2010). In adulthood and under normal conditions, NPCs are latent and their activity is mainly to maintain their own population through self-renewal (Meletis et al., 2008; Barnabe-Heider et al., 2010). However, upon injury, they become activated and migrate to the site of injury where they can generate new glial cells (Horner et al., 2000; Barnabe-Heider et al., 2010). Studies by our group and others have demonstrated that activated NPCs predominantly differentiate into astrocytes after SCI, with limited number differentiating into new oligodendrocytes (Mothe and Tator, 2005; Meletis et al., 2008; Barnabe-Heider et al., 2010; Karimi-Abdolrezaee et al., 2012). Moreover, the newly formed NPCs derived astrocytes contribute to glial scar formation following SCI (Meletis et al., 2008; Sabelstrom et al., 2013).

Recent studies have shown that limited ability of NPCs for oligodendrocyte differentiation in SCI milieu may be attributed to unavailability, or modified expression of essential growth factors for oligodendrocyte development (Karimi-Abdolrezaee et al., 2006; Gauthier et al., 2013). Several studies have addressed this possibility by administering growth factors to optimize the post-SCI microenvironment to support survival and differentiation of transplanted and endogenous NPCs into oligodendrocytes as well as remyelination (Karimi-Abdolrezaee et al., 2006; Ohori et al., 2006; Furusho et al., 2012; Gauthier et al., 2013). In our NPC transplantation studies in subacute and chronic SCI, sustained infusion of a cocktail of growth factors including EGF, bFGF, and PDGF-AA was able to significantly enhance the long-term survival of NPCs in the injured spinal cord (Karimi-Abdolrezaee et al., 2006, 2010). Importantly, transplanted NPCs were able to survive and integrate within the host tissue and differentiate into mature myelinating oligodendrocytes and remyelinated axons (Karimi-Abdolrezaee et al., 2006). EGF and bFGF are known to play essential role in NPC survival and proliferation (Weiss et al., 1996). *In vivo* delivery of these growth factors into animal models of contusive SCI was associated with increased proliferation in ependymal layer where NPCs reside. However, despite increased proliferative activity, no significant change in oligodendrogenesis were seen, which could be due to the lack of PDGF-AA in this growth factor regimen (Kojima and Tator, 2000, 2002).

PDGF-AA promotes the proliferation of glial progenitor cells and can trigger differentiation and survival of newly formed oligodendrocytes (Raff et al., 1988; Butt et al., 1997). Moreover, bFGF in synergy with PDGF can regulate proliferation of adult OPCs (Lachapelle et al., 2002; Frost et al., 2003). Interestingly, a developmental study on FGF receptor 1 and 2 double knockout mice ($Fgfr1^{-/-}$, $Fgfr2^{-/-}$) showed normal OPC proliferation, differentiation and initiation of myelination. However, these FGF receptor null animals demonstrated defective myelin thickening during postnatal period and remained defective throughout their adulthood (Furusho et al., 2012). This evidence suggests that FGF signaling can regulate myelin sheath thickness (Furusho et al., 2012).

Mash1, a transcription factor known to promote neural differentiation, have also been implicated in endogenous oligodendrocyte differentiation (Parras et al., 2004; Ohori et al., 2006). Retroviral induction of Mash1 expression in endogenous spinal cord NPCs following SCI resulted in increased oligodendrocyte differentiation and formation of new oligodendrocyte progenitor cells following a complete transection rat model of SCI. Although a small number of new Mash1 expressing oligodendrocytes expressed markers of mature oligodendrocytes, the majority of these cells remained NG2+ expressing progenitor cells and did not fully mature into myelinating oligodendrocytes even weeks after injury (Ohori et al., 2006).

Neuregulin-1 (Nrg-1) is another growth factor known to promote OPCs survival, migration, and differentiation into mature myelinating oligodendrocytes (Vartanian et al., 1999; Miller, 2002). Nrg-1 is known to play essential roles in oligodendrocyte and SC myelination (Brinkmann et al., 2008). Our group has recently demonstrated that the rapid and long lasting downregulation of Nrg-1 following contusive SCI is an underlying cause of inadequate oligodendrocyte differentiation (Gauthier et al., 2013). Restoring the reduced levels of Nrg-1 in the injured spinal cord enhanced tissue preservation, oligodendrocyte differentiation of spinal cord NPCs, and increased oligodendrocyte and axonal survival following SCI (Gauthier et al., 2013). Collectively, these studies show the necessity of micro-environmental optimizations in order to improve endogenous and exogenous replacement of oligodendrocytes and axon remyelination following SCI.

Inhibition of Remyelination after SCI

Current evidence shows that remyelination is additionally limited by inhibitory modifications in the post-SCI niche caused by secondary injury mechanisms particularly in chronic SCI (Larsen et al., 2003; Kuhlmann et al., 2008; Karimi-Abdolrezaee et al., 2010; Lau et al., 2012; Lukovic et al., 2015). Newly formed oligodendrocytes often fail to fully ensheath and myelinate the injured spared axons following injury resulting in incomplete remyelination (Salgado-Ceballos et al., 1998). These inhibitory signals are primarily associated with myelin debris, activated glial cells, and infiltrating leukocytes following injury (Ji et al., 2006; Kotter et al., 2006; Fancy et al., 2009; Pohl et al., 2011; Plemel et al., 2013; Smith et al., 2014; Tepavcevic et al., 2014).

Presence of myelin debris and insufficient clearance by microglia and macrophages contributes to incomplete remyelination by inhibiting OPCs differentiation and maturation *in vitro* and *in vivo* (Kotter et al., 2006; Nave, 2010; Plemel et al., 2014). Recent *in vitro* studies by Plemel et al. (2013) indicate that exposure to myelin debris prevents OPCs maturation and their transition to a myelinating phenotype (Plemel et al., 2013). This was demonstrated by a significant decrease in the number of mature oligodendrocytes and was accompanied by increased expression of two proteins, namely inhibitor of differentiation (ID) 2 and ID4 that are known to block oligodendrocyte maturation (Plemel et al., 2013). It has been shown that myelin clearance and remyelination become less sufficient with aging due to changes in macrophage secretory and phagocytic activity (Shields et al., 1999; Miron and Franklin, 2014). A study using a technique known as "heterochronic parabiosis" where the circulation of a young animal is infused into an older animal, demonstrated improvements in remyelination in the old animal which is presumably due to better functioning of young circulating monocytes for myelin debris clearance (Miron and Franklin, 2014). Myelin debris is a potent inhibitory component of injured spinal cord that impairs regeneration and remyelination. Thus, proper myelin clearance is an important step for remyelination process (Kotter et al., 2006).

Other molecules and pathways known to inhibit myelination include LINGO (leucine rich repeat and Ig domain-containing, Nogo receptor interacting protein), Wnt signaling, and Semaphorin 3A (Sema3A) (Ji et al., 2006; Fancy et al., 2009; Ye et al., 2009; Syed et al., 2011; Boyd et al., 2013). LINGO-1 is a component of Nogo receptor signaling complex (Ji et al., 2006). In a hemisection model of SCI, application of LINGO-1 antagonist (LINGO-1-Fc) promoted functional recovery (Ji et al., 2006). Dysregulation of Wnt signaling in OPCs also inhibits myelination during development and repair (Fancy et al., 2009; Ye et al., 2009). Wnt signaling is activated in differentiating OPCs following chemically induced demyelination and in samples from MS patients (Fancy et al., 2009). Following demyelination, upregulation of T-cell factor 4 (tcf4), a Wnt pathway mediator, is significantly upregulated in differentiating OPCs and inhibit oligodendrocyte maturation and myelination (Fancy et al., 2011). Similarly, Sema3A negatively affects OPC maturation and recruitment in demyelinating conditions (Syed et al., 2011). Its level increases significantly after SCI, reaching its peak at one week following injury (Kaneko et al., 2006). Increased expression of Sema3A has also been observed in MS and experimental demyelination models (Piaton et al., 2011; Boyd et al., 2013). Sema3A overexpression delays recruitment of OPCs to the demyelination site through a chemo-repulsive mechanism (Piaton et al., 2011). Use of Sema3A inhibitor improved tissue preservation, remyelination and functional recovery following SCI (Kaneko et al., 2006).

Collectively, these findings demonstrate that endogenous remyelination was impeded by the inhibitory microenvironment following injury and activated astrocytes and microglia/macrophages seem to play pivotal roles in this inhibition. We will discuss recent studies on the role of resident glial cells and peripherally recruited immune cells in modulating

oligodendrocyte replacement and remyelination following CNS injury.

Role of Glial Cells in Myelination

Astrocytes and CNS Myelination

Astrocytes play critical role in several aspects of myelination in pathologic CNS including clearance of myelin debris, modulating the activity of oligodendrocytes, myelin maintenance, and renewal (Sorensen et al., 2002; Kakinuma et al., 2004; Iacobas and Iacobas, 2010; Moore et al., 2011; Siebert and Osterhout, 2011; Schulz et al., 2012; Bracchi-Ricard et al., 2013; Pendleton et al., 2013; Skripuletz et al., 2013; Brambilla et al., 2014). Using a cuprizone model of rodent demyelination, Skripuletz et al. (2013) demonstrated that astrocytes contribute to the clearance of myelin debris by inducing the recruitment of microglia into demyelinated lesion sites (Skripuletz et al., 2013). Astrocytes impose their modulatory effects through upregulation of CXCL10, a chemokine that is known to play a role in T-cell chemoattraction in CNS autoimmune disorders such as MS (Sorensen et al., 2002).

Intercellular connections between astrocytes and oligodendrocytes are critical for the proper physiology of oligodendrocytes. While there are no gap junctions between oligodendrocytes themselves, they are connected to astrocytes through gap junctions, which make oligodendrocytes indirectly interconnected (Nagy et al., 1997, 2001; Menichella et al., 2003). Evidence shows that gap junctions are essential for proper myelin physiology in the CNS (Menichella et al., 2003). Four different types of connexins have been identified in oligodendrocytes (Cx29, 32, 45, 47). Cx29, Cx32, and Cx47 are known to be expressed by oligodendrocytes that in conjunction with Cx26, 30, 43 on astrocytes, form the astrocyte-oligodendrocyte gap junction complex (Scherer et al., 1995; Altevogt et al., 2002; Menichella et al., 2003; Nagy et al., 2007). Double knockout mice models lacking Cx47 and Cx32 die postnatally due to severe apoptotic oligodendrocyte death, hypomyelination, and axonal degeneration (Menichella et al., 2003). This evidence suggests a critical role for astrocyte and oligodendrocytes inter-cellular signaling in myelin physiology.

Astrocytes provide trophic support to oligodendrocytes by producing growth factors. In an ethidium bromide (EB) induced rat model of spinal cord demyelination, Talbott et al. (2005) were able to show despite recruitment of OPCs to the site of injury, they failed to mature and remyelinate axons in the areas that astrocytes were absent. Astrocytes are known to produce PDGF and LIF, which are supportive for oligodendrocyte survival at progenitor and mature stages, respectively (Barres et al., 1993; Gard et al., 1995).

While supportive of myelination in the normal CNS, astrocytes can play detrimental roles in CNS remyelination following pathology (Rosen et al., 1989; Kakinuma et al., 2004; Pendleton et al., 2013; Brambilla et al., 2014; Hammond et al., 2014). Astrocytes contribute substantially to the extracellular matrix of the CNS. Following injury, they are activated and form a glial scar, which is inhibitory to the repair and regeneration of the CNS. The inhibitory influence of scar is mediated mainly through chondroitin sulfate proteoglycans (CSPGs), which have known inhibitory effects on axonal regeneration, axonal conduction, remyelination, and cellular therapies in SCI (Massey et al., 2008; Karimi-Abdolrezaee et al., 2010, 2012; Haylock-Jacobs et al., 2011; Lau et al., 2012; Cua et al., 2013; Cregg et al., 2014; for review see Dyck and Karimi-Abdolrezaee, 2015). Our recent evidence shows that CSPGs inhibit the ability of NPCs to proliferate, spread their cell processes, survive and differentiate into oligodendrocytes (Dyck et al., 2015). Degradation of CSPGs with chondroitinase ABC promotes oligodendrocyte differentiation and myelination of both transplanted NPCs (Karimi-Abdolrezaee et al., 2010) and endogenous precursor cell populations (Karimi-Abdolrezaee et al., 2012). The detrimental effect of CSPGs upregulation by astrocytes is also observed in MS lesion where the CSPGs aggrecan, neurocan, and versican as well as hyaluronan accumulate at the borders of active demyelinating lesions (Back et al., 2005; Chang et al., 2012). *In vivo* and *in vitro* observations have shown that CSPGs limit the ability of OPCs to migrate, mature and myelinate axons (Kuhlmann et al., 2008; Lau et al., 2012; Pendleton et al., 2013). Removal of CSPGs is correlated with enhanced remyelination in MS lesions (Lau et al., 2012). Collectively, these data identify the inhibitory role of activated astrocytes and scar-associated CSPGs, in modulating NPCs and OPCs integration, migration, maturation and myelination in SCI and MS conditions (Dyck and Karimi-Abdolrezaee, 2015). In addition to the inhibitory ECM produced by astrocytes, reactive astrocytes can also be detrimental to remyelination in demyelinated CNS through the secretion of Endothelin-1 (Hammond et al., 2014). Endothelin-1 is shown to inhibit the differentiation of OPCs into mature myelinating oligodendrocytes through the activation of Notch signaling.

Taken together, these data demonstrate the complex role of astrocytes in the CNS. The presence of astrocytes is required to produce healthy myelin, however, the detrimental effects of activated astrocytes and their production of inhibitory ECM molecules following injury limits the ability of the CNS in self-repair and axon remyelination. Thus, developing interventions to moderate the inhibitory effects of scar-associated molecules is a vital therapeutic strategy for CNS repair and remyelination following injury.

Macrophages/Microglia and CNS Myelination

Emerging evidence indicates that macrophages and microglia also play critical roles in modulating demyelination and remyelination through their antigen presenting ability and production of cytokines, chemokines and growth factors (for review see Gordon, 2003; Mosser, 2003; Martinez et al., 2008). After CNS injury or infection, microglia/macrophages undergo phonotypical changes and become polarized into pro-inflammatory "classically activated" M1 or anti-inflammatory "alternatively activated" M2 phenotypes (as reviewed by Mosser, 2003; Martinez et al., 2008; Kigerl et al., 2009). Although both M1 and M2 cell types are activated microglia/macrophages, they play distinct roles in CNS injury and repair. Pro-inflammatory M1 microglia/microphages are characterized by the production of

cytokines such as interleukin (IL)- 1β, IL-6, IL-12, tumor necrosis factor (TNF)-α (Mosser, 2003; Cherry et al., 2014; Kroner et al., 2014; Peferoen et al., 2015) and reactive oxygen and nitrogen species such as nitric oxide (NO) (Mosser, 2003; Peferoen et al., 2015). Conversely, M2 microglia/microphages are a source of anti-inflammatory factors such as arginase-1 (Arg-1) and IL-10, which are known for their role in the development of type II adaptive immune responses (Anderson and Mosser, 2002; Miron and Franklin, 2014). Generally, accumulating evidence has identified a pro-regeneration role for M2 microglia/macrophages including a supporting role in overcoming axonal growth inhibition imposed by CSPGs and myelin debris (Kigerl et al., 2009).

A recent study also demonstrated phonotypical changes in macrophages/microglia following lysolecithin-induced demyelination in mice (Miron et al., 2013). Using specific M1/M2 markers, Miron et al. (2013) identified a "switch" from M1 to M2 phenotype following demyelination. This transformation occurred between day 3 to 10 post-lysolecithin-induced demyelination when M1 dominant population of $CD68^+$ macrophages/microglia adopted a M2 dominant phenotype identified by Arg-1 expression. This time window was closely correlated with a regenerative stage at which OPCs were recruited to the site of lesion, and differentiated into mature myelinating oligodendrocytes (Miron et al., 2013). Further *in vitro* and *in vivo* investigations confirmed a supportive role for M2 microglia/macrophages in remyelination. Adding M2 conditioned media into OPCs cultures increased oligodendrocyte differentiation and maturation (Miron et al., 2013; Miron and Franklin, 2014). Selective depletion of M1 macrophages by intralesional injection of gadolinium chloride reduced the proliferation rate of OPCs without affecting their migration and remyelination capacity. Interestingly, in a rat model of lysolecithin demyelination, M2 depletion was associated with delayed oligodendrocyte differentiation and nodal reconstruction (Miron et al., 2013) suggesting the pro-myelinating role of M2 macrophages.

Activation of microglia through intraspinal injection of lipopolysaccharide (LPS), a Toll-like receptor-4 (TLR-4) agonist, caused a significant increase in $NG2^+$ cell proliferation and oligodendrocyte differentiation. However, activating microglia using intraspinal injection of zymosan, a TLR-2 agonist, showed oligodendrocyte loss without increase in $NG2^+$ cell proliferation (Schonberg et al., 2007). Interestingly, the extent of SC remyelination remained unaffected (Kotter et al., 2001). Of note, these studies also revealed that the timing of macrophages response is a key factor as the early presence of the macrophages was important for remyelination while delayed macrophage depletion did not impair remyelination (Kotter et al., 2001).

Several mechanisms have been proposed for the positive role of macrophages in remyelination. Among the main proposed mechanisms are removal of myelin debris (Ousman and David, 2000) and production of growth factors known to promote oligodendrocyte differentiation such as Insulin like Growth Factor (IGF)-1 and TGF-β1 (Hinks and Franklin, 1999; Woodruff and Franklin, 1999; Hsieh et al., 2004). However, recent studies by Kotter et al. in demyelinating models suggest that failure in remyelination is mainly attributed to the loss of macrophages-derived promoting factors rather than their role in myelin clearance since this function can be covered by the resident microglia (Kotter et al., 2001; Miron et al., 2013).

Altogether, evidence indicates that the type of immune response is a determining factor that can promote or inhibit remyelination in demyelinating CNS lesions (as reviewed by Mosser, 2003; Wee Yong, 2010). Accordingly, targeted immunomodulatory strategies rather than complete anti-inflammatory treatments appears to be a more effective strategy for promoting remyelination in autoimmune demyelinating conditions such as MS. Further research was needed to elucidate the mechanisms involved in immune response after demyelinating CNS conditions and the factors that promote remyelination (Jackson et al., 2011).

Schwann Cells and Spinal Cord Remyelination

Following SCI or demyelination, endogenous SCs invade the injury site and contribute to remyelinating the demyelinated axons (Blakemore, 1975; Bunge et al., 1994; Beattie et al., 1997; Guest et al., 2005). SCs entry to the injured spinal cord occurs during the first week of injury in parallel to clearance of myelin and glial debris by microglia/macrophages (Bunge et al., 1994; Beattie et al., 1997). SCs enter through dorsal funiculi via dorsal root entry zone or lateral funiculi from the rootlets that become adhered to the lateral spinal cord after injury (Jasmin et al., 2000). In chemical models of demyelination in rodents, remyelination by endogenous SCs and oligodendrocytes progress simultaneously and fully myelinate demyelinated axons by 4 weeks following the insult. However, the extent of oligodendrocytes remyelination is smaller when compare to peripheral myelin formed by SCs and is restricted to the edges of the lesion (Blakemore, 1975). The limited degree of oligodendrocyte remyelination has been attributed to the absence of astrocytes in chemically demyelinated lesions, as oligodendrocytes are dependent on astrocytes for remyelination (Blakemore, 1975; Blakemore and Patterson, 1975).

Entry of SCs to the injured spinal cord is normally limited by glia limitans formed by astrocytes. Following insult, SCs invade the spinal cord through the regions where glia limitans is disrupted. When the glial limitans is re-established by astrocytes, SC invasion becomes progressively limited (Blakemore, 1976). Interestingly, studies have shown that oligodendrocytes gradually replace SCs in remyelinated axons and the transition from peripheral to central myelination occurs without any loss of function (Jasmin et al., 2000). However, other studies showed that SCs persist even chronically following SCI and continue myelinating axons (Hill et al., 2003). Subsequent studies investigated the transition from SCs to oligodendrocytes remyelination and found no change in SC myelination despite increasing oligodendrocyte myelination in EB and radiation (X-EB) demyelination model (Gilson and Blakemore, 2002). There is also evidence that transplantation of OECs, SCs, and bone marrow stromal cells can promote migration of SCs from dorsal roots into the injury site (Hill et al., 2006;

Biernaskie et al., 2007). Therefore; enhanced remyelination or other beneficial effects observed after cell transplantation can be partially attributed to migrating SCs particularly in studies with poor survival of transplanted cells. This evidence suggests that SCs serve as emergency responders and protect demyelinated spinal cord axons at the time when oligodendrocytes are unable to remyelinate efficiently.

Cell-Based Strategies for Remyelination after SCI

Over the past decade, efforts from our group and others have been made to enhance oligodendrocyte replacement after SCI by cell transplantation or activating endogenous stem/progenitor cells (Hofstetter et al., 2005; Keirstead et al., 2005; Karimi-Abdolrezaee et al., 2006, 2010, 2012; Parr et al., 2008; Gauthier et al., 2013; Sparling et al., 2015). Cell transplantation in particular has shown promising results in enhancing SCI repair through multiple mechanisms including cell replacement, trophic support, immunomodulation, and remyelination (Ogawa et al., 2002; Okano et al., 2003; Cummings et al., 2005; Hofstetter et al., 2005; Karimi-Abdolrezaee et al., 2006, 2010; Rossi et al., 2010; Tetzlaff et al., 2011; Vaquero and Zurita, 2011; Hawryluk et al., 2012, 2014). Using different cell types (**Table 1**), these studies have suggested that remyelination is a key mechanism in promoting functional recovery following SCI and demyelinating conditions (Karimi-Abdolrezaee et al., 2006; Sasaki et al., 2006; Eftekharpour et al., 2007; Yasuda et al., 2011; Hawryluk et al., 2014).

Neural Stem and Progenitor Cells

Potential of transplanting NPCs or glial progenitor cells in promoting remyelination has been explored in a wide variety of pathological conditions such as SCI, genetically myelin deficient rodent models, and MS (Karimi-Abdolrezaee et al., 2006; Eftekharpour et al., 2007; Cao et al., 2010; Karimi-Abdolrezaee et al., 2010; Sharp et al., 2010; All et al., 2012; Sun et al., 2013; Hawryluk et al., 2014). These studies have collectively demonstrated the ability of transplanted NPCs to differentiate into myelinating oligodendrocytes and ensheath demyelinated axons. Our studies in mutant *Shiverer* mice and rat SCI revealed that NPC-derived oligodendrocytes integrate with demyelinated and dysmyelinated axons and successfully remyelinate them (Karimi-Abdolrezaee et al., 2006; Eftekharpour et al., 2007) (**Figure 2**). When we transplanted brain-derived NPCs into the spinal cord of subacutely injured rats, we found that survival and oligodendrocyte differentiation of NPCs was limited in the injury microenvironment (Karimi-Abdolrezaee et al., 2006; Eftekharpour et al., 2007) Improving the microenvironment of engrafted NPCs with a cocktail of growth factors (EGF, bFGF, PDGF-AA) considerably promoted their long-term survival, tissue integration, and oligodendrocyte differentiation and remyelination (Karimi-Abdolrezaee et al., 2006, 2010). Importantly, in adult *Shiverer* mice transplanted with NPC, we found evidence of myelination and normal reconstruction of the node of Ranvier in chronically dysmyelinated axons (Eftekharpour et al., 2007). In the areas that NPC-derived oligodendrocytes enwrapped and myelinated the *Shiverer* axons, restoration of a normal configuration of paranodal and juxtaparanodal structures was achieved accompanied by improved axonal function in myelinated axons (**Figure 2**). Similarly, in our rat SCI studies, evidence of NPC-derived remyelination was confirmed with immunoelectron microscopy against YFP expression in transplanted YFP-NPCs (Karimi-Abdolrezaee et al., 2006). Of note, in these studies, transplantation of NPCs resulted in improved locomotor recovery evident by significant improvements in BBB and grid walking test as well as foot print analysis (Karimi-Abdolrezaee et al., 2006).

Subsequent studies by Windrem et al. (2008) demonstrated similar outcomes following transplantation of human glial progenitor cells into the brain of immune-deficient neonatal *Shiverer* mice. In this study, transplanted cells successfully differentiated into myelinating oligodendrocyte and functionally myelinated the dysmyelinated host axons in forebrain and brainstem (Windrem et al., 2008). In agreement with our studies, immunohistological and electrophysiological evidence revealed reconstruction of the node of Ranvier in transplanted neonatal *Shiverer* mice and restoration of transcallosal conduction velocity (Windrem et al., 2008). Moreover, transplanted mice showed increased lifespan and decreased seizure rate, which is frequently seen in *Shiverer* mice (Windrem et al., 2008). Collectively, these studies provided proof-of-concept evidence that NPC-derived oligodendrocytes can functionally remyelinate chronically demyelinated axons in SCI and demyelinating lesions.

Recent studies have provided further evidence that implicates remyelination as a key mechanism for neurological improvement observed after transplantation of NPCs in models of SCI (Yasuda et al., 2011; Hawryluk et al., 2014). Yasuda et al. (2011) transplanted *Shiverer*-derived NPCs that lack the capacity for myelination into the injured spinal cord of NOD/SCID immune-deficient mice. Neuroanatomical, functional, and electrophysiological analyses demonstrated better outcomes in the injured mice transplanted with wild-type NPCs compared to the mice that received *Shiverer* NPCs (Yasuda et al., 2011). This work and similar study by Hawryluk et al. (2014) suggest that remyelination is a key mechanism by which NPCs contributes to the functional recovery following transplantation in SCI.

Genetically Modified NPCs for Remyelination

As mentioned earlier, in the post-SCI microenvironment, transplanted stem cells exhibit limited capacity for survival and migration and they primarily differentiate into astrocytes at the expense of oligodendrocytes and neurons (Hofstetter et al., 2005; Karimi-Abdolrezaee et al., 2006, 2010). Genetic modifications in NPCs have been made to induce expression of specific classes of transcription or growth factors in order to enhance their survival and differentiation or modulate the hostile microenvironment of SCI (Coumans, 2001; Linker et al., 2002; Murray et al., 2002; Hwang et al., 2009; Yang et al., 2009; Cao et al., 2010; Zhang et al., 2012). Using this approach, impact of oligodendrocyte transcription factor,

TABLE 1 | List of selected cell therapies for promoting remyelination following spinal cord injury (SCI) and multiple sclerosis (MS).

Reference	Cell type	Injury model	Outcome
Hofstetter et al. (2005)	Adult rat spinal cord NPCs (Naïve or transduced to express Neurogenin-2)	Thoracic contusive rat SCI, (Subacute)	Increased myelination and white matter sparing in Ngn2-NPC group. Improved BBB and grid-walking in Ngn2-NPC group.
Keirstead et al. (2005)	hESC derived OPCs	Thoracic contusive rat SCI, (Subacute and chronic)	Significant remyelination occurred in subacute OPCs transplantation. Improved functional recovery was observed after subacute transplantation.
Karimi-Abdolrezaee et al. (2006)	Adult brain NPCs + growth factor cocktail	Thoracic compressive SCI (subacute and chronic)	Significant oligodendrocyte replacement and remyelination in subacute transplantation (2 weeks post-injury). Significant functional improvement in subacute therapy (BBB, grid-walking and footprint analysis). Chronic transplantation was not successful (8 weeks post-injury).
Karimi-Abdolrezaee et al. (2010)	Adult brain NPCs + ChABC and growth factors	Thoracic compressive SCI (chronic)	Significant improvement in remyelination and functional recovery in transplanted animals.
Eftekharpour et al. (2007)	Adult brain NPCs	adult *Shiverer* mice	Myelination of chronically dysmyelinated axons happened in transplanted group. Reconstruction of nodes of Ranvier and enhanced axonal conduction.
Sasaki et al. (2006)	Adult OECs from	Adult rat model of spinal cord X-EB demyelination	Transplanted OECs integrated with host tissue and remyelinated axons. Nodes of Ranvier were reconstructed and conduction velocity was significantly restored.
Yasuda et al. (2011)	Adult NPCs derived from wild-type or *Shiverer* mice	Thoracic contusive SCI in adult NOD/SCID mice	Both cell types survived after transplantation and exhibited similar differentiation potential. Only wild-type NPC group demonstrated preserved or enhanced myelination and significant functional and electrophysiological recovery.
Hawryluk et al. (2014)	Adult mice brain NPCs from wild-type or *Shiverer* mice	Rat thoracic contusive SCI using 23 g clip compression	*Shiverer* NPC transplanted group showed no significant remyelination and no significant change in functional recovery while wild-type NPC transplanted group demonstrated significant remyelination by transplanted cells and significant functional recovery.
Kumagai et al. (2009)	Primary and secondary neurospheres (PNS and SNS) from CCV-ES cell line derived NPCS	Mouse thoracic contusive SCI	PNS and SNS survived in host tissue. Transplanted SNS but not PNS showed remyelination, axonal regeneration and functional recovery.
Sharp et al. (2010)	hESC derived OPCs	Rat cervical midline contusive SCI	Significant white and gray matter sparing. Significantly higher properly oligodendrocyte remyelinated axons.
Sun et al. (2013)	mESC derived OPCs	Radiation induced rat cervical spinal cord demyelination	Transplanted cells survived and integrated into the host tissue, migrated to the injured tissue and differentiated into oligodendrocytes. Improvement of forelimb locomotor function.
Windrem et al. (2008)	Human GRPs	Neonatal *Shiverer* mice cross bred with immune-deficient *rag2* null mice	Multifocal anterior and posterior fossa delivery of hGRPs showed significant improvement in survival, neurological function and seizure frequency in neonatal *Shiverer* mice.
Hwang et al. (2009)	hNPCs expressing Olig2	Adult rat contusive SCI	Transplanted animals exhibited enhanced myelination in white matter and improved functional recovery.
Cao et al. (2010)	CNTF expressing adult rat spinal cord OPCs	Thoracic contusive SCI	Enhanced remyelination and functional recovery in transplanted animals.
Utzschneider et al. (1994)	Adult spinal cord glial cells	Neonatal congenitally myelin deficient rats	Improvement in conduction velocity of axons in transplanted region was observed.
Walczak et al. (2011)	Human glial restricted progenitors	Adult rat chemical focal demyelination model and neonatal rag2$^{-/-}$*shiverer* mice	Transplanted rats showed preserved electrophysiological conduction across spinal cord. Despite extensive remyelination in neonatal *Shiverer* mice marginal myelination observed in transplanted adult rats.

(Continued)

TABLE 1 | Continued

Reference	Cell type	Injury model	Outcome
Totoiu et al. (2004)	Neonatal mouse glial committed progenitors	Mouse HMV induced CNS demyelination	Remyelination in transplanted animals was evident with significant axonal preservation and locomotor recovery.
Hatch et al. (2009)	hESC derived OPCs	JHMV induced CNS demyelination	Transplanted cells failed to survive beyond 2 weeks. Focal remyelination and subtle functional recovery was observed that was attributed to inflammatory modulation and trophic support provided by transplanted cells.
Piao et al. (2015)	hESC derived OPCs	Athymic nude rat model of radiation induced brain demyelination	Transplantation to several spots along cerebellum and forebrain showed significant remyelination and cognitive and motor improvement.
Imaizumi et al. (2000)	Adult OECs or Schwann cells (SCs) from pigs expressing human complement inhibitory protein, CD59	Adult rat dorsal column transection	Remyelination of demyelinated axons was observed with improvement in conduction velocity in transplanted animals.
Radtke et al. (2004)	Adult porcine OECs	EBr or lysophosphatidyl choline induced demyelination in African green monkey spinal cord	Transplanted cells integrated with host tissue and remyelinated axons.
Boyd et al. (2004)	LacZ expressing OECs injected into the cystic cavity	Rat model of thoracic contusive SCI	Transplanted cells did not migrate from the injection site. SCs migrated into the cystic cavity. No direct evidence of remyelination by LacZ labeled OECs was observed. OECs mainly enveloped groups of axons myelinated by SCs.
Sasaki et al. (2011)	Rat OECs	Rat model of X-EB induced spinal cord demyelination (acute and subacute)	Transplanted cells integrated with host tissue and remyelinated axons. Reconstruction of nodes of Ranvier and improved axonal conduction velocity were achieved. Remyelination was higher in acutely transplanted group.
Tsuji et al. (2010)	MEF-iPSCs derived NPCs	Mouse model of contusive SCI	Transplanted cells showed multilineage differentiation into oligodendrocytes, astrocytes and neurons.
Wang et al. (2013)	Human skin derived iPSC derived OPCs	*Shiverer* mice neonatal brain	Transplanted cells integrated with host tissue and differentiated into astrocytes and MBP expressing oligodendrocytes. *Shiverer* axons were remyelinated and node of Ranvier was reconstructed. No tumorigenicity was observed. Increased lifespan of *Shiverer* mice was observed.
Nori et al. (2011)	hiPSC derived neurospheres	Thoracic contusive SCI in NOD-SCID mice	Grafted cells differentiated into neurons, astrocytes and oligodendrocytes. Myelin content of the tissue was increased with significantly better functional recovery in transplanted group.
Kobayashi et al. (2012)	hiPSC-NS/PCs	Adult common marmoset primate model of contusive SCI	Improved functional recovery in open field, bar grip and cage climbing tests in transplanted group.

Olig2 has been investigated in myelin physiology and repair. Olig2 is known to play important role in oligodendrocyte differentiation during development (Takebayashia et al., 2000). Using a contusive model of rat SCI, Hwang et al. (2009) demonstrated that retroviral overexpression of Olig2 in transplanted F3 human NPCs enhanced oligodendrocyte replacement accompanied by a significant increase in white matter sparing, decreased lesion size, increased myelin thickness and improved locomotor recovery compared to non-transduced F3 NPCs. In another study, using transgenic mice overexpressing Olig2 in $SOX10^+$ oligodendrocytes, Wegener et al. (2015) confirmed that Olig2 overexpression enhanced OPC differentiation, maturation, and migration as well as remyelination following chemical demyelination. Studying demyelinated lesions in human MS brain samples revealed that $Olig2^+$ remyelinating oligodendrocytes are more abundantly present in active lesion borders compared to chronic plaques (Wegener et al., 2015). This evidence identifies a role for Olig2 in promoting oligodendrocyte differentiation and remyelination.

A neuroprotective role for ciliary neurotrophic factor (CNTF) in enhancing oligodendrocyte differentiation and maturation has been established in the CNS and PNS (Hagg and Varon, 1993; Linker et al., 2002; Naumann et al., 2003). Genetic modifications in adult rat spinal cord OPCs to express CNTF attenuated oligodendrocyte apoptosis resulting in improved survival, maturation and myelinating capacity of OPCs (Cao et al., 2010). CNTF expressing OPCs transplanted rats also showed significant

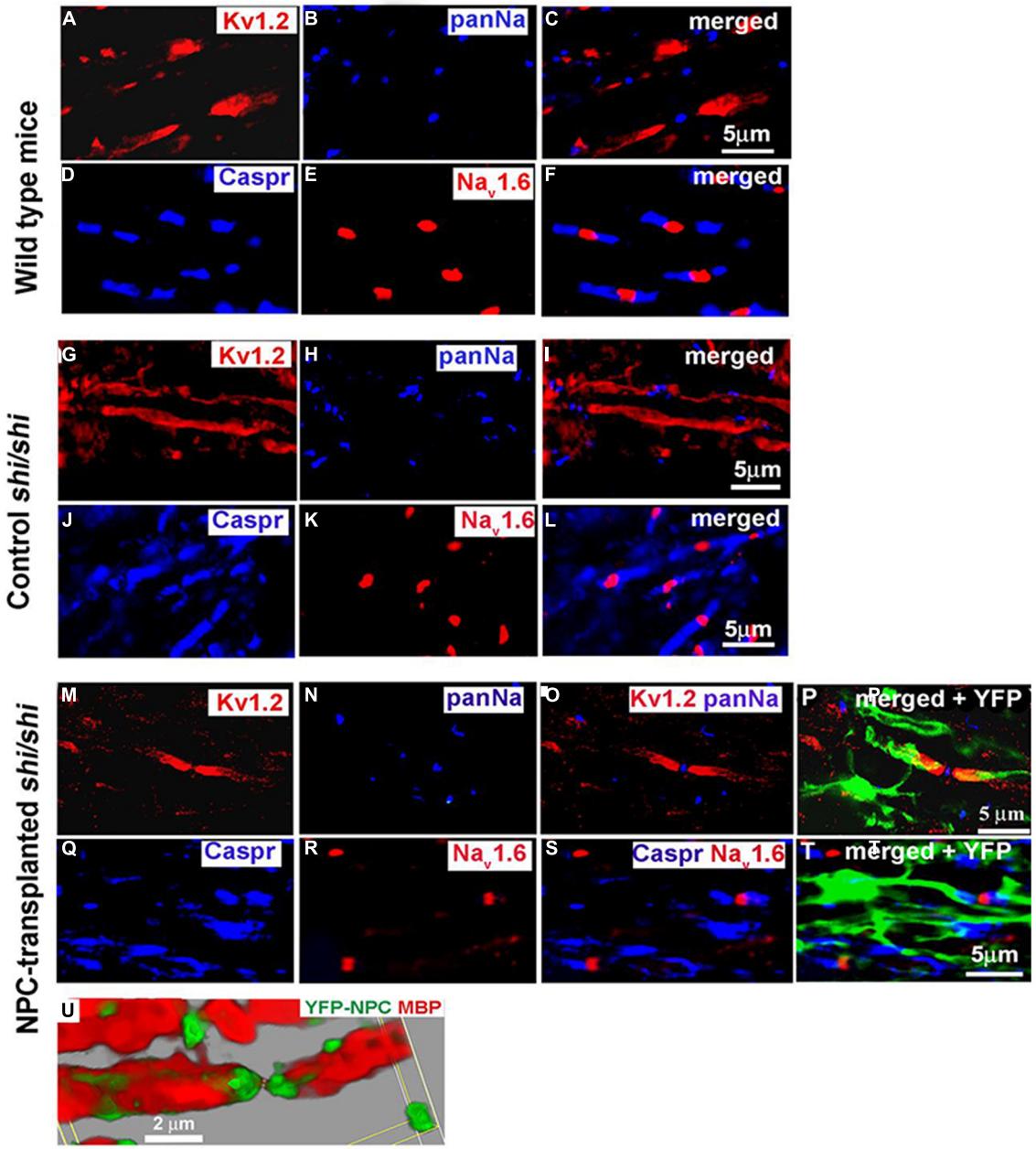

FIGURE 2 | Transplanted adult NPCs (aNPCs) promote the aggregation of K^+ channels and the formation of nodes of Ranvier in the spinal cord axons of *shi/shi* mice. Confocal immunostaining of Kv1.2 subunits (red) and pan-Na^+ hannels (blue) in the spinal cord of wild-type mice **(A–C)**, control *shi/shi* mice **(G–I)**, and transplanted *shi/shi* mice **(M–P)** is depicted. Kv1.2 subunits were clearly localized to the juxtaparanodal regions of wild-type spinal cord axons **(A–C)**, confirmed with nodal pan-Na^+ immunostaining. In *shi/shi* mice, Kv1.2 immunostaining was abnormally distributed along the axonal internodes **(G–I)**, but Na^+ clusters were observed as aberrant nodal aggregates. Six weeks after aNPCs transplantation, spinal cord segments of *shi/shi* mice showed restoration of Kv1.2 subunit clusters **(M–P)**. YFP-positive processes of transplanted aNPCs were observed in close association with axons containing restored K^+ channels aggregates **(P)**. Nodal localization of Na^+ channels was further confirmed using Nav1.6 (red) immunostaining in wild-type **(D–F)**, control *shi/shi* **(J–L)**, and transplanted *shi/shi* **(Q–T)**. Caspr (blue) immunostaining was used to identify the paranodal area. A 3D reconstruction clearly shows a node of Ranvier that is bordered by an MBP-expressing NPC derived oligodendrocyte. Note that the processes of YFP-labeled oligodendrocytes avoid the nodal region **(U)**. From Eftekharpour et al. (2007).

improvements in their axonal signal conduction and hind limb locomotor recovery compared to the group that received normal OPCs (Cao et al., 2010).

Altogether, current evidence suggests that NPCs can be engineered to act as environmental modulators in addition to their role in cell replacement. This strategy presents

a therapeutic avenue for improving microenvironment and optimizing the outcome of cell transplantation in CNS trauma and demyelinating diseases.

Glial-Restricted and OPCs

Due to challenges with oligodendrocyte differentiation of NPCs, transplantation of differentiated glial-restricted precursors (GRP) and OPCs has been pursued in SCI and demyelinating conditions (Utzschneider et al., 1994; Keirstead et al., 2005; Walczak et al., 2011; Sun et al., 2013). Early studies demonstrated the potential of bi-potential O2A progenitor cells for differentiation into oligodendrocytes or type 2 astrocytes (De Los Monteros et al., 1993). When postnatal O2A progenitor cells, genetically modified to express β-galactosidase, were transplanted into the spinal cord following x-ray induced demyelination, they showed the capacity to form oligodendrocytes and successfully remyelinated axons in demyelinated lesion (Groves et al., 1993). Interestingly, no evidence of astrocyte differentiation was observed after transplantation of O2A progenitors (Groves et al., 1993) suggesting the influence of demyelinated host tissue on cell fate specification of transplanted cells. Successful remyelination also achieved following transplantation of brain-derived mouse GRPs into the spinal cord of mice with virally induced demyelination lesion that was associated with improved functional recovery (Totoiu et al., 2004).

Application of OPCs has also shown promising results in supporting remyelination. The potential of human ESC derived OPCs for remyelination has been evaluated in animal models of SCI and demyelination (Keirstead et al., 2005; Sharp et al., 2010; Piao et al., 2015). These studies uncovered that subacute stage of injury is the optimal time window for OPCs transplantation in the context of traumatic SCI. Keirstead et al. (2005) compared remyelination and functional recovery following transplantation of hESC derived OPCs at 7 days (subacute) and 10 months (chronic) after contusive SCI in rats. Subacute transplantation was associated with successful remyelination by transplanted OPCs and functional recovery while chronic transplantation did not improve remyelination (Keirstead et al., 2005). In this study, contribution of invading SCs to remyelination was also assessed (Keirstead et al., 2005). The number of SC-remyelinated axons was significantly increased in injured animals and decreased to normal levels after transplantation of hESC derived OPC. In order to quantify the contribution of SCs and OPCs in remyelination, authors relied on the difference in the thickness of myelin to differentiate between central vs peripheral myelination. However, use of immunological labeling for SCs or ultrastructural criteria of SC myelination would be a more precise approach to determine the contribution of SCs in spinal cord remyelination after injury. In addition to SCs, endogenous OPCs and NPCs also contribute to remyelination which was not investigated in this study.

One important outcome of the Keirstead study was the inability of transplanted OPCs to remyelinate chronically demyelinated axons despite their survival and the capacity to differentiate into oligodendrocytes (Keirstead et al., 2005). This evidence suggests the inherited inhibitory nature of chronic lesions for axonal ensheathment and remyelination. Interestingly, our groups observed the same outcomes after transplantation of NPCs in chronic SCI (Karimi-Abdolrezaee et al., 2006). Our subsequent studies revealed that dense deposits of CSPGs in the chronic glial scar is a potent limiting factor to survival, integration, oligodendrocyte differentiation, and remyelination of both endogenous and transplanted NPCs (Karimi-Abdolrezaee et al., 2010, 2012; Dyck et al., 2015). As described in earlier sections, removal of CSPGs by ChABC allowed NPC-mediated remyelination of chronically demyelinated spinal cord axons. These studies suggest that successful application of OPCs or NPCs in chronic SCI requires multifaceted interventions to modulate the inhibitory milieu of the established glial scar. Although other factors may play a role in this inhibition, CSPGs seems to be a major obstacle (Dyck and Karimi-Abdolrezaee, 2015).

Efficacy of transplanted OPCs has also been investigated in chronic demyelinating conditions. Transplantation of mouse ESC derived OPCs into a radiation induced rat model of cervical spinal cord demyelination 4 months after radiation therapy, showed successful survival, and migration of these cells toward the lesion and their capacity for oligodendrocyte remyelination (Sun et al., 2013). Moreover, significant myelin and tissue preservation as well as improved forelimb locomotor function were observed in OPC transplanted animals (Sun et al., 2013). Transplantation of hESC derived OPCs transplanted into athymic nude rat model of radiation induced brain demyelination, promoted remyelination of the demyelinated brain and cerebellum and ameliorated cognitive and motor deficits in the injured animals, with no evidence of tumor formation following transplantation (Piao et al., 2015). Absence of neuronal differentiation and insignificant astrocyte differentiation in transplanted cells indicates a defining role for remyelination by oligodendrocytes in white matter repair and cognitive and motor improvement (Piao et al., 2015).

In addition to their role in replacing remyelinating oligodendrocytes, OPCs are known to enhance axo-neuronal growth and survival by producing growth factors such as brain derived neurotrophic factor (BDNF), IGF-1, glial derived growth factor (GDNF), neuregulins (NRGs), and neurotrophins (Du and Dreyfus, 2002; Dai, 2003; Wilkins et al., 2003) that potentially contributed to the functional improvement observed in these studies. Based on current evidence, transplanted OPCs or NPCs have additional benefits beyond their known role in cell replacement and myelination including improving the host environment with providing trophic support and their immunomodulatory effects.

Olfactory Ensheathing Cells

Olfactory ensheathing cells (OECs) have been extensively examined for their potential for remyelination after SCI and demyelinating lesions (Ramon-Cueto and Nieto-Sampedro, 1994; Imaizumi et al., 2000; Kato et al., 2000; Akiyama et al., 2004; Radtke et al., 2004; Boyd et al., 2005; Sasaki et al., 2006, 2011; Lankford et al., 2008, 2014; Radtke, 2008; Tabakow et al., 2013). OECs are supporting cells that ensheath the

axons of olfactory neurons. They have become a popular choice for cell transplantation due to their accessibility. Unlike oligodendrocytes or SCs, OECs do not normally myelinate axons in the nervous system (Sasaki et al., 2011). However, evidence suggests that following transplantation into the injured CNS, OECs demonstrate the ability to remyelinate axons and can promote functional recovery in animal models of SCI (Imaizumi et al., 2000; Kato et al., 2000; Akiyama et al., 2004; Radtke et al., 2004; Sasaki et al., 2006, 2011; Lankford et al., 2008; Radtke, 2008; Tabakow et al., 2013; Lankford et al., 2014).

Studies by Sasaki et al. (2011) showed evidence of remyelination by transplanted rat OECs in a rat model of X-EB induced demyelination in the thoracic spinal cord. Unlike chemical demyelination, X-EB models allow long lasting demyelination due to elimination of oligodendrocytes and astrocytes in the lesion area; therefore, excluding the potential contribution of endogenous myelinating cells. In this model, remyelination by transplanted OECs was investigated after acute and subacute OECs transplantation. Engrafted OECs were shown to integrate with the host tissue and remyelinated axons with predominant characteristic of P0 peripheral myelination (Sasaki et al., 2006). Immunoelectron microscopy at 2 and 3 weeks following transplantation confirmed OECs-derived remyelination of host demyelinated axons and reconstruction of the normal organization of the nodes of Ranvier (Sasaki et al., 2006). Functionality of OEC remyelination was confirmed by improved conduction velocity through electrophysiological recording of the spinal cords at 3 weeks after transplantation (Sasaki et al., 2006). While considerable myelination was observed following both acute and subacute lesions, the extent of myelination was considerably higher in acutely transplanted group (Sasaki et al., 2006).

Another study in nonhuman primate demonstrated the ability of OECs to remyelinate spinal cord axons following demyelination lesions (Radtke et al., 2004). In this study, porcine OECs were transplanted into chemically induced demyelinated adult female African green monkey spinal cord 3 days after the induction of lesion (Radtke et al., 2004). Evidence of remyelination was observed at 3-5 weeks after transplantation in 62.5% of the transplant recipients. Endogenously remyelinated axons were evident in the vicinity of OECs-myelinated axons in the lesion while no remyelination was observed in non-transplanted group at this time-point. However, this study was not able to clearly distinguish between OECs and SCs derived remyelination (Radtke et al., 2004).

Since SCs and OECs demonstrate similar characteristics, their behavior and response have been compared in demyelinated spinal cord in co-transplantation studies (Lankford et al., 2008, 2014). Interestingly, these studies suggest distinct migratory and proliferative properties for OECs compared to SCs. Lankford et al. (2008) injected a mixture of SCs and OECs at 1 week after X irradiation into the rat spinal cord. Only transplanted OECs migrated through white and gray matter of the irradiated spinal cord. Four weeks after transplantation, a new EB demyelination was induced in the spinal cord. Tissue analysis revealed that "pre-loaded" OECs but not SCs were able to migrate toward the new EB lesion and populate the demyelinated site. Subsequent studies by the same induced demyelination using X irradiation in the juvenile rats hippocampus confirmed a better migratory ability for OECs in populating the lesion compared to SCs similar to the pattern observed in the adult host (Lankford et al., 2008, 2014).

While extensive evidence suggests a direct role for OECs in axonal ensheathing and remyelinating, there are also some reports that have questioned their myelinating capability in SCI (Takami et al., 2002; Boyd et al., 2004). Boyd et al. (2004), using Lacz expressing OECs, were able to track transplanted OECs in the spinal cord of injured rats. While labeled OECs were evident in the lesion at 3 weeks post-transplantation, electron micrographs of the injured spinal cord showed no direct evidence of axonal ensheathment and myelination by LacZ labeled OECs (Boyd et al., 2004). Transplanted LacZ labeled OECs mainly enveloped a group of axons myelinated by SCs. The authors concluded that OECs support SCI repair by other mechanisms such as providing permissive substrate for axon growth and SC remyelination (Boyd et al., 2004). In contrast to these observations, another study using superparamagnetic iron oxide labeling demonstrated the ability of both transplanted SCs and OECs for remyelination in X-EB spinal cord demyelination (Dunning et al., 2004). Subsequently, Dombrowski et al. (2006) transplanted GFP expressing OECs into the injured sciatic nerve and using immunoelectron microscopy showed that transplanted GFP+ OECs formed peripheral type myelin around axons. However, one question that remained undetermined in these studies was the degree of SC contamination in their OEC culture. Degree of SCs contamination in OEC cultures is an important consideration that may underlie different outcomes seen in these studies.

Regardless of their direct role in axonal remyelination, OECS are shown to recondition injury environment by producing a host of trophic factors including NGF, BDNF, and CNTF that can support endogenous repair (Ramon-Cueto and Avila, 1998; Chuah and West, 2002; Martin et al., 2002; Au and Roskams, 2003). Studies have shown that transplanted OECs enhance functional recovery through enhanced angiogenesis and immunomodulatory effects at acute and subacute stages post-transplantation (López-Vales et al., 2005). López-Vales et al. (2005) demonstrated that transplantation of OECs results in upregulation of Cox-2 and vascular endothelial growth factor (VEGF) following SCI.

Collectively, while application of OECs has shown promising results in experimental models of SCI and demyelination, the ability of OECs for CNS remyelination is still a matter of debate due to their similarities with SCs and potential contamination of OEC cultures with SCs. Further *in vitro* and *in vivo* studies using specific markers of OECs and SCs is required to specifically distinguish these two cell populations and confirm the myelinating capacity of OECs.

Induced Pluripotent Stem Cells

While cell translation is a promising approach for enhancing remyelination following injury, graft rejection due to the host

immune reaction poses a major challenge to the success of cell-based therapies (Nakamura and Okano, 2013). Successful transplantation in animal models has required immunosuppression, wherein chance of the graft rejection had been minimized using immunosuppressive therapy or genetically immune-deficient models (Cummings et al., 2005; Karimi-Abdolrezaee et al., 2006, 2010). Importantly, immune reaction is a hallmark of pathologic CNS that plays essential roles in tissue injury and repair (Eyo and Dailey, 2013; Cherry et al., 2014). Hence, use of autologous sources of stem cells seems to be a logical solution to warrant the long-term survival of transplanted cells without compromising the host CNS immune response. Moreover, autologous transplantation preclude ethical and technical limitations in using embryonic stem cells or fetal derived tissues that has been a major hurdle in translating this promising repair strategy into clinical arenas (Lo and Parham, 2009; Zarzeczny and Caulfield, 2009; Nakamura and Okano, 2013; King and Perrin, 2014).

Generation of induced pluripotent stem cells (iPSCs) using autologous somatic cells has opened new avenues for developing clinically feasible cell transplantation approaches (Saito et al., 2005; Takahashi and Yamanaka, 2006; Yamanaka and Takahashi, 2006; Qi and Pei, 2007; Yamanaka, 2008; Nagy, 2009; Zhou et al., 2010; Liu et al., 2012; Nguyen et al., 2014). Since the introduction of iPSCs technology in stem cell research, several studies have explored the therapeutic potential of transplanting iPSCs-derived NPCs or OPCs in SCI for inducing remyelination as well as the safety of this approach with regards to tumorigenesis (Miura et al., 2009; Tsuji et al., 2010; Nori et al., 2011; Kobayashi et al., 2012; Wang et al., 2013). Tsuji et al. (2010) transplanted allogenic NPCs generated from mouse embryonic fibroblast (MEF)-derived iPSCs into a mouse model of contusive SCI at 9 days post injury. The safety of this approach was previously confirmed by observing no teratoma formation after 24 weeks of transplantation into NOD/SCID mouse brain (Miura et al., 2009). Six weeks following transplantation, MEF-iPSC derived NPCs showed multilineage capacity and differentiated into oligodendrocytes, astrocytes, and neurons (Tsuji et al., 2010). Functional analysis suggested improved neurological recovery in the transplanted animals, which was accompanied by enhanced remyelination as well as regeneration of the raphe-spinal fibers in transplanted animals (Tsuji et al., 2010). Similar observations have been made using a variety of adult tissue derived iPSCs (Nakamura and Okano, 2013). Transplantation of selected non-tumorigenic clone of adult tail tip fibroblast derived iPSCs into a mouse model of contusive SCI resulted in significant functional recovery (Tsuji et al., 2010; Nakamura and Okano, 2013). In mouse (Nori et al., 2011) and primate (Kobayashi et al., 2012) models of SCI, transplanted NPCs derived from human adult facial skin derived iPSCs were able to differentiate into neurons, oligodendrocytes, and astrocytes which resulted in enhanced functional recovery accompanied by axonal growth (Nori et al., 2011; Kobayashi et al., 2012; Nakamura and Okano, 2013).

Remyelination has been considered as a mechanism underlying the functional improvement observed in iPSCs transplantation strategies (Wang et al., 2013). A recent study by Wang et al. (2013) demonstrated that transplanted OPCs generated from autologous skin derived human iPSCs of keratinocyte and fibroblast origin, were able to differentiate into mature myelinating oligodendrocytes and myelinate human fetal cortical neurons *in vitro* (Wang et al., 2013). In immune-deficient *Shiverer* mice, human iPSC-derived OPCs integrated with the host neonatal brain tissue after transplantation, differentiated to MBP expressing oligodendrocytes, remyelinated the dysmyelinated *Shiverer* axons, and constructed the nodes of Ranvier (Wang et al., 2013). No tumorigenicity was observed in this study and transplanted *Shiverer* mice exhibited increased lifespan (Wang et al., 2013).

Taken together, emerging evidence shows the potential and feasibility of transplanting iPSCs derived NPCs and OPCs for promoting oligodendrocyte replacement and remyelination in demyelinating conditions such as MS and traumatic CNS injury. While promising, the risk of tumorigenicity and proper cell differentiation of this therapy have yet remained to be carefully investigated. In **Table 1**, we have listed cell therapies that have been developed to promote oligodendrocytes replacement and remyelination after SCI and MS conditions.

Conclusion

The myelin sheath is an essential component in the CNS and PNS that ensures rapid signal transduction in axons through the nervous system. Myelinated fibers show a highly specialized and organized structure at the node of Ranvier that is vital to their proper functioning. Damage to oligodendrocytes due to trauma or disease results in demyelination that causes aberrant localization and expression of ion channels associated with axonal dysfunction. Although considerable remyelination occurs spontaneously by endogenous CNS progenitor cells, the quality and extend of myelination is not optimal to restore structural and physiological properties of injured axons. Emerging research evidence has uncovered several mechanisms by which oligodendrocyte differentiation and remyelination are regulated within the microenvironment of injury. Inhibitory role of activated glial cells and insufficient expression of promoting factors are major limiting factors that impede remyelination. Over the past years, extensive research efforts have been made to therapeutically promote remyelination following CNS trauma or demyelinating disease. Outcomes of the recent pharmacological and cell-based therapies indicate the impact of remyelination as a mechanism for improving function after injury. Further investigations are needed to develop effective and feasible repair strategies with potential for clinical translation.

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Extrinsic and intrinsic regulation of axon regeneration at a crossroads

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Repair of the injured spinal cord is a major challenge in medicine. The limited intrinsic regenerative response mounted by adult central nervous system (CNS) neurons is further hampered by astrogliosis, myelin debris and scar tissue that characterize the damaged CNS. Improved axon regeneration and recovery can be elicited by targeting extrinsic factors as well as by boosting neuron-intrinsic growth regulators. Our knowledge of the molecular basis of intrinsic and extrinsic regulators of regeneration has expanded rapidly, resulting in promising new targets to promote repair. Intriguingly certain neuron-intrinsic growth regulators are emerging as promising targets to both stimulate growth and relieve extrinsic inhibition of regeneration. This crossroads between the intrinsic and extrinsic aspects of spinal cord injury is a promising target for effective therapies for this unmet need.

Keywords: spinal cord injury, CNS regeneration, PNS, conditioning lesion, RhoA, cAMP

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Introduction

The adult mammalian central nervous system (CNS) has a poor ability to regenerate and restore function after injury. The pioneering work of Aguayo and colleagues showing that CNS neurons can grow into peripheral nerve grafts, but stop when they re-encounter the CNS led to the predominance of the hypothesis that the failure of axons to regenerate is attributed to the presence of inhibitory factors in the CNS microenvironment (David and Aguayo, 1981; Yiu and He, 2006). Indeed, it is now recognized that CNS myelin contains myelin-associated inhibitors (MAIs) including Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) that collapse axonal growth cones and inhibit growth. The deposition of chondroitin sulfate proteoglycans (CSPGs) by reactive astrocytes also presents a formidable barrier to axon regeneration through sites of injury (Yiu and He, 2006). Targeting these extrinsic inhibitory factors has led to modest improvements in axonal plasticity and functional recovery after CNS injury. Important work spearheaded by Marie Filbin and colleagues revealed a critical role for the intrinsic state of CNS neurons in mediating extrinsic inhibition of axon regeneration. Filbin and colleagues demonstrated that an age-associated decline in neuronal cAMP underlies a gain of sensitivity to myelin-mediated inhibition (Cai et al., 2001). The notion that the neuron-intrinsic growth state can regulate the sensitivity of the injured axon to extrinsic factors is also supported by the enhanced axon regeneration in the spinal cord that can be elicited by a preconditioning lesion of the peripheral nerve processes (Mar et al., 2014a). More recently, it has been shown that stimulating intrinsic growth potential by neuronal knockout of negative regulators of growth, including phosphatase and tensin homolog (PTEN) and suppressor of cytokine signaling 3 (SOCS3), can induce striking long-distance axon regeneration after CNS injury (Liu et al., 2010; Sun et al., 2011). Furthermore, engraftment of neural stem cells into transected rodent spinal cords can result in impressive long-distance growth of grafted cells (Lu et al., 2012). These studies provide proof-of-concept that robust neuron-intrinsic growth potential is able to overcome

the inhibitory nature of the injured CNS. Here we will review some novel therapeutic strategies that have been developed based on our expanding knowledge of the molecular basis of intrinsic axon growth and extrinsic outgrowth inhibition (**Figure 1**). We will also highlight several recent examples of intrinsic regulators that dually affect the neuronal response to the inhibitory CNS environment. These findings have blurred the distinction between an extrinsic or an intrinsic origin of CNS regeneration failure and support the idea that a dynamic interplay between the two determines whether an axon regenerates. A better understanding of this crossroads between extrinsic and intrinsic regulation of axon regeneration will contribute to the conception of therapies to stimulate CNS repair.

Extrinsic Influences on Neural Repair

Myelin-associated Inhibitors

The identification of the inhibitory ligands and neuronal receptors that mediate myelin-dependent inhibition of regeneration has facilitated the development of selective antagonists to neutralize the inhibitory effect of myelin proteins. MAIs in CNS myelin bind to either paired immunoglobulin repeat B (PirB; Atwal et al., 2008) or Nogo receptor (NgR), which forms a complex with LINGO-1 (LRR and Ig containing Nogo Receptor interacting protein) and p75NTR (p75 neurotrophin receptor) or TROY (tumor necrosis factor receptor superfamily member 19; Yiu and He, 2006). One strategy to target these interactions has been to generate peptide mimics of ligands to compete for receptor binding. An early example of this is the Nogo extracellular peptide (NEP1–40), a peptide derived from the Nogo-66 NgR-binding region of Nogo to compete with the MAIs for NgR binding (Grandpré et al., 2002). NEP1–40 was shown to abrogate Nogo-dependent neurite outgrowth inhibition and promote corticospinal tract regeneration after dorsal hemisection SCI (Grandpré et al., 2002; Li and Strittmatter, 2003), but a later replication study did not observe significant effects on axon regeneration (Steward et al., 2008). Vaccines (Huang et al., 1999) and monoclonal antibodies (Zörner and Schwab, 2010) have also been developed to neutralize the MAIs, with reported improvements on regeneration and functional recovery. However, deletion of NgR or Nogo, MAG and OMgp in mice has had more variable effects on regeneration and recovery (Zheng et al., 2003, 2005; Lee et al., 2009, 2010; Cafferty et al., 2010). While deletion of Nogo or NgR has negligible to modest effects on axon regeneration after dorsal hemisection SCI, a unilateral pyramidotomy injury to the CST results in an impressive collateral sprouting response of uninjured axons into the denervated side of the spinal cord (Cafferty and Strittmatter, 2006). Furthermore, a soluble NgR decoy receptor, NgR(310)ecto-Fc that neutralizes Nogo, MAG, and OMgp shows promise in promoting axonal sprouting and functional recovery in acute and chronic SCI in rodents (Ji et al., 2005; Li et al., 2005; Wang et al., 2011).

The Glial Scar

The astrocytic response to CNS injury is characterized by the production of CSPGs, which present a potent barrier to axon regeneration (Yiu and He, 2006). The inhibitory action of CSPGs has been attributed to the abundance of negatively charged glycosaminoglycan (GAG) chains that decorate the protein core, which are thought to act as a poor substrate and electrostatically repel growth cones. Protein tyrosine phosphatase sigma (PTPsigma), leukocyte common antigen related phosphatase (LAR) and NgR have been identified as neuronal receptors that functionally interact with and mediate CSPG-dependent inhibition of neuron growth (Shen et al., 2009; Fisher et al., 2011; Dickendesher et al., 2012). Chondroitinase ABC (chABC), a bacterial enzyme that digests the GAG chains, abrogates CSPG-dependent neurite outgrowth inhibition *in vitro* and improves neurite sprouting and functional recovery after SCI (Bradbury et al., 2002). The efficacy of chABC alone and in combination with other approaches in promoting recovery after SCI has been independently confirmed in multiple laboratories and is a promising approach for future translation (Alilain et al., 2011; García-Alías et al., 2011; Lee et al., 2012). Mechanistically, contrary to the view that CSPGs repel growth cones, recent studies from the Silver group suggest that CSPGs in fact tightly adhere to and confine growth cones (Filous et al., 2014; Lang et al., 2015). After SCI, axons closely associate with NG2, a transmembrane CSPG expressed by a subset of oligodendrocyte progenitor cells. The presence of synaptic markers at these sites of apposition suggests the formation of stable "synapse-like" contacts between neurons and NG2+ cells (Filous et al., 2014). Furthermore, DRG neurons preferentially adhere to CSPG substrates in *in vitro* stripe assays. Silver and colleagues propose a model whereby CSPGs function to capture and arrest regenerating growth cones after injury instead of repelling them (Filous et al., 2014). Perhaps unexpectedly, deletion of NG2 in mice did not enhance axon regeneration after dorsal column crush, but rather resulted in more pronounced axonal dieback away from the injury site. This was attributed to the loss of NG2-mediated capture of axons after injury.

Pharmacological targeting of CSPG receptors is a strategy to relieve CSPG-dependent capture of growth cones that has shown potential in recent studies. Systemic administration of cell-penetrating peptide mimetics of the extracellular and intracellular regions of LAR by subcutaneous injection have been shown to enhance serotonergic axon sprouting and functional recovery after SCI in mice (Fisher et al., 2011). A recent report from the Silver group demonstrates that PTPsigma can be targeted in a similar fashion with a cell–permeable peptide, termed intracellular sigma peptide (ISP), to enhance functional recovery after contusive spinal cord injury in rats (Lang et al., 2015). ISP was designed to bind to the cytoplasmic wedge domain of PTPsigma. Using a CSPG gradient crossing assay, Silver and colleagues provide evidence that PTPsigma-dependent interactions with CSPGs maintain growth cones in a dystrophic state and that treatment with ISP allows for growth cone release. Based on the proposed mechanism that the interaction between PTPsigma and CSPGs acts as a "molecular velcro", it is surprizing that ISP is sufficient to overcome growth cone immobilization because ISP targets the cytoplasmic domain of PTPsigma, presumably sparing the physical interaction between

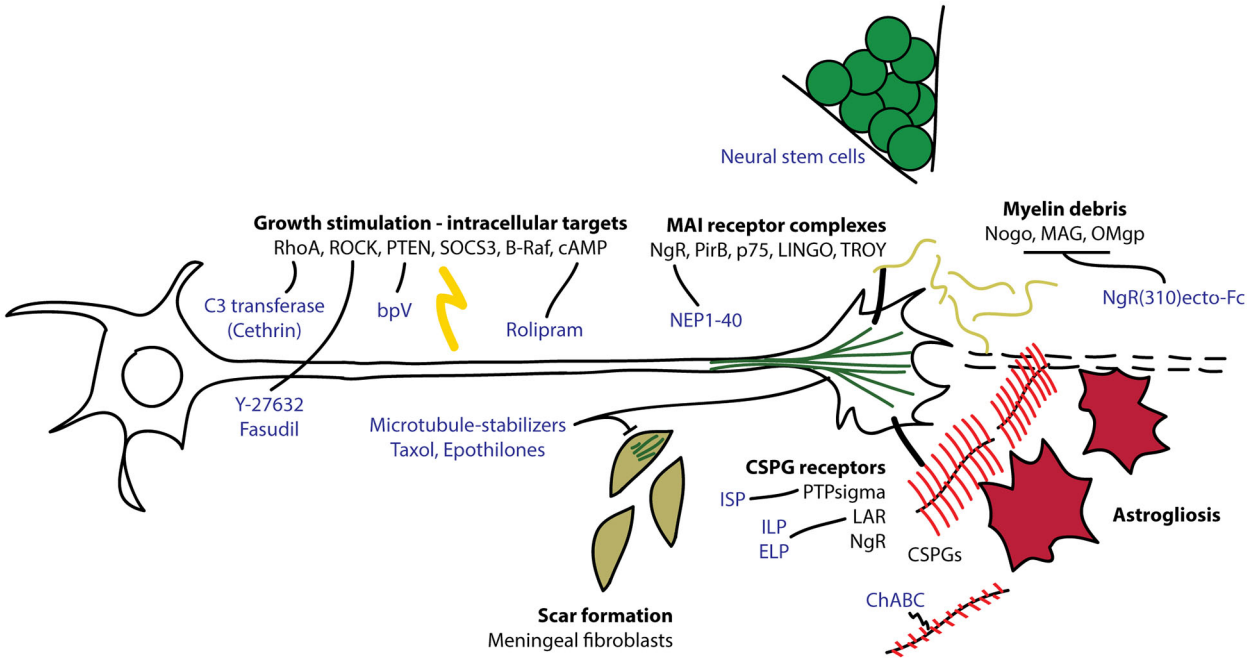

FIGURE 1 | Overview of intrinsic and extrinsic molecules involved in axon regeneration after CNS injury. Myelin debris from damaged oligodendrocytes and chondroitin sulfate proteoglycans (CSPGs) produced by reactive astrocytes interact with receptors on the growth cones to interfere with axon extension. Intracellular signaling molecules regulate the ability of the neuron to regenerate its damaged axon through the lesion environment. C3 transferase (Cethrin) inhibits RhoA to promote axon extension and cell survival. Fasudil and Y-27632 inhibit Rho kinase (ROCK) to promote axon extension. Bisperoxovanadium (bpV) inhibits phosphatase and tensin homolog (PTEN) to promote neuroprotection. Rolipram inhibits phosphodiesterase 4 (PDE4) to stabilize cAMP and promote axon extension. Microtubule stabilizers, taxol and epothilones, reduce fibrotic scarring and promote axon extension. Chondroitinase ABC (ChABC) digests CSPG glycosaminoglycan (GAG) chains (in red), relieving CSPG-dependent growth inhibition. Intracellular sigma peptide (ISP) inhibits protein tyrosine phosphatase sigma (PTPsigma) and intracellular/extracellular leukocyte common antigen related phosphatase (LAR) peptides (ILP/ELP) inhibit leukocyte antigen-related receptor (LAR) to neutralize the inhibitory effect of CSPGs and promote axon sprouting. NgR(310)ecto-Fc competes for binding to Nogo, myelin-associated glycoprotein (MAG) and Oligodendrocyte myelin glycoprotein (OMgp) to relieve myelin-dependent growth inhibition. Nogo extracellular peptide 1–40 (NEP1–40) competes for NgR binding to neutralize inhibitory signaling through NgR.

CSPGs and the receptor. The relative contribution of the receptor-ligand interaction and downstream signaling events to growth cone immobilization remain unclear. Daily subcutaneous injection of ISP following spinal cord contusion in rats resulted in delayed beneficial outcomes, particularly in resumption of bladder function (Lang et al., 2015). While these studies have identified CSPG receptors as potential targets, future work could focus on developing small molecule inhibitors to attain better CNS penetrance and distribution for earlier and more efficient targeting after injury. The idea that CSPGs are critical for growth cone arrest suggests that these approaches may also be efficacious in chronic SCI.

Intrinsic Influences on Neural Repair

The Conditioning Lesion

Injury to axons in the PNS is followed by neuronal expression of regeneration-associated genes (RAGs), a response that is quite limited in CNS neurons (Mar et al., 2014a). It has therefore been hypothesized that a failure to initiate a growth program contributes to unsuccessful regeneration in the CNS. The positive effect of a peripheral conditioning lesion on CNS regeneration has been attributed to the engagement of a RAG expression response that stimulates growth (Blesch et al., 2012). One of these RAGs, c-Jun, has been shown to be critical for axon regeneration, as its deletion impairs axon regeneration and results in exacerbated cell death after peripheral nerve injury in mice (Raivich et al., 2004). The conditioning lesion has also recently been shown to enhance the axonal transport of a multitude of signaling and cytoskeleton regulators into the central process (Mar et al., 2014b). Among these include members of the 14-3-3 adaptor proteins, which have important roles in neuroprotection, axon guidance and cell growth (Shimada et al., 2013; Kaplan et al., 2014). Elevation of cAMP is also a critical component of the conditioning lesion effect and injection of cAMP into dorsal root ganglia can mimic the pro-regeneration effect of a conditioning lesion (Qiu et al., 2002). Interestingly, cAMP also regulates the sensitivity of neurons to MAIs and CSPGs (Cai et al., 2001). The Filbin group demonstrated that an age-associated decline of neuronal cAMP correlates with an acquisition of responsiveness to myelin-mediated inhibition of growth and that pharmacological elevation of cAMP renders adult neurons insensitive to myelin and CSPGs (Cai et al., 2001). This reinforces the concept that extrinsic inhibitors rely

on the intrinsic sensitivity of the neuron in order to inhibit growth and suggests that targeting neuron-intrinsic signaling can relieve the influence of the inhibitory microenvironment. Supporting a direct link between cAMP and responsiveness to myelin, a recent study shows that enhanced cAMP and protein kinase A (PKA) signaling stimulates the proteolysis of Nogo through activation of the ubiquitin ligase praja2 (Sepe et al., 2014). cAMP is a promising target for development and rolipram, a phosphodiesterase 4 (PDE4) inhibitor that elevates intracellular cAMP, has been shown to enhance regeneration and recovery after SCI in rodents (Lu et al., 2004; Nikulina et al., 2004; Pearse et al., 2004; Kadoya et al., 2009; Costa et al., 2013).

MAI Signaling

The actin-regulating small GTPase RhoA and downstream effector Rho kinase (ROCK) are extensively studied mediators of neurite outgrowth inhibition in the CNS. MAIs have been shown to increase the levels of active GTP-bound RhoA (Dubreuil et al., 2003) and one study suggests that the dual Rac and Rho guanine exchange factor (GEF), Kalirin9, functionally interacts with p75 in complex with NgR1 to stimulate RhoA activation in response to receptor activation (Harrington et al., 2008). Nogo also stimulates the phosphorylation and membrane translocation of ROCK (Alabed et al., 2006). Inhibition of ROCK stimulates neurite outgrowth on myelin and infusion of the small molecule ROCK inhibitors Y-27632 or fasudil into the injured rat spinal cord improves locomotor recovery (Fournier et al., 2003; Sung et al., 2003). However, while ROCK inhibition enhances neurite outgrowth on myelin, a persistent myelin-induced reduction of growth relative to enhanced baseline growth on permissive substrates is often reported (Fournier et al., 2003; Alabed et al., 2006; Ahmed et al., 2009), suggesting that ROCK inhibition fails to fully de-sensitize neurons to myelin-dependent growth inhibition. It is therefore difficult to attribute the positive effects of ROCK inhibition in SCI to a reduction in the sensitivity of the axons to myelin or simply to an intrinsic stimulation of outgrowth. Our group has identified a Nogo-induced interaction between RhoA and collapsin response mediator protein 4 (CRMP4) as a functional signaling event that can be targeted with a competitive peptide antagonist called C4RIP to block myelin-induced neurite outgrowth inhibition (Alabed et al., 2007). Interestingly, C4RIP does not enhance baseline neurite outgrowth (Alabed et al., 2007), suggesting the specific involvement of a CRMP4-RhoA complex engaged by MAIs. RhoA can be specifically inhibited with the *Clostridium botulinum* exoenzyme C3 transferase, which ADP-ribosylates and inactivates RhoA. Treatment of animals with C3 improves locomotor recovery in contusion and dorsal hemisection injury models (Boato et al., 2010). C3 has a dual effect of enhancing neurite outgrowth in the presence of myelin (Dergham et al., 2002) and promoting cell survival after SCI (Dubreuil et al., 2003). Interestingly, C3 has also been shown to stimulate neurite outgrowth and induce Erk and Akt phosphorylation independent of its ability to inhibit RhoA, suggesting that its mechanism-of-action has yet to be fully elucidated (Auer et al., 2012). Detrimental effects of C3 (Fournier et al., 2003; Sung et al., 2003) observed in other studies are potentially due to endotoxin contamination and lack of a cell-penetrating peptide (McKerracher and Higuchi, 2006). The positive outcomes with C3 have translated into a phase I/II clinical trial with Cethrin (BA-210), a cell-permeable form of C3 that is delivered locally over the dura mater in a fibrin sealant during spinal surgery. Results from the Cethrin trial have shown promising improvements of locomotor function compared to historical statistics (McKerracher and Anderson, 2013).

Growth Stimulation

Recent studies have provided a strong rationale for targeting intracellular growth regulators as a means to both drive neuron growth and relieve sensitivity to extrinsic inhibition of growth. Initial studies have explored strategies to stimulate intrinsic growth potential by genetically manipulating the expression of master regulators of cellular growth that are considered classical tumor suppressors or oncogenes. PTEN is a tumor suppressor that antagonizes the PI3K-Akt-mTOR pathway (Song et al., 2012). The He lab reported an age-associated down-regulation of mTOR activity in adult cortical neurons and showed that conditional knockout of PTEN and consequent increase in mTOR activity in mouse cortex promotes regeneration of corticospinal axons after dorsal hemisection injury (Liu et al., 2010). Another study showed that combined deletion of PTEN and SOCS3, a negative regulator of the JAK/STAT pathway, synergize to promote axon regeneration in injured optic nerve (Sun et al., 2011). These studies were noted for extraordinarily extensive long-distance axon regeneration that had not been observed with any prior intervention. These positive outcomes have resulted in a greater focus on manipulation of intrinsic regulation of neuron growth as an approach for drug development. Chronic targeting of tumor suppressors poses a concern for oncogenesis, but an acute regimen may be an effective strategy to initiate a regenerative response after injury. One study found that systemic administration of the PTEN inhibitor bisperoxovanadium (bpVpic) twice daily for 1 week improved forelimb motor function and was neuroprotective (Walker et al., 2012).

A complementary strategy is to enhance the activity or expression of growth-promoting molecules. A recent study showed that expression of a point-mutated kinase activated version of the B-Raf oncogene (V600E B-Raf) results in robust regeneration of retinal axons after optic nerve injury and regeneration of dorsal root ganglion neurons into the dorsal root entry zone after crush injury (O'Donovan et al., 2014). Further enhancement of regeneration was observed with combined knockout of PTEN. How these manipulations influence neuronal responses to extrinsic inhibition are poorly understood. It was recently shown that codeletion of Nogo and PTEN does not cause more corticospinal axon sprouting after unilateral pyramidotomy, but results in improved long-distance axon regeneration after dorsal hemisection, compared to PTEN deletion alone (Geoffroy et al., 2015). The lack of a combinatorial effect of PTEN and Nogo deletion on

sprouting suggests that perhaps PTEN deletion affords maximal de-sensitization to Nogo. One study provides evidence that PTEN knockout neurons are less sensitive to MAG-dependent neurite outgrowth inhibition (Perdigoto et al., 2011), while another study has shown that Erk, a downstream effector of Raf, can also relieve MAG-dependent inhibition (Gao et al., 2003). It remains unclear whether these pathways are directly involved in MAI signaling or whether they act in parallel to alter MAI signal-transduction efficiency. Interestingly, a recent study shows that myelin and CSPGs stimulate the expression of pro-growth immediate early genes through serum response factor (SRF) and Erk activation. This may function as a protective response because expression of constitutively active SRF was shown to overcome myelin and CSPG—dependent neurite outgrowth inhibition (Stern and Knoll, 2014).

Neural Stem Cells

The ability of implanted neural stem cells to survive and functionally integrate into injured host spinal cord in rodents also suggests that the inhibitory nature of the injured CNS can be overcome by neurons with vigorous growth capacity (Lu et al., 2012, 2014). In studies from the Tuszynski group, implanted neural stem cells were shown to extend long axons throughout the grey and white matter of transected host spinal cords, establishing an electrophysiological bridge across the injury. A recently published study confirmed the ability of grafted neural stem cells to integrate into host spinal cords, but also noted the presence of ectopic colonies of donor cells throughout the spinal cord and brain stem in half of the animals (Steward et al., 2014). This highlights the caution that must be exercised in the development of neural stem cell therapies, as implanted cells can give rise to tumors and exuberant synaptic connections could result in unfavorable behavioral and sensory side effects including neuropathic pain (Hofstetter et al., 2005). Nonetheless, these studies also serve as proof-of-principle that neurons with high growth capacity are capable of extensive growth in the injured CNS despite the presence of inhibitory factors.

Conclusion

Both the extrinsic and intrinsic aspects of the injury response together forge the basis for regeneration failure. Peripheral conditioning lesions combined with chABC and NgR(310)ecto have been shown to provide superior benefit to single interventions, supporting the idea that simultaneously stimulating neuron-intrinsic growth potential and neutralizing extrinsic inhibition can provide maximal efficacy in promoting neural repair (Wang et al., 2012). SCI therapies that take both of these contributors into consideration can come in the form of drug combinations or single multi-action drugs. The use of multi-action drugs is an attractive strategy to simultaneously manipulate several pathophysiological features of the injury response. For example, work from the Bradke group has shown that local administration of low dose taxol, a potent microtubule-stabilizing drug, exerts manifold beneficial effects by reducing CSPG production and fibrosis, inhibiting meningeal fibroblast migration and stimulating axon extension after SCI (Hellal et al., 2011). A recent report from the Bradke group shows that systemic administration of another microtubule stabilizer, epothilone B, similarly inhibits scarring and promotes axon regeneration (Ruschel et al., 2015). Both drugs are approved chemotherapeutics, however epothilone B, but not taxol, is CNS-penetrant. CNS-penetrant multi-action pharmaceutical agents that can be given systemically hold the most promise for translation into practical and effective treatments for SCI. The repurposing of approved drugs for indication in SCI is an attractive approach to achieve accelerated approval for this serious unmet need. However, the identification of new drugs that have multiple activities, including modulation of astrogliosis, neuroprotection and neurite outgrowth induction, may offer superior efficacy in stimulating CNS repair, but will also pose challenges for safety and tolerability.

Author Contributions

AK, SOT and AEF wrote the manuscript.

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REVIEW
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Regulating Axonal Responses to Injury: The Intersection between Signaling Pathways Involved in Axon Myelination and The Inhibition of Axon Regeneration

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Following spinal cord injury (SCI), a multitude of intrinsic and extrinsic factors adversely affect the gene programs that govern the expression of regeneration-associated genes (RAGs) and the production of a diversity of extracellular matrix molecules (ECM). Insufficient RAG expression in the injured neuron and the presence of inhibitory ECM at the lesion, leads to structural alterations in the axon that perturb the growth machinery, or form an extraneous barrier to axonal regeneration, respectively. Here, the role of myelin, both intact and debris, in antagonizing axon regeneration has been the focus of numerous investigations. These studies have employed antagonizing antibodies and knockout animals to examine how the growth cone of the re-growing axon responds to the presence of myelin and myelin-associated inhibitors (MAIs) within the lesion environment and caudal spinal cord. However, less attention has been placed on how the myelination of the axon after SCI, whether by endogenous glia or exogenously implanted glia, may alter axon regeneration. Here, we examine the intersection between intracellular signaling pathways in neurons and glia that are involved in axon myelination and axon growth, to provide greater insight into how interrogating this complex network of molecular interactions may lead to new therapeutics targeting SCI.

Keywords: myelination, axon regeneration, spinal cord injuries, Schwann cell, oligodendrocytes, signaling pathways, radial growth, adaptive myelination

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MYELINATING GLIA OF THE CNS AND PNS

Neuron-glia interactions have been fundamental to the structure and function of the brain throughout evolution (Herculano-Houzel, 2014). Oligodendrocytes (OLs) in the central nervous system (CNS) and Schwann cells (SCs) in the peripheral nervous system (PNS) ensheathe axons with myelin for the promotion of saltatory conduction (Nave and Werner, 2014). In the case of OLs, they extend their processes spirally inward, around the axons, in a corkscrew-like manner to lay down a multi-lamellar, compact, lipid rich sheath (myelin sheath; myelin from myelós, Greek for marrow) on the axons. Formation of the myelin sheath occurs in an outside

to inside fashion by a process involving homotypic fusion of myelinophore organelles within the confines of their processes (Ioannidou et al., 2012; Snaidero et al., 2014; Szuchet et al., 2015). OLs represent almost 75% of the neocortical glial population, and each OL is capable of laying down myelin on 40–60 short axonal segments of multiple CNS axons with varying diameter (Matthews and Duncan, 1971; Lubetzki et al., 1993; Shaham, 2006; Pelvig et al., 2008; Fields et al., 2015). In the CNS, the renewal of myelinating OLs comes from oligodendrocyte precursor cells (OPCs). OPCs, activated by specific mitogens [e.g., platelet derived growth factor (PDGF) and neurotrophin-3 (NT3)], and differentiating factors [e.g., thyroid hormone T3, insulin growth factor-1 (IGF-1), transforming growth factor-β1 (TGF-β1) and stromal derived factor-1 (SDF-1)] proliferate and progress through a pre-myelinating phase to eventually become myelinating OLs (Boulanger and Messier, 2014). CNS myelination begins prenatally and proceeds gradually at the level of axonal tracts in a rostral to caudal—dorsal to ventral gradient (Schreyer and Jones, 1982; Almeida et al., 2011; Wang and Young, 2014). In mice, the generation of a surprisingly large numbers of OLs occurs even in adulthood, which contribute towards a distinct remodeling of the myelin sheath and remyelination after CNS injury (Powers et al., 2013; Young et al., 2013). Studies have also suggested that, in humans, neocortical myelination is protracted, lending support to the idea that the presence of myelination and remyelination in the adult is relevant across species (Miller et al., 2012; Glasser et al., 2014; Shafee et al., 2015). Accumulating evidence suggests that not only do actively growing axons become myelinated, but also axons which have reached their target can undergo continual myelin remodeling, a capability that persists when the axon is injured (Yeung et al., 2014).

In the PNS, SCs are derived from neural crest cells that pass through precursor and immature stages to eventually become myelinating or non-myelinating SCs (Jessen and Mirsky, 2005). In rodents, Schwann cell precursors (SCPs) are observed in spinal nerves by E12-E14 (Jessen et al., 1994; Dong et al., 1999). Survival of SCPs usually requires growth factors like PDGF, NT-3, endothelin, fibroblast growth factor (FGF) and IGF (Woodhoo et al., 2004). SCPs continue to proliferate under mitogens such as axonal neuregulin 1 (NRG1) and TGFβ to become immature SCs that envelop a large group of axons *en masse* (Ridley et al., 1989; Morrissey et al., 1995; Woodhoo and Sommer, 2008). Axonal caliber and glia-axonal contact are critical in deciding the myelinating and non-myelinating, inter-convertible fates of SCs (Weinberg and Spencer, 1975; Aguayo et al., 1976; Trapp et al., 1988; Voyvodic, 1989; LeBlanc and Poduslo, 1990). Through the process of radial sorting, that continues postnatally, immature SCs differentiate and establish a 1:1 relationship with peripheral axons and spirally ensheathe and myelinate large diameter axons, whereas some mature SCs, termed Remak cells, remain associated with multiple, small diameter axons without myelinating them (Feltri et al., 2015).

Myelination is a multistage process with considerable overlap among its different phases. In general, these phases involve: (1) the migration and ensuing differentiation of glial precursors into mature myelinating glia; (2) the initial recognition of the axon, axon-glia contact, axonal segment selection and subsequent ensheathment of the target axonal segments by the myelinating glia; (3) the initiation of myelin-associated protein expression in the myelinating glia and finally; (4) the compaction and maturation of the myelin sheath (Szuchet et al., 2015). Further fine-tuning of the myelination process involves the generation of functional axonal domains such as nodes of Ranvier, paranodes and juxtaparanodes.

There is a striking difference, however, in the structural proteins that make up the myelin of the CNS and the PNS. CNS myelin produced by OLs is compact, rich in glycolipid (e.g., galactocerebroside) and sulfolipid-sulfatide, has a higher concentration of proteolipid protein (PLP) and consists of unique glycoproteins, such as the myelin-associated inhibitors (MAIs) including myelin oligodendrocyte glycoprotein (OMgP/MOG; Nave and Trapp, 2008; Jahn et al., 2009). In contrast, myelin protein zero (P0/MPZ) and peripheral myelin protein (PMP22) constitute characteristic structural proteins of peripheral myelin (Patzig et al., 2011). Despite these structural and composition differences, axonal signaling plays an important role in the regulation of both OL and SC development, myelin biogenesis and their ability to myelinate CNS and the PNS axons, respectively (Barres and Raff, 1999; Nave and Trapp, 2008; Taveggia et al., 2010). In humans, OPC maturation takes place almost 3 months before the onset of myelination (around 40 weeks), reiterating the need for specialized signaling mechanisms between OLs and axons for the initiation of myelination (Brody et al., 1987; Kinney et al., 1988; Back et al., 2002). In contrast, SCPs and immature SCs appear at around 12 weeks of fetal development, and mature SCs commence peripheral myelination 2 weeks later, first at the motor roots, then the sensory roots (Cravioto, 1965). Most of the peripheral myelination completes within 1 year of birth, whereas CNS myelination continues well past the first decade of life (Jakovcevski et al., 2009; Bercury and Macklin, 2015).

Injury to CNS axons, in contrast to that of PNS axons, leads to impaired axonal regeneration as a result of the actions of various intrinsic and extrinsic factors (Afshari et al., 2009). These factors adversely affect the gene programs that govern the expression of regeneration-associated genes (RAGs) and the production of a diversity of extracellular matrix molecules (ECMs), leading to structural alterations in the axon that perturb the axonal growth machinery or lead to the formation of extraneous barriers to axonal regeneration at the site of lesion (Kaplan et al., 2015). Here, the role of myelin (both intact and debris) in altering injured axon growth responses has been the focus of both targeted therapeutic approaches and transgenic mouse studies, in which components of myelin, specifically MAIs, have been blocked, or are genetically knocked out (Raisman, 2004; Schwab and Tuszynski, 2010; Lee and Zheng, 2012). However, there has been less attention on how myelination of the injured axon, whether by endogenous or exogenously transplanted glia as a therapeutic approach, may alter axon regeneration. Combinatorial approaches involving

the modulation of the: (1) properties of glial scar; and (2) MAI signaling and transplantation of myelination-competent cells, with or without trophic factors, have all yielded limited axonal regeneration caudal to the injury site in various spinal cord injury (SCI) models (Deumens et al., 2005). Understanding the pathways involved in myelination and how these pathways may directly play a role in or intersect with, signaling cascades involved in axon growth or its inhibition, may provide new avenues for developing regenerative therapies after CNS injury. The current review examines the distinct signaling pathways implicated in axon-glia communication during myelination, and discusses how these same pathways play a role in altering axonal growth responses after injury.

THE INTERSECTION OF SIGNALING PATHWAYS REGULATING MYELINATION AND AXONAL GROWTH

Notch Signaling

Notch (notch1 and notch2) is a transmembrane receptor and Delta, Delta-like (Dll-1, 3, 4), Serrate/Jagged (jagged 1, jagged 2), F3/Contactin and NB3 (Contactin-6) are its known ligands (Andersson et al., 2011). Notch ligands that are present on axons play an instructive role in the development of various glia, including OLs (Gaiano and Fishell, 2002; Givogri et al., 2002; Stump et al., 2002), and regulate the differentiation of SCPs and the proliferation of SCs, but postnatally can also act in an inhibitory fashion towards SC mediated myelination (Woodhoo et al., 2009). Axons harbor extracellular notch ligands jagged1, F3/contactin and NB3 near the paranodes, whereas OLs and SCs express the notch receptors 1 and 2 (Stidworthy et al., 2004; Woodhoo et al., 2009). Adult rodent and human brain shows expression of notch receptors (notch1, notch2) and its ligand (jagged 1; Berezovska et al., 1998; Stump et al., 2002; Chen et al., 2005). Axonal jagged1 blocks OPC differentiation into OLs within the adult rodent brain (Grandbarbe et al., 2003; Park and Appel, 2003). However, evidence also suggests that notch signaling can enhance myelination in a ligand-dependent manner (Hu et al., 2003).

Notch signaling (**Figure 1**) begins with trans-binding of the ligands to notch receptors. This event leads to proteolytic cleavage of the notch extracellular truncated domain, first by a disintegrin and metalloprotease (ADAM), and then by γ-secretase, which releases the notch intracellular domain (NICD) to permit its translocation to the nucleus (Andersson et al., 2011). In the nucleus, in conjunction with the RBPJ/MAML (recombining binding protein suppressor of hairless/ mastermind-like) transcription activation complex, NICD acts to de-repress notch target genes such as *Hes/Hesr/Dec* (hairy/enhancer; hairy/enhancer related; differentiated embryo chondrocyte 1; Andersson et al., 2011).

Hu et al. (2003) proposed a switch model in which axonal jagged-1 expression initially blocks OPC differentiation in a *Hes1* dependent manner. Then, as jagged1 expression decreases with development, the interaction of F3/contactin with notch1, notch2 or NB3 with notch1, could then promote nuclear translocation of NICD, and the transcription of myelin-associated genes, myelin-associated glycoprotein (MAG) and CNPase, in a RBPJ/*Hes1* independent manner (Wang et al., 1998; Hu et al., 2003; Popko, 2003; Cui et al., 2004). This non-canonical notch activation was found to be exclusively dependent on F3/Contactin/NB3, and was mediated by Deltex1 (DTX1), an E3 ligase also known to antagonize c-Jun N-terminal kinase (JNK), and promote the degradation of NICD, as well as MAPK/ERK kinase kinase 1 (MEKK1; Liu and Lai, 2005; Zhang P. et al., 2010). However, given the role of DTX1/p300 in functioning as a non-canonical downstream transcriptional regulator of notch, the exact mechanism of transcriptional activation of myelin-associated genes by DTX1 remains to be elucidated (Yamamoto N. et al., 2001). It is plausible that parallel activation of other axon-glia signaling might post-translationally influence the activity and interactions of DTX1, and direct it towards promyelination signaling. In an apotransferin-induced-cortical remyelination model, notch activation correlated with F3/Contactin expression (Aparicio et al., 2013). Increasing expression of F3/contactin and NB3 during the early postnatal period has been documented in the rat spinal cord (Cui et al., 2004). This apparent change of ligands for the same receptor (notch1) promotes maturation of OLs on demand from axons (Givogri et al., 2002; Hu et al., 2003). In such a scenario, if axons continue to maintain a high expression of jagged1, they can potentially tip the balance of myelination signals towards its inhibition.

Among the 30 members of the ADAM family of proteolytic enzymes, ADAM10 and ADAM17 play an important role in myelination within the PNS (van Tetering et al., 2009; La Marca et al., 2011; Palazuelos et al., 2014). ADAM10 cleaves notch1 in a ligand-dependent manner, whereas cleavage of notch by ADAM17 is ligand-independent (Bozkulak and Weinmaster, 2009). In addition, cytoplasmic notch activity is known to be negatively regulated by numb (Puca and Brou, 2014). The exact mechanism of numb-mediated negative regulation of notch signaling is not known, but multiple mechanisms are proposed, including its interference with NICD endocytosis, NICD nuclear translocation, and notch/RBPJ/MAML-mediated transcription of genes (Giebel and Wodarz, 2012; Flores et al., 2014).

Notch1 is also required for the timely differentiation of neuronal progenitors, and cells that are deficient in notch1 undergo premature neurogenesis, but die by apoptosis before completing the terminal differentiation into post-mitotic neurons (Lutolf et al., 2002). Expression of notch1 in 6 DIV (day *in vitro*) mouse E16–18 cortical neurons, and neurite forming NB2A cells, inhibits neurite extension (Berezovska et al., 1999; Franklin et al., 1999). Notch signaling was also demonstrated to inhibit axonal regeneration in C.*elegans* after axotomy, and preventing notch activation post-injury resulted in enhanced regeneration (El Bejjani and Hammarlund, 2012). Further, numb was shown to reverse notch-mediated axon growth inhibition in 6 DIV cultures of E16–18 mouse cortical neurons, highlighting the importance of negatively regulating notch signaling to promote neuritogenesis (Berezovska et al., 1999; Puca and Brou, 2014). In mice, subsequent to compressive SCI, the expression of numb was observed to be predominantly

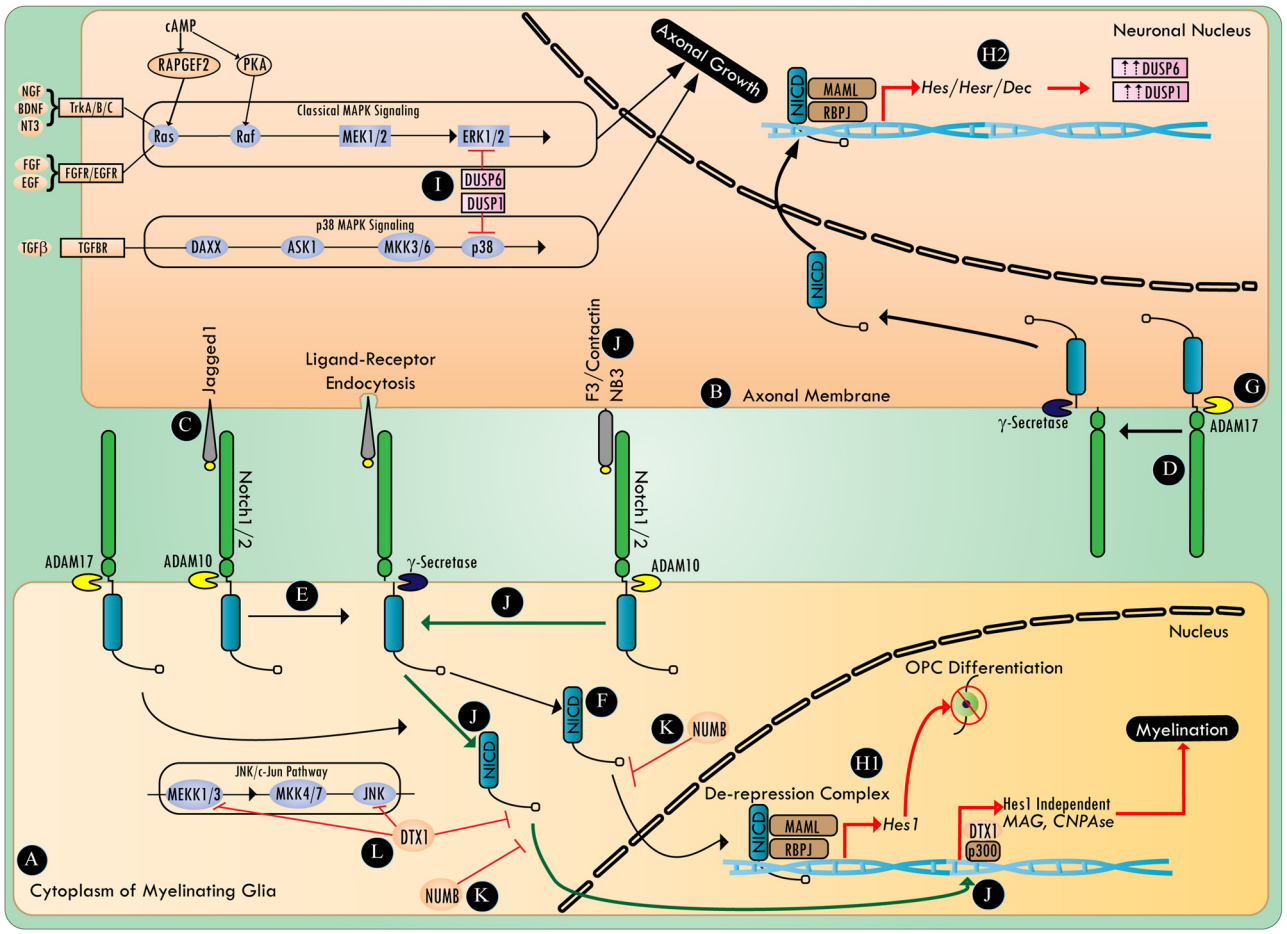

FIGURE 1 | The involvement of Notch in the regulation of myelination and axonal growth. Myelinating glia **(A)** and the axonal membrane **(B)** both harbor notch ligands **(C)** and notch receptors **(D)**. Proteases (e.g., ADAM10 and γ-secretase) activate the canonical notch signaling pathway **(E)** by processing the ligand bound notch receptor to release notch intracellular domain (NICD) **(F)**. The protease ADAM 17 can activate canonical notch signaling in a ligand independent manner **(G)**. NICD undergoes translocation to the nucleus and binds to a transcriptional de-repression complex containing RBPJ/MAML transcription factors **(H1,H2)**. In myelinating glia, canonical notch signaling activates a cascade of downstream genes (e.g., *Hes1*), halting OPC differentiation **(H1)**. Whereas, in neurons, canonical notch signaling upregulates DUSP1 and DUSP6 in a *Hes/Hesr/Dec* dependent manner **(H2)**. DUSP1 and DUSP6 inhibit axonal growth and neuritogenesis by negatively regulating the p38 MAPK and $ERK_{1/2}$ pathways, respectively **(I)**. Novel notch ligands (e.g., F3/contactin or NB3) activate non-canonical notch signaling **(J)** that recruits the NICD/Deltex1 (DTX1)/p300 transcription complex, thus activating genes that are essential for myelination. The notch antagonist, numb, inhibits various steps in both the canonical and non-canonical notch signaling pathway **(K)**, whereas DTX1 plays a critical regulatory role in both non-canonical notch signaling and the c-Jun N-terminal kinase (JNK) signaling pathway **(L)**.

upregulated in both NeuN-positive neurons and GFAP-positive astrocytes, in rostral as well as caudal spinal cord, for up to 10 mm from the lesion (Chen et al., 2005; Wilhelmsson et al., 2012). However, following injury to the nervous system, output of the notch signaling pathway with the effects of this increased expression of numb remains unknown.

In a mouse SCI compression model, notch1 expression significantly increased both at the mRNA and protein level (Yamamoto S. et al., 2001; Chen et al., 2005). In contrast to numb, notch1 mRNA expression was identified for up to 10 mm rostral and caudal to the injury, exclusively within neurons (Chen et al., 2005). Notch1 mRNA expression was detected beginning 2 days post injury and was still apparent at 14 days post injury (Chen et al., 2005). Notch-mediated inhibition of axonal regeneration appears to be ligand-independent, a finding that could potentially explain the absence of an improvement in hind limb motor function when the jagged1 antagonist (Jagged1-Fc-Chimera) was administered intravenously in mice immediately following a 50 kdyn thoracic T10 contusion SCI (Fassbender et al., 2011; El Bejjani and Hammarlund, 2012). Interestingly, inactivation of γ-secretase using its inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester), was sufficient to overcome notch-mediated axonal growth inhibition in *C.elegans* following LASER-assisted axotomy (El Bejjani and Hammarlund, 2012). However, DAPT, which eventually decreases the available NICD pool, was ineffective when applied 2 h after LASER-assisted

axotomy, suggesting an alteration in the post-injury molecular milieu, that could provide resistance to notch modulation through putative transcriptional events and post-translational modifications.

The signaling cascade involved in axon growth inhibition via notch activation needs further elucidation (**Figure 1**). In a RBPJ dependent manner, notch signaling has been shown to lead to the dephosphorylation of $ERK_{1/2}$, and the inhibition of the Ras/Raf/$MKK_{1/2}$/$ERK_{1/2}$ pathway through the upregulation of MAPK phosphatase (Lip-1/MKP-3/DUSP6), and the antagonism of p38 MAPK by the up-regulation of MKP-1 (DUSP1; Muda et al., 1996; Berset et al., 2001; Kondoh et al., 2007). Notch signaling can regulate p38 MAPK/JNK by positively regulating the mTOR pathway in a c-myc dependent manner to increase the expression of MKP1 (DUSP1) via Akt (Protein Kinase B; PKB) signaling (Chan et al., 2007; Rastogi et al., 2013). The role of the p38 MAPK pathway and the downstream effector of the JNK pathway, c-jun, are now being recognized as the critical molecules required for resetting SC fate towards a reparative phenotype (Arthur-Farraj et al., 2012; Yang et al., 2012). The p38 MAPK and JNK pathways are also specifically important in axonal regeneration (Nix et al., 2011). However, work in C.*elegans* has demonstrated that notch can negatively influence axonal regeneration without affecting the DLK-1/MEK_{4-7}/JNK pathway (El Bejjani and Hammarlund, 2012). Reports have also suggested that there are cross talks between NICD and the canonical β-catenin pathway, as well as NF-κB, HIF1, and TGFβ-BMP signaling pathways (Andersson et al., 2011; Bonini et al., 2011). Hence, notch mediated down regulation of axonal growth could primarily be due to its negative regulation of the p38 MAPK and ERK pathways, and its effects on myelination could be due to a complex modulation of the transcriptional network involved with myelin-associated gene expression and its indirect modulation of Akt signaling (Flores et al., 2008).

Conversely, multiple reports have highlighted that there is post-translational regulation of NICD by MAPK/ERK, which influences the transcriptional output of canonical notch signaling (Stockhausen et al., 2005; Tremblay et al., 2013; Yamashita et al., 2013). In addition, the MAPK signaling pathway shares multiple substrates with the cyclic AMP/PKA cascade and in turn can be regulated by cyclic AMP in a PKA-dependent (PKA/RhoA or PKA/PTP) or PKA-independent (Epac/Rap1 or Epac/Rit) manner (Gerits et al., 2008). Anecdotal reports of cyclic AMP being able to upregulate jagged 1 levels in osteoblasts, in a PKA-dependent manner, supports the potential existence of a wider notch regulatory network in neural cells (Weber et al., 2006). Examining the cross talk between notch signaling and cyclic AMP after injury, under conditions where levels of cyclic AMP are dramatically reduced (Pearse et al., 2004; Hannila and Filbin, 2008; Lau et al., 2013), may reveal novel players at the intersection of those signaling pathways.

In summary, notch signaling (**Figure 1**) constitutes an emerging component of axon-glia communication during injury. Notch interaction with its ligands plays an important role in modulating myelination, and warrants further work to better define these relationships, and to identify the intermediaries involved in these processes. In particular, nodes involving the interaction of notch with MAPK signaling (e.g., DUSPs) and cytoskeletal network may offer unique therapeutic targets for enhancing remyelination repair and axonal regeneration.

Neuregulin-ErbB Signaling

NRG1 is a member of the neuronal growth and differentiation factor family best known to be critical for SC development (Birchmeier and Nave, 2008). NRG1, the most studied of the four neuregulin genes, produces at least 15 different isoforms from multiple transcription start sites and alternative splicing (Nave and Trapp, 2008). All the six main isoforms of NRG1: type I (Heregulin: HRG; soluble), II (glial growth factor: GGF), III [sensory motor neuron-derived factor (SMDF); transmembrane], IV, V and VI have a similar epidermal growth factor (EGF)-like domain but distinct N-terminal regions. Expression of NRG1 has been detected in the uninjured spinal cord, as well as following SCI, in neurons, axons and OLs (Vartanian et al., 1999; Gauthier et al., 2013). NRG1 binds to membrane spanning receptor tyrosine kinases (RTKs), ErbB3 and ErbB4, which are part of the EGF receptor superfamily (Iwakura and Nawa, 2013). CNS expression of ErbB3, and to some extent that of ErbB4, is observed exclusively in OLs (Sussman et al., 2005; Makinodan et al., 2012). ErbB receptor subunit expression is present in the adult brain, spinal cord as well as dorsal root ganglia (DRGs; Bermingham-McDonogh et al., 1996; Martínez et al., 2004; Pearson and Carroll, 2004). In the PNS, SCs predominantly express ErbB2 and ErbB3 (Garratt et al., 2000). Binding of NRG1 to ErbB3 or ErbB4 leads to the activation of multiple signaling pathways (**Figure 2**), ensuing heterodimerization with ErbB2, since ErbB3, which contains a pseudokinase domain, cannot activate downstream effectors (Weiss et al., 1997; Maurel and Salzer, 2000; Burgess et al., 2003). Proteolytic cleavage of the NRG1 N-terminal domain by ADAM17 or β-secretase, is a prerequisite for NRG1/ErbB2/3 signaling (Ronchi et al., 2015).

Much of our understanding regarding the role of NRG1 in the process of myelination comes from experiments with knockout mice (Brinkmann et al., 2008; Newbern and Birchmeier, 2010). Conditional knockout of NRG1 in cortical projection neurons, before the onset of cortical myelination, did not lead to any changes in the myelin assembly of the subcortical white matter or spinal cord, whereas parallel observations within CNS-PNS border zones suggested that SC development and its myelination program were altered (Dragatsis and Zeitlin, 2000; Michailov et al., 2004; Brinkmann et al., 2008). In nestin-cre driven NRG1 knockout mice, changes in the CNS were again largely unremarkable, though animals did exhibit early lethality (Brinkmann et al., 2008). However, in spinal cord explants obtained from the NRG1 knockout mice, a selective and severe reduction in OL development was observed that could be rescued with recombinant NRG1 (Vartanian et al., 1999). Conversely, when NRG1 type I or type III were overexpressed under a Thy1.2 driver in mice, hypermyelination was seen in thin (0.4 μm) neocortical fibers, without an overt change in OL numbers

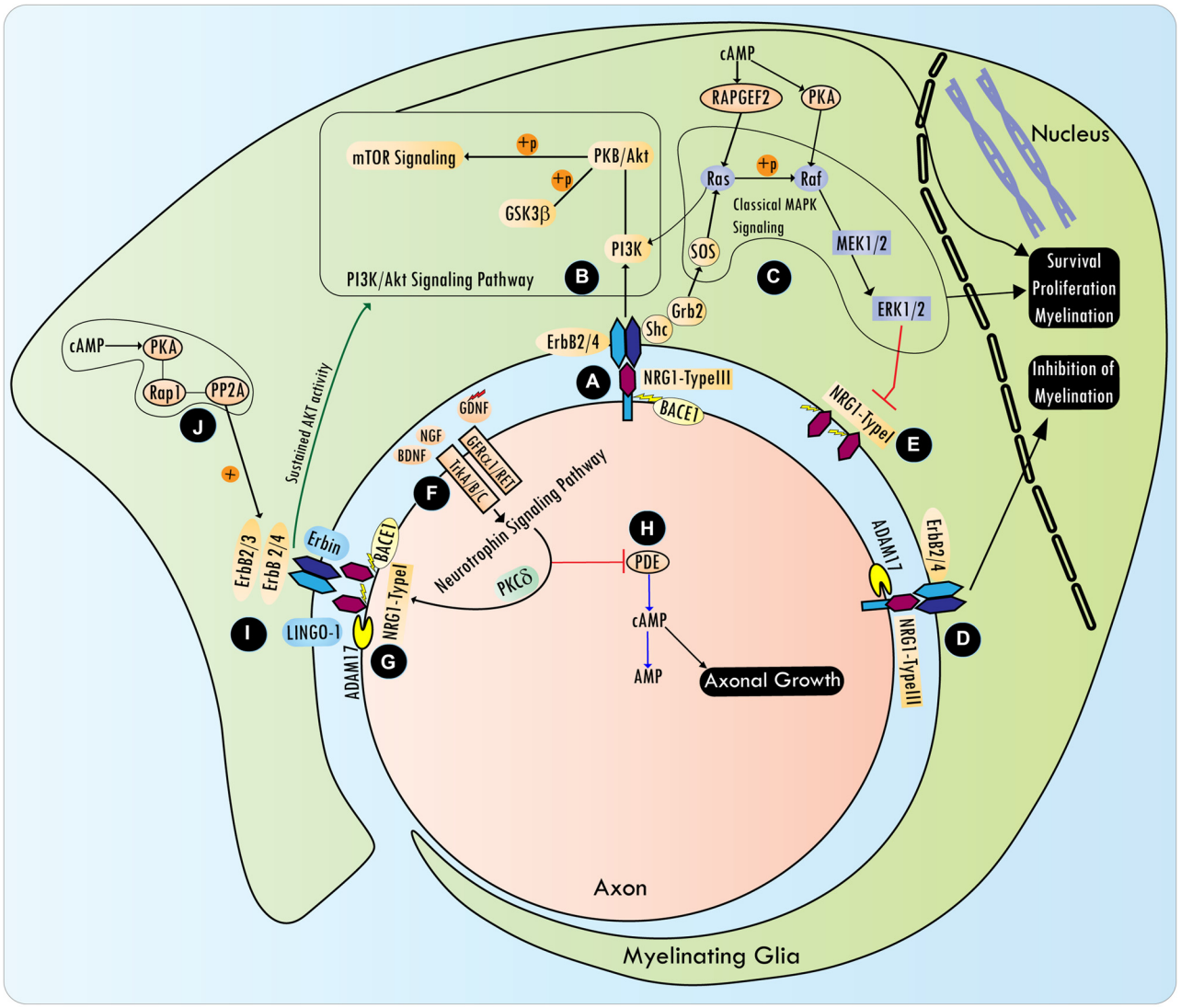

FIGURE 2 | NRG/Erb signaling in the control of peripheral myelination and axonal growth. BACE1 processed axonal NRG1-type III interacts with ErbB receptors **(A)**, and promotes cell survival, proliferation and myelination by activating various signaling pathways, including mTOR via PI3K/Akt **(B)** and $ERK_{1/2}$ via Ras/Raf **(C)**. However, NRG1 type III cleavage by ADAM17 conveys inhibitory signals to the myelination program **(D)**. In Schwann cells (SCs), axonal NRG type III (transmembrane) represses NRG type I (soluble) isoform expression via $ERK_{1/2}$ **(E)**. Rapid axonal NRG1 release can be spatially regulated by neurotrophic factors [e.g., Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF)], that are released by SCs, and act via TrkA, TrkB or GFRα1/RET receptors, respectively, on axonal surfaces **(F)**. Neurotrophins induce the coordinated efforts of PKCδ and cell surface proteases such as BACE1 and ADAM, which cleave a precursor form of axonal NRG1 to release its active soluble form **(G)**. In addition, neurotrophins also activate the Shc/PI3K/Akt survival pathway and Shc/Ras/$ERK_{1/2}$ differentiation pathways. Activation of ERK by neurotrophins can inhibit PDE4 (phosphodiesterase) activity, which leads to the antagonism of cyclic AMP hydrolysis **(H)**. Elevating intracellular cyclic AMP is critical to overcoming axon growth inhibition by myelin-associated molecules, such as myelin-associated glycoprotein (MAG), which is present in non-compact myelin. NRG1/ErbB signaling is further regulated by the adaptor protein Erbin and ErbB2 spatial localization determinant protein, LINGO-1, a component of the MAI-Nogo receptor-signaling complex **(I)**. Cyclic AMP can sustain NRG1 type I-mediated transient Akt phosphorylation via PKA dependent phosphorylation of the ErbB receptor, a mechanism that involves Rap1b-PP2A signaling **(J)**. NRG1/ErbB signaling is dispensable for the myelination program in the CNS, and a detailed understanding of the NRG1/ErbB interaction network following the CNS injury is lacking.

(Brinkmann et al., 2008). In the same study, NRG1 type III overexpression lead to premature myelination in the mouse optic nerve (Brinkmann et al., 2008). Closer examination of the OLs in the optic nerve of these mice showed that they exhibited an increase in soma size and a widened territory of coverage by their processes, suggesting that NRG1 overexpression could produce subtle changes in OL morphology (Brinkmann et al., 2008). OLs have been shown to respond in culture to soluble NRG1 by producing galactocerebroside and myelin basic protein (MBP; Vartanian et al., 1997, 1999; Fernandez et al., 2000; Calaora et al., 2001). Thus, discrepancies between *in vivo* and cell culture observations with OLs in NRG1 knockout animals highlight

some limitations of the models used, though they do suggest that NRG1 may play a role in OL function and myelination at later stages, but it is largely dispensable for myelination during CNS development.

On the contrary, loss of ErbB2 produces a severe reduction in OL numbers, as well as an impairment in their axon ensheathing capability (Park et al., 2001). The development of a transgenic mouse in which a dominant negative ErbB2 was expressed specifically in OLs, through use of a MBP promoter, showed that competitive antagonism of ErbB2 resulted in widespread hypomyelination and defects in OL differentiation (Kim et al., 2003). Similarly, the prevention of ErbB2 translocation to lipid rafts by LINGO-1, a component of the MAI-Nogo receptor-signaling complex, prevents OL differentiation (Lee et al., 2014). In the PNS, effective NRG1-ErbB myelination signaling needs Erbin expression in SCs, a leucine rich repeat (LRR) and PDZ domain-containing adapter protein belonging to the LAP family that interacts with ErbB2 (Borg et al., 2000; Tao et al., 2009). Erbin also appears to be an essential component for peripheral axon remyelination after injury (Liang et al., 2012). Interfering with Erbin expression inhibits NRG1 mediated Akt activation (Tao et al., 2009). These findings collectively suggest an important role of ErbB2, not only in the maturation of myelinating glia during development, but also for their functioning in repair when the nervous system is injured. In the CNS, ErbB3 knockout, in contrast, has no effect on OL differentiation and myelination (Schmucker et al., 2003). OLs from mice with an OL specific knockout of ErbB3 and ErbB4 were still observed to myelinate the CNS in a timely manner as compared to their wild type controls (Brinkmann et al., 2008). On the contrary, activation of ErbB3 leads to OL proliferation and differentiation, whereas ErbB4 activation leads to the suppression of OL maturation (Sussman et al., 2005; Makinodan et al., 2012). Further studies with the selective targeting of ErbB receptors during injury remain to be undertaken.

Neurotrophic factors secreted by SCs can spatially regulate rapid axonal NRG1 release (Esper and Loeb, 2004). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) released by SCs act via TrkA, TrkB or GFRα1/RET receptors respectively, on axonal surfaces. These neurotrophins provide trophic support to underlying axons and drive NRG1/ErbB signaling in SCs within the vicinity of axons, as well as regulate their myelination ability (Hoke et al., 2003; Ascano et al., 2009; Esper and Loeb, 2009). Neurotrophins induce the coordinated actions of PKCδ and cell surface proteases such as BACE1 and ADAM, which cleave a precursor form of axonal NRG1 to release its active soluble form (Loeb et al., 1998; Esper and Loeb, 2009). In addition to activating PKC via the PLCγ pathway, neurotrophins activate the Shc/PI3K/Akt survival and Shc/Ras/$ERK_{1/2}$ differentiation pathways (Ohira and Hayashi, 2009). Activation of ERK by neurotrophins can inhibit PDE4 (phosphodiesterase 4) activity, which leads to the antagonism of cyclic AMP hydrolysis (**Figure 2**). Elevating intracellular cyclic AMP is critical to overcoming axon growth inhibition by myelin-associated molecules, such as MAG, which are present in non-compact myelin (Gao et al., 2003; Patzig et al., 2011). NGF differentially regulates myelination in the CNS and the PNS, inhibiting myelination by OLs, while promoting myelination by SCs (Chan et al., 2004). Though work remains to improve our understanding of the modulation NRG1/ErbB signaling by neurotrophins in OLs, this signaling interaction has important implications for transplantation experiments after SCI that involve the use of genetically modified SCs with the overexpression of neurotrophins.

Axonal NRG1 interacts with ErbB2/3 to activate various signaling pathways (**Figure 2**; Ras/$ERK_{1/2}$, NF-κB, Ras/PI3K/Akt, Shc/Ras/PI3K, Shc/Shp2/Src/FAK and PLCγ/Calcineurin) that promote cell survival, proliferation and myelination (Newbern and Birchmeier, 2010; Heermann and Schwab, 2013). PI3K/Akt signaling downstream of NRG1/ErbB can serve as an additional mechanism to promote myelination, even at later stages of the development (Flores et al., 2008; Goebbels et al., 2010). Cyclic AMP, when used as a mitogen in SC cultures, can sustain NRG1 type I (heregulin)-mediated Akt phosphorylation via PKA dependent phosphorylation of the ErbB receptor, a mechanism that involves Rap1b-PP2A signaling (Monje et al., 2006; Hong et al., 2008). Conversely, constitutively active Akt (possibly acting through mTOR) can enhance myelination in the CNS without affecting OL numbers, but does not seem to have any role in the PNS myelination (Flores et al., 2008; Narayanan et al., 2009). The NRG1/ErbB signaling pathway also has an extensive cross talk (covered below) with other signaling pathways originating from notch, neuronal merlin, β integrins and GPR126/cyclic AMP adhesion G protein (Pietri et al., 2004; Woodhoo et al., 2009; Mogha et al., 2013; Schulz et al., 2014; Petersen et al., 2015). Recently, a nuclear variant of ErbB3 has been identified, which is under transcriptional control of NRG1; siRNA knockdown of nuclear-ErbB3 in SC-neuron co-cultures lead to a nearly 50% reduction in myelin segments, prompting a re-evaluation of the role of ErbB3 in myelination (Adilakshmi et al., 2011).

The current understanding of the involvement of NRG in peripheral regeneration following injury is that the axonal NRG type III (transmembrane) represses NRG type I (soluble) isoform expression in SCs via $ERK_{1/2}$ (Stassart et al., 2013). After the injury, the autocrine/paracrine NRG1 signals originating from SCs take over to promote de-differentiation and myelination by denervated SCs (Raphael et al., 2011; Stassart et al., 2013; Mei and Nave, 2014). However, studies in peripheral nerve injury also provide evidence that following injury, axonal NRG1, though not essential, is required for remyelination in a rate limiting fashion (Fricker et al., 2009, 2011, 2013).

Following injury, in the PNS, NRG1/ErbB isoforms show a differential expression pattern during the degeneration and regeneration phases (Ronchi et al., 2015). Intriguingly, NRG1 type III (b/c) is upregulated in the regeneration phase of peripheral nerve injury via a reversible switch between ADAM17 to BACE1 dependent cleavage (Ronchi et al., 2015). ADAM17, a ligand-independent activator of notch signaling, has previously been shown to produce NRG1 type III cleavage that is inhibitory

to SC myelination (Bozkulak and Weinmaster, 2009; La Marca et al., 2011). BACE1, on the other hand, is known to produce a promyelinating cleavage product of NRG1 type III, but this product was recently deemed non-essential for the peripheral myelination program (Willem et al., 2006; Velanac et al., 2012). These studies highlight the dichotomy and dynamic nature of decision making between myelination and axon growth programs in the PNS. Whether similar mechanisms operate following injury to the CNS is currently unknown. Following injury, ErbB2 protein levels are upregulated by 3rd day and persist for 4 weeks, whereas ErbB3 expression is upregulated at around 7 days and persists until 4 weeks (Ronchi et al., 2015). Intriguingly, the protein level of ErbB2 does not reflect the pattern of mRNA expression, which is downregulated after PNS injury. This can be related to a relative resistance of ErbB2 to degradation by several debated mechanisms that include efficient recycling, regulation of its endocytosis and HSP90/Cdc37 induced stabilization, or post-transcriptional regulation by the ERK/PDE/cAMP/PKA network (Bertelsen and Stang, 2014). Together, these studies suggest that NRG/ErbB signaling could be regulated in a complex manner, especially after injury, and more work needs to be done to iron out the discrepancies that exists among various studies.

Within 1 day following moderate thoracic contusion SCI in mice with the OSU device, NRG1 type I expression increases at the lesion site, which after 2 weeks, returns to control levels (Lasiene, 2009). Conversely, the expression of NRG1 type III was found to remain significantly decreased at all time points following the injury (Lasiene, 2009). In the same study, intrathecal infusion of NRG1 type III but not NRG1type I-β1 (Ser2-Lys246) into the mouse spinal cord lead to a transient increase in myelin sheath thickness in the axons caudal to the injury site (Lasiene, 2009). On the contrary, a study using a compressive thoracic SCI model in rats observed a downregulation of NRG1 type I expression following injury, along with no change in ErbB2/3/4 receptor expression (Gauthier et al., 2013). In a similar SCI model (compression) sustained intrathecal infusion of NRG1 type I-β1 (Thr176-Lys246) to the spinal cord was able to increase the expression of CNPase (a marker of OLs) and NF200 (axonal neurofilament), which was reversed by an ErbB2/4 inhibitor (Gauthier et al., 2013). In addition, a study administering subcutaneous NRG1 (soluble Type II; GGF2; Nrg1-β3) 24 h after thoracic contusion SCI in rats (weight drop, 10g/2.5 cm height) and mice (60 kdyn, T9), for a delivery period of 7 days, showed an increase in adult OL number and a subsequent increase in the myelination of spared axons (Whittaker et al., 2012). These findings highlight the differential characteristics of NRG1 isoforms. In extrapolating the usefulness of different NRG1 isoforms for therapeutic purposes, it is important therefore that NRG1 domains and amino acid stretches are adequately identified, in addition to the SCI model system used for a research study (Cheriyan et al., 2014). It is likely that some amino acid stretches on NRG1 domains might interact with pathways countering OL survival and myelination programs. A detailed evaluation of this research direction remains to be undertaken.

Studies to date suggest that the requirement of NRG1 *in vivo* is different for the PNS and the CNS. In the PNS, NRG1 is essential for SC differentiation and myelination, however, for the CNS, even though NRG1 is capable of altering the myelination process, it is dispensable (Brinkmann et al., 2008). However, after the injury there is considerable evidence that NRG1/ErbB signaling might play an important role in re-myelination and the survival of glia in both the CNS and the PNS (Lasiene, 2009; Whittaker et al., 2012; Gauthier et al., 2013). Even though there is anecdotal *in vitro* evidence that NRG1-β1 can promote neurite outgrowth, a majority of studies report that NRG1 promotes neuronal survival and acts as a neuroprotectant in adult injured tissues (Bermingham-McDonogh et al., 1996; Zhang et al., 2004; Edwards and Bottenstein, 2006; Iaci et al., 2010; Li et al., 2012; Whittaker et al., 2012). Accumulating evidence suggests that different isoforms of NRG1 may hold the key to understanding their precise role in CNS myelination and axon regeneration following injury. Specifically, there is a need to revisit the role of NRG1 isoforms, and the signaling cascade emanating from lipid-raft-inserted ErbB2 for its role in modulating the myelination program. Emerging understanding of NRG1/ErbB signaling necessitates further experiments to determine its broader clinical significance.

β-Secretase and Metalloprotease Signaling

Neuronally expressed β-site amyloid precursor protein (APP) cleaving enzyme1 (BACE1), a transmembrane protein and an asparatyl protease that is upregulated during PNS myelination, is another player in NRG1/ErbB signaling (Willem et al., 2006). BACE1, as well as ADAM10 and 17, can cleave NRG1 (type I and type III) at their C-termini to their EGF domains, whereas BACE1 and ADAM17 can also cleave NRG1 type III at its N-terminus to its EGF domain, releasing them from the neuronal membrane and assisting in their paracrine signaling (Montero et al., 2000; Syed and Kim, 2010; Luo et al., 2011; Fleck et al., 2013). NRG1 N-terminal cleavage releases α-sEGF and β-sEGF by ADAM17 and BACE1 respectively, whereas C-terminal cleavage by ADAM or BACE1 releases α/β-CTF, which undergoes rapid turnover (Fleck et al., 2013). Both α/β-sEGF can activate ErbB3 phosphorylation and promote downstream Akt signaling (Fleck et al., 2013). Crude N-terminal fragment of NRG1 (NTF) activates Akt in a similar fashion (Luo et al., 2011). However, BACE1-mediated processing of NRG1 was found to be crucial for myelination in SC-DRG co-culture studies (Luo et al., 2011). BACE1 released β-sEGF was sufficient to rescue peripheral hypomyelination in BACE1 mutant zebrafish (Fleck et al., 2013). Recently, for the first time, a study observed NRG1 cleavage to switch between two enzymes (ADAM17 to BACE1 and back to ADAM17) over the course of regeneration, after rat sciatic nerve crush as well as in a neurotmesis repair paradigm (Ronchi et al., 2015).

BACE1 knockout mice show a specific defect in myelination and not in axonal ensheathment. This is exemplified by the decreased expression of MBP and PLP proteins, enriched in OL-laid compact myelin, and normal expression levels of MAG, a protein enriched in the periaxonal membrane, in

BACE1 knockout mice (Hu X. et al., 2006). Expectedly, in BACE1 knockout mice, full length NRG1 was increased and a reduced activation of PI3K-Akt was noted (Hu X. et al., 2006). However, a re-evaluation of the BACE1 knockout showed more specific defects in remyelination following cuprizone induced demyelination (Treiber et al., 2012). CNS hypomyelination in BACE1 knockout mice was observed previously in hippocampal and optic nerves, while work by Treiber et al. (2012), in the corpus callosum, suggested region specific differences in the myelination program in the brain, as observed in other studies (Tomassy et al., 2014). Recently, breeding BACE1 knockout mice to mice expressing a constitutively active Akt (Akt-DD), specifically in OLs, showed rescue of BACE1-induced CNS hypomyelination by Akt, reinforcing that NRG1-ErbB/Akt signaling is downstream of BACE1 (Hu et al., 2013). In the PNS, BACE1 was present on both axons and SCs, and identified as important for the proper myelination of axons (Fleck et al., 2012; Hu et al., 2015).

Understanding the substrates of BACE1 during different phases of regeneration can provide unique insights into the PNS regeneration program. Studies have indeed applied quantitative proteomics to identify the substrates of BACE1 in cell models and zebrafish (Hemming et al., 2009; Hogl et al., 2013). At least 24 unique proteins accumulate in the membrane fractions of brain from BACE1 knockout zebrafish, suggesting them to be putative substrates (Hogl et al., 2013). Many of the molecules are involved in axonal growth, guidance and sprouting such as NCAM, L1, Plexin A3 and Glypican1 (Jakeman et al., 2006; Zhang et al., 2008; Bai and Pfaff, 2011; Shen, 2014). These findings stress the need for further work to understand the importance of BACE1 specific substrates in clinically related injury models of the PNS and CNS.

Fibroblast Growth Factor-2 (FGF2) Signaling

FGF2 isoforms have been known to be differentially regulated in the DRG and sciatic nerve (Meisinger and Grothe, 1997). DRG neurons express both FGFR1 and FGFR2 (Grothe and Nikkhah, 2001; Hausott et al., 2011). OPCs and differentiated OLs express FGF receptors in a developmentally-regulated manner. OPCs express FGFR1 and FGFR3, and differentiated OLs express FGFR1. On the other hand, paranodal myelin shows clusters of FGFR2 on lipid rafts of OLs (Bansal, 2002; Bryant et al., 2009). Downstream signaling for FGFR1/2 seems to occur via Raf-MEK-$ERK_{1/2}$ and PI3K/Akt/mTOR pathways, but it is plausible that FGF1/2 signals are also transmitted by other receptors (β1 integrins), and affected via other various intracellular signaling pathways (Grothe and Nikkhah, 2001; Hausott et al., 2011; Ornitz and Itoh, 2015).

Various FGF isoforms exert differential effects on OLs and OPCs. Specifically, FGF-2 promotes proliferation and inhibits differentiation of OPCs *in vitro*, but promotes process elongation, cell cycle re-entry, and decreases MBP protein expression in mature OLs (Fortin et al., 2005). Even though FGF2 signaling for OPC proliferation *in vivo* and *ex vivo* seems to be dispensable, a decrease in MOG, MBP transcription, and thickness of the myelin sheath have been observed after the long term absence of both FGFR1/2 in OLs (Furusho et al., 2012). On the contrary, administration of FGF2 to SCs in culture decreases the expression of P0 mRNA as well as its protein. In addition, FGF2 was able to inhibit the positive regulation of the myelin sheath component protein P0 via Forskolin, an activator of adenylyl cyclase (Morgan et al., 1994). These studies highlight the differential effect of FGF signaling in CNS and PNS glia, and point towards co-regulators that could be driving such a differential response.

Intrathecal injection of bFGF (30 min to 1 h post spinal contusion injury, infusion for 7 days), has been shown to produce positive effects on tissue preservation (Lee et al., 1999; Rabchevsky et al., 1999). Recently, acute subcutaneous administration (within 30 min of injury, then every 2 days post SCI) of human FGF2 was carried out in a rat spinal cord hemisection injury model showing decreased gliosis and a concurrent decrease in chondroitin sulfate proteoglycans (CSPGs; Goldshmit et al., 2014). Additionally, increased neuronal progenitor and radial glial numbers, as well as a change in the morphology of the glial scar and glial cell morphology (bipolar), towards that are supportive for axonal regeneration, were observed with FGF2 (Goldshmit et al., 2014). Combinatory treatment of a spinal thoracic transection injury in rats with a SC-fibrin bridge, along with recombinant human FGF2, produced a 3–4 fold increase in surviving NeuN positive cells in the adjacent host cord as compared to control (Meijs et al., 2004). However, recombinant human FGF2 failed to produce any significant growth of axons into the bridge, and did not lead to improved functional recovery (Meijs et al., 2004). By overexpressing the different isoforms of FGF2 in SCs and using them in a sciatic nerve graft, Haastert et al. (2006) demonstrated that FGF2–21/23 kDa (High Molecule Weight FGF2; HMW-FGF2) isoform preferentially promoted myelination whereas FGF2–18 kDa (Light Molecule Weight FGF2; LMW-FGF2) isoform was observed to be inhibitory to the myelination of regenerated axons (Haastert et al., 2006). In addition, the studies suggested that LMW and HMW FGF2 differentially regulated sensory and motor neuron regeneration and functional recovery (Haastert et al., 2006; Allodi et al., 2013). Other studies have shown that SCs overexpressing FGF2 enhance peripheral nerve regeneration (Danielsen et al., 1988; Fujimoto et al., 1997; Timmer et al., 2003). It is plausible, however, that FGF2 has pleotropic effects on myelination and axonal regeneration. Studies in FGF2 knockout mice support this view; where following sciatic nerve crush injury, distal to the crush site, at least 5× the number of regenerating axons were present as compared to control. This improvement is seen along with increased myelination and axon diameter, as well as enhanced sensory recovery, which the authors ascribed to an enhanced myelin clearance (Jungnickel et al., 2004, 2010).

In summary, these findings support that there exists a differential, isoform specific role of FGF2 in axon regeneration. Further experiments are required to understand the role of FGF2 isoforms and the modulation of FGFR endocytosis in relation to axonal regeneration and myelination following SCI (Goldshmit et al., 2012; Adeeb and Mortazavi, 2014).

Insulin Growth Factor (IGF) Signaling

IGF-I and II are polypeptides that play an important role in the development and maturation of neurons and glia, particularly projection neurons and cerebellar neurons (Andersson et al., 1988; Liu et al., 2000). Neurons secrete IGF-1 during activity via somatodendritic exocytosis in a syaptotagmin-10 dependent manner (Cao et al., 2011). IGF works through IGF receptor 1 and 2 (IGF1R, IGF2R), which are expressed on OLs. IGF receptor (IGF1R) abundance decreases with age (Garofalo and Rosen, 1989). IGF-1 signaling seems to be crucial for OPC survival, proliferation, and differentiation as well as CNS myelination (D'Ercole et al., 2002). Similarly in the PNS, IGF-1 promotes survival, proliferation and differentiation of SCs by inducing myelin-associated P0 protein expression (Cheng et al., 1996; Stewart et al., 1996; Cheng and Feldman, 1997; Sondell et al., 1997). In OL-specific IGF-1 knockout mice (Olig1-cre and PLP-cre driver), a severe reduction in $CC1^{+}$ mature OLs as well as NG2+ OLs was observed, along with a decrease in the myelin-associated protein PLP (Zeger et al., 2007). In global IGF-1 knockout mice, cortex, hippocampus and diencephalon show the maximum effect of decreased myelination, whereas brainstem and cerebellum show modest demyelination (Ye et al., 2002). However, post-demyelination, a local increase of IGF1 mRNA levels in the spinal cord failed to produce any OL-mediated myelination (O'Leary et al., 2002). The relevance of IGF-1 signaling with respect to myelination of long axonal tracts in the spinal cord and long relay neurons has yet to be established (Lee et al., 1992; Bibollet-Bahena and Almazan, 2009; De Paula et al., 2014). These findings highlight the need to search for co-modulators of CNS IGF signaling. Interestingly, SCs express IGF-1 and IGF-II, and are the most abundant source of IGF-1 supply in extra-ocular muscles (Kerkhoff et al., 1994; Feng and Von Bartheld, 2010). However, besides IGF1 expression, SCs also express IGF-binding proteins such as IGFBP4 and 5, which modulate IGF1 action. IGFBP4 was shown to inhibit IGF1 action, whereas IGFBP5 works along with IGF1 to promote myelination (Clemmons, 1998; Hammarberg et al., 1998; Cheng et al., 1999a,b).

IGF-1 specifically enhances axonal growth of pyramidal, brainstem and spinal motor neurons (Dobrowolny et al., 2005; Ozdinler and Macklis, 2006). On the contrary, IGF-1 is ineffective for the axonal growth of callosal projection neurons and retinal ganglion neurons (Goldberg et al., 2002; Catapano et al., 2004). Similarly, transplantation of IGF-1 secreting bone marrow stromal cell grafts into a dorsal column hemisection lesion in the rat, led the regeneration of raphespinal and cerulospinal, but not corticospinal axons (Hollis et al., 2009). Together these findings suggest that additional molecules could regulate the downstream response of IGF1 in a cell-specific manner. Recently, the β subunit of IGF1R was observed to be expressed on the distal axons of adult retinal ganglion cells, and negative modulation of IGF1R expression/activity led to nearly 20-fold decrease in axonal regeneration potential of the RGC neurons in culture (Dupraz et al., 2013). Similarly, following transection of the ventral funiculus in the rat lumbar spinal cord, the expression of both IGF-1 and IGFBPs (2, 5 and 6) were found to be upregulated in the ventral and ventrolateral gray matter as well as in the scar tissue (Hammarberg et al., 1998). The combination of the IGF-1 secreting cell graft with the infusion of an IGFBP inhibitory non-peptide ligand (NBI-317712) improved the survival of corticospinal neurons after an internal capsule injury in rats (Hollis et al., 2009). These findings reiterate that further understanding of the downstream bottlenecks of IGF1 signaling could provide novel tools for therapeutic intervention.

Downstream signaling from IGF-1 occurs via the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways. Recently, ubiquitin ligases MDM4/2, along with the transcription factor p53, were observed to form an inhibitory complex that blocked IGF1R signaling (Joshi et al., 2015). Nutilin3, an anticancer drug in development that blocks MDM2-p53 inhibitory interactions, has been shown to enhance axon regeneration in a IGF1R dependent manner (Joshi et al., 2015). Suppressing p53 levels or p53 activity also enhances the reprogramming efficiency of fibroblasts to convert into dopaminergic neurons (Liu et al., 2014; Rasmussen et al., 2014). Together, these findings suggests that in long projection neurons, IGF signaling might be amenable to transcriptional modulation, and calls for further exploration of the mechanisms that increase the expression of IGF1R, decrease the expression of inhibitory IGFBP (thus preventing sequestration of useful IGF) or that enhance IGF1R-mediated signaling.

Integrin Signaling

Integrins belong to a family of cell surface receptors that recognize ECM proteins such as fibronectin, laminin, collagen, vitreonectin, and which can also bind to other integrin family members (DeSimone et al., 1987; Hynes, 1987). The α and β subunits of integrins come in various forms, and binding of a combined αβ integrin to the ECM promotes a positive feedback loop between integrin clustering, intracellular cytoskeleton assembly and ECM organization (Giancotti and Ruoslahti, 1999). OLs have been observed to express various combinations of αβ integrins, including $\alpha_6\beta_1$ (Milner et al., 1997). The expression levels of integrins change throughout OPC development and during the generation of mature OLs. In addition, axons themselves considerably affect integrin subunit expression on OLs (Milner and Ffrench-Constant, 1994; Milner et al., 1997). The ligand for $\alpha_6\beta_1$ integrin, laminin2α, is known to be expressed on retinal projections, dendritic spines, and differentially on myelinating axons of the brainstem and proximal spinal cord (Morissette and Carbonetto, 1995; Tian et al., 1997; Colognato et al., 2002). Laminin is also abundant on the SC basal lamina (Bunge et al., 1986).

Binding of ligand to the β-integrin receptor leads to auto-phosphorylation of focal adhesion kinase (FAK). The FAK/Paxillin complex then recruits the SRC family of protein kinases (Fyn, Src, Lyn and especially Lck) to transfer the signal to CRK/$p130^{Cas}$, which in turn recruits the small GTPase Rac1 to activate NF-κB or JNK pathways (Chen et al., 2000; Schaller, 2001; Iwahara et al., 2004; Ness et al., 2013). In the PNS, Rac1 activated integrin signaling in SCs is crucial for radial sorting and subsequent myelination of PNS axons (Nodari et al., 2007). Binding of ligands to β-integrin can also directly activate the PI3K/Akt survival pathway via FAK.

In a mouse model, the overexpression of dominant negative β1 integrin (dnβ1) reduced FAK activation (p-Tyr_{397}) by 43% in optic nerves as compared to controls, but not in the spinal cord, suggesting a region-specific involvement of integrin signaling (Camara et al., 2009). In addition, an increase in the minimum axon diameter required to initiate myelination was noted in the optic nerves of the dnβ1 mice. This is very similar to NRG levels influencing the myelination program (covered below). FAK knockout mice showed similar findings (Forrest et al., 2009). Selective knockout of FAK in OLs, using a PLP-cre driver led to a reduction in the number of myelinated fibers in P14 mouse optic nerves (Forrest et al., 2009). However, at P28, both wild type and mutant dnβ1 and FAK knockout mice, show a comparable number of myelinated axons in the optic nerves, suggesting a transient and region-specific FAK influence. Contrary to the transient nature of the myelination defect in dnβ1 integrin mice, α_6 integrin knockout mice show hypomyelination due to the apoptotic cell death of OLs (Colognato et al., 2002). Interestingly, OL apoptosis was rescued by NRG1β1 (neuregulin), and OPCs from the α_6 integrin knockout mouse, when differentiated on laminin2α ($\alpha_6\beta_1$ integrin ligand) coated plates in the presence of NRG1β1, were able to myelinate appropriately (Colognato et al., 2002). NRG1β1-induced survival and differentiation in the presence of the laminin2α depends on the MAPK signaling pathway, with subsequent phosphorylation (Ser_{112})-mediated inhibition of Bad, a pro-apoptotic molecule, in a PI3K-independent manner (Fang et al., 1999; Colognato et al., 2002). These findings suggested that NRG1β1 was able to switch the dependence of the integrin response from PI3K to MAPK.

β1 integrins interact with the extracellular domain (L1-Fc) of F3/Contactin, a protein enriched on axons that activates Fyn kinase by repressing its inhibitory phosphorylation of Fyn-pTyr_{531} (Laursen et al., 2009). Supporting this finding, OL-specific knockout of contactin-1, using a PLP-cre driver, led to a 46% decrease in OL processes and impaired myelin membrane expansion (Colakoglu et al., 2014). Taken together, these findings implicate an integrated role of integrin-FAK pathway in deciding the onset and delay of myelination, and a cross talk with other molecular pathways could potentially influence its region specificity.

Laminin/β1 integrin signaling assists neuritogenesis by mediating microtubule assembly as well as stabilization in axons (Lei et al., 2012). Recent studies suggest that the integrin ligand, laminin, does this by switching $\alpha_3\beta_1/\alpha_7\beta_1$ integrin-mediated F-actin dynamics/exocytic signaling from the Ena/VASP/WAVE/VAMP2 pathway to FAK/SRC/Cdc42/Rac/Arp2/3/VAMP7 complex-dependent signaling (Krause et al., 2003; Gupton and Gertler, 2010; Havrylenko et al., 2015). Loss of β1 integrin leads to decreased pLKB1 (Ser431) and SAD-A/B kinase levels, and alters microtubule stabilization via the FAK/SAD pathway (Lei et al., 2012). The mechanistic aspects of LKB1 phosphorylation are elusive. However, PKA might phosphorylate LKB1, since $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins are known to function as AKAPs (PKA specific A-Kinase anchoring proteins), and Ser431 in LKB1 is a PKA consensus phosphorylation site (Lim et al., 2008; Lei et al., 2012). These findings suggest that integrin-associated Src kinases can integrate signals from axons as well as the basal lamina and interact with a larger network of partners intracellularly.

MAIs further regulate these cell-specific integrin pathways. MAG stimulates asymmetric clathrin and calcium-mediated endocytosis of β1 integrins at the growth cone, and MAG likely performs a similar function elsewhere in axons (Hines et al., 2010). Since MAG is usually present at the axon-glial junction, it is plausible that the dynamic regulation of the surface expression of MAG couples integrin signaling with the myelination program at the axon-glial junction.

In summary, integrin signaling has a profound influence on both myelination and axon regeneration. Axons and glia have been demonstrated to mutually activate and modulate the integrin signaling pathway (Eva et al., 2012; Eva and Fawcett, 2014).

Cell Adhesion Molecules (CAMs)

CAMs upon the plasma membrane are critical to neuron-glia interactions, bringing neuronal (in this case axonal) and oligodendrocyte membranes together. CAMs either activate signaling pathways themselves or help juxtapose signaling complexes (Pollerberg et al., 2013). In addition, CAMs play an important role in the patterning of axonal functional domains (e.g., node of Ranvier, paranode, juxtaparanodes) with the involvement of adjacent glia (Normand and Rasband, 2015). There are at least four groups of CAMs described, which differ largely in their functional requirement for calcium. Of these, classic cadherins are calcium-dependent cell-to-CAMs that work in tandem with catenins (α, β and p120), their cytoplasmic binding partners, which connect them to the actin cytoskeleton (Takeichi, 2007).

In the nervous system, OLs, SCs and axons express the prototype molecule, N-cadherin, in an age-dependent manner. When N-cadherin function was blocked by a peptide, only 50% of Purkinje cell axons were myelinated in an organotypic cerebellar slice culture model, suggesting its important role in myelination (Schnadelbach et al., 2001). The cytoplasmic domain of cadherin can sequester β-catenin, thus modulating the levels of available β-catenin for the canonical Wnt signaling pathway, as well as β-catenin's association with APC (adenomatous polyposis coli), a molecule implicated in the stabilization of microtubules (Hansen et al., 2008). Studies suggest that the Wnt signaling pathway could be an essential pro-myelination cascade (Tawk et al., 2011). Association of the cadherin cytoplasmic domain with α/β catenin and p120 is positively regulated by GSK3β and CaMKII, while it is negatively regulated by Src, Fer, abl and EGFR kinases (Nelson, 2008). Following cervical spinal cord unilateral hemisection, activation of the Wnt signaling pathway in corticospinal axons antagonizes regeneration via the Wnt1/Wnt5a/Ryk signaling complex (Liu et al., 2008; Lewallen et al., 2011; Tawk et al., 2011). The cytoplasmic cadherin domain also regulates both RhoGTPase activity, by interacting with p120, and actin dynamics, by interacting with α catenin (Cavallaro and Dejana, 2011). Conversely, Rho GTPase regulates the clustering of cadherins on the cell surface (Fukata and Kaibuchi, 2001; Grosheva et al., 2001). Cadherins can also bind to several growth factor receptors, including FGFR, and modulate

their intracellular signaling, either by activating them in a ligand-independent mechanism, or by recruiting components for signaling units, which include adaptor proteins (Shc), kinases (Src, CSK) and phosphatases (SHP2, RPTPβ/η; Cavallaro and Dejana, 2011).

Nectins are the second group of calcium-independent CAMs that interact with themselves on the same cell (homophilical) or with nectin present on another cell (heterophilic; Takai et al., 2003). Further, a third group, nectin-like molecules (Necl), specific to nervous tissue, resemble nectins in structure and function and are expressed on axons (Kakunaga et al., 2005). Necl-1 has a strict neuronal expression in the cortex, retina, cerebellum and spinal cord (Kakunaga et al., 2005; Park et al., 2008). Necl-1 regulates time dependent critical aspects of myelination (Park et al., 2008; Zhu et al., 2013). Necl-1 knockout mice showed delayed axonal myelination of both the optic tract and spinal cord, which normalizes by 60 days post-birth (Park et al., 2008). Given a complex network of interactions and ligands, discerning the exact roles of CAMs in myelination would be an elaborate engagement, and has been excellently reviewed by Pollerberg et al. (2013).

The IgSF (immunoglobulin superfamily) is the fourth group of CAMs that do not depend on calcium and are present on axons. Growing axons express at least nine types of IgSF CAMs (NCAM1/2, L1-CAM, Contactin1/2, NRCAM, ALCAM, CHL1, and Neurofascin; Pollerberg et al., 2013). The IgSF members have a complex interaction network; they not only interact with themselves, but also with other CAMs in a homotypical or heterotypical manner. Extracellularly, IgSF CAMs have been noted to interact with CSPGs (neurocan, phosphacan), ECM components (Tenascin C and R, MMP14), β1 integrins, growth receptors (GDNF/GFRα1, FGF-R, TrkB), semaphorin receptors (Neuropilin 1, Plexin A1), ephrin receptors (Eph A 3/4/7), sodium and potassium channels (SCN1B, Kir 3.3), and with some unusual interacting partners, such as prion protein (PrPC), APP and extracellular GAPDH (Pollerberg et al., 2013). Intracellularly, IgSF CAMs routinely interact with cytoskeletal components such as ankyrin and the microtubule-associated protein, doublecortin (DCX; Rader et al., 1996; Kizhatil et al., 2002).

NCAM, one of the prominent members of IgSF CAM, is known to be involved in neural differentiation, axonal guidance and branching (Walsh and Doherty, 1997). Expression of unmodified NCAM persists during myelination (Bartsch et al., 1989). In contrast, post-translational modification of NCAM by the addition of sialic acid (PSA-NCAM) has been reported to negatively regulate myelination, with axons undergoing myelination only when they do not express PSA-NCAM, suggesting that inhibitory signals strongly regulate the myelination program (Charles et al., 2000; Fewou et al., 2007; Jakovcevski et al., 2007). The role of NCAM in myelination and neuroprotection could be more complex given its unconventional partners. For example, NCAM is known to interact with FGF and GDNF (GFRα1) receptors. Thus a trans-interaction with similar glial receptors can activate growth and differentiation pathways, driving differential myelination via FGF2 signaling (Jacobsen et al., 2008). Following transection SCI in rodents, NCAM levels increase in the dorsal spinal cord, motor neurons and corticospinal tract fibers (Tzeng et al., 2001). Investigations of contusive SCI in NCAM knockout mice revealed extensive neuronal apoptosis, decreased 5HT axon regeneration, and defective ERK and GAP43 signaling when compared to wild-type controls (Zhang S. et al., 2010). However, how NCAM, both native and surface modified, can alter ERK/GAP43 signaling and be neuroprotective in the injury scenario is unclear.

L1-CAM, a member of the IgSF CAM family, is intricately involved in axon growth and guidance during development (Cohen et al., 1998; Kenwrick and Doherty, 1998). Strong expression of L1-CAM has been found on unmyelinated optic nerve axons that are not in contact with glial cells, whereas it was absent from axon-glial contact regions (Bartsch et al., 1989). Trans-interactions of axonal L1-CAM can activate integrin signaling on glia, regulating axon-glia interactions and myelination (Silletti et al., 2000; Guseva et al., 2011). Transplantation of genetically engineered SCs producing L1, or secreting its chimeric form, L1-Fc, into the SCI lesion in mice, led to enhanced serotonergic fiber sprouting into, and across, the lesion (Lavdas et al., 2010). In addition, in transgenic mice that overexpressed L1 in neurons, severe contusion SCI studies suggested that L1-CAM could enhance catecholaminergic fiber regeneration and sprouting (Jakovcevski et al., 2013). It has been found that MBP, a myelin-associated protease present in both CNS and PNS myelin, is capable of cleaving L1-CAM to promote neuritogenesis (Lutz et al., 2015). This suggests that, similar to β1 integrins, myelin-associated proteins also can modulate CAM-mediated interactions.

Neurons express contactin, another member of the IgSF CAM family, which interacts heterophilically with L1-CAM, NRCAM and neurofascin (Falk et al., 2002). Since contactins do not have an intracellular domain, their signaling requires additional recruited molecules (Rios et al., 2000; Charles et al., 2002; Traka et al., 2002, 2003; Gautam et al., 2014). Contactin functions as a non-canonical notch ligand, and likely has numerous cell surface interaction partners (Hu Q. D. et al., 2006). Contactin2, another member of contactin superfamily, present on both SCs and OLs at the juxtaparanodal region, was recently discovered to be a BACE1 substrate, and an interaction partner of neuronal Caspr2 (a contactin-neurofascin interaction modulator) at paranodal areas (Rios et al., 2000).

Axons and glia harbor a host of molecules (including gliomedin, NrCAM and various isoforms of neurofascin), which take part in the maturation of the nodes of Ranvier on myelinated axons (Thaxton and Bhat, 2009). Myocilin, a Wnt and ErbB2/3 signaling regulator, interacts with gliomedin, NrCAM, and neurofascin (NF186; Kwon et al., 2013). Knocking out myocilin upregulates DLG1-PTEN in sciatic nerves (Cotter et al., 2010; Kwon et al., 2013). The DLG1-PTEN interaction negatively regulates peripheral myelin thickness, likely by stabilizing PTEN and decreasing Akt activation (Cotter et al., 2010). DLG1-mediated negative regulation of myelination was found to be transient, whereas a fine-tuning of PI3K-Akt-mediated mTOR signaling by another protein, DDIT4 (DNA damage-inducible transcript 4 protein), was found to lead to a sustained negative

regulation of peripheral myelin thickness (Noseda et al., 2013). Conversely, the interactions of glial NrCAM and axonal contactin, as well as of glial neurofascin and axonal NrCAM have been shown promote neurite outgrowth in cultured tectal neurons (Morales et al., 1993; Grumet, 1997).

In summary, CAMs have dual roles in myelination and axonal growth that, at present, remain underinvestigated, particularly following injury. CAMs communicate with multiple downstream signaling pathways in a cell-specific manner. Conversely, axo-glial signaling pathways and myelin-associated proteins that share the same spatial domains can modulate CAM actions. Together, the advantages and disadvantages of CAMs, prompt a nuanced approach in their use for combinatorial therapies targeting myelination and axon regeneration.

Chondroitin Sulfate Proteoglycans (CSPGs)

CSPGs constitute one of the important extrinsic factors limiting CNS axon regeneration following injury (Davies et al., 1997; Silver and Miller, 2004). OPCs, astrocytes and macrophages account for the predominant expression of CSPGs, which following SCI undergo tremendous modulation (Jones et al., 2002). CSPGs on the cell surface are often released from the membrane and become a part of the extracellular matrix (Carulli et al., 2005). The majority of CSPGs present in the CNS belong to the lectican family. Aggrecan, versican, neurocan, brevican, phosphacan, neuroglycan-D, NG2 and phosphacan constitute predominant CSPGs of the CNS (Carulli et al., 2005). Aggrecan is expressed primarily by neurons, versican by astrocytes, NG2 by OPCs and macrophages whereas neurocan and brevican are expressed by all neural cells (neurons, astrocytes and OPCs; Jones et al., 2002; Dyck and Karimi-Abdolrezaee, 2015). Structurally, CSPGs contain a protein core and glycosaminoglycan (GAG) side chains that routinely undergo excessive branching or modifications like sulfation (Carulli et al., 2005). Modifying the GAG chain lengths (using enzyme Cha'se ABC) or their sulfation modulates CSPG permissiveness for axon regeneration (Zuo et al., 1998; Wang et al., 2008).

CSPGs interact with a multitude of axonal and growth cone surface receptors such as Nogo receptor 1 (NgR1), Nogo receptor 2 (NgR2), leukocyte common antigen (LAR) and protein tyrosine phosphase σ (PTPσ). Possibly with more players (such as p75NTR), CSPG-receptor interaction can negatively regulate axon regeneration by activating the Rho/ROCK signaling pathway (Dergham et al., 2002; Monnier et al., 2003). A recent study suggests that extracellular CSPGs, by an as yet unknown mechanism, can regulate local intra-axonal translation of RhoA, thus ensuring an enhancement of Rho/ROCK signaling in the axon (Walker et al., 2012). Interestingly, studies implicate NG2 (expressed on OPCs) to be the predominant CSPG following dorsal hemisection SCI (Plant et al., 2001; Jones et al., 2002). NG2-positive OPCs surround corticospinal axons within the injury environment after SCI (Jones et al., 2002). Based upon this interaction, NG2 could then serve as an important reason for impaired axon regeneration following injury. However, even though NG2 *per se* inhibits axon growth, both postnatal and adult NG2 expressing cells are growth permissive (Dou and Levine, 1994; Ughrin et al., 2003; Yang et al., 2006; Busch et al., 2010). This paradox could be due to the co-expression of axonal growth permissive substrates (e.g., laminin and fibronectin) on NG2 positive cells (Yang et al., 2006). In addition, SCs have been shown to express CSPGs that are sensitive to treatment with chondroitinase (Cha'se) ABC, and which retard DRG axon growth (Kuffler et al., 2009). Together these findings suggest that glial CSPGs, both membrane-bound and ECM-associated, can interact with several membrane receptors on the axon, growth cone and soma to modulate the axon growth machinery. Whether membrane bound CSPGs can activate distinct molecular pathways in axons as compared to those associated with the ECM remains unknown.

OPCs show an increase in PTP expression during their differentiation (Ranjan and Hudson, 1996). CSPGs negatively regulate OL process outgrowth and the OL myelination program by interacting with PTPσ (Pendleton et al., 2013). In one study, aggrecan, neurocan and NG2, all inhibited OL process outgrowth, and downregulated MBP expression, and this effect was reversed by Cha'se ABC (Pendleton et al., 2013). In addition, a combination of CSPGs (neurocan, versican, phosphacan and aggrecan) led to a stronger phenotype of OL process inhibition and MBP expression in OLs (Siebert and Osterhout, 2011). Treatment of OPCs with Cha'se ABC also enhanced their spontaneous differentiation towards O4-positive OLs (Karus et al., 2016). This effect of Cha'se ABC could be mediated by its ability to eliminate phosphacan/RPTPβ/ζ from the OPC surface (Karus et al., 2016). Moreover, Cha'se ABC stimulated neural precursor cells in the mouse spinal cord to differentiate into OLs in large numbers (Karus et al., 2016). Intracellularly, CSPG-mediated changes in the outgrowth and alterations in the myelination program of OPCs and OLs was mediated by PTPσ driven Rho/ROCK activation (Pendleton et al., 2013). Together these findings suggest that similar molecular pathways activate CSPG-mediated axon growth inhibition and the OL myelination program. Therefore, it is important that signaling intermediaries/downstream of Rho/ROCK are identified so as to permit future therapeutic targeting of CSPGs' differential effects on myelination and axon regeneration.

THE PHYSICAL PROPERTIES OF AXONS AND THE CONGRUENCE OF MYELINATION AND AXONAL GROWTH

Axonal Caliber and Radial Growth of Axons

Axon diameters vary in the CNS (0.1–24 μm), and accordingly, their cross sectional area and volume (Barazany et al., 2009; Perge et al., 2012). In an elegant experiment, Voyvodic showed that altering the target size of postganglionic, unmyelinated sympathetic axons altered their caliber, as well as the responses of SCs towards them, suggesting a strong correlation between

axonal caliber and myelination (Voyvodic, 1989). As a corollary to Voyvodic's (1989) work, axons that differ in their caliber above the physiological limit (100 nm) appear to reflect the information they carry to and from their target (Faisal et al., 2005; Perge et al., 2012). In the PNS, axons less than 700 nm in diameter are not myelinated but are ensheathed by a SC, or reside in a Remak bundle (Garbay et al., 2000). Such a threshold for ensheathment and myelination is much lower in the CNS, where OLs myelinate axons of around 200 nm diameter and axon-like nano fibers of $\geq$ 400 nm in diameter *in vitro* (Waxman and Bennett, 1972; Lee et al., 2013). OLs have been observed to produce myelin components without the presence of axons, but intriguingly, in *in vitro* co-cultures with neurons, OLs myelinate only axons even when the dendrite diameters vary from 200 to 8000 nm, suggesting a role for axon-specific signals in myelination (Ulfhake and Cullheim, 1981; Dubois-Dalcq et al., 1986; Claiborne et al., 1990; Lubetzki et al., 1993). Indeed, SCs that do not myelinate cervical sympathetic axons were observed to myelinate sural nerve axons, which routinely undergo myelination, reiterating axon-specific signals for myelination (Aguayo et al., 1976). Intriguingly, a linear relation between the cross-sectional area of the myelin sheath and axon diameter (see G-ratio) was observed, suggesting a role for the axonal surface in regulating myelin sheath volume (Berthold et al., 1983; Paus and Toro, 2009). G-ratio is the ratio of axonal diameter to fiber diameter (including myelin sheath), used as a measure for optimal myelination. An axon can influence its myelination by communicating its information load via specific molecular signals to their ensheathing cells (Fraher and Dockery, 1998). Supporting this view, in the PNS, axons directly control the thickness of their myelin sheath via the regulation of the expression of NRG1 type III on the axonal membrane (Michailov et al., 2004; Taveggia et al., 2008).

Conversely, myelination can affect axonal caliber in the PNS and the CNS, effectively modulating their radial growth. Myelinated axons have been consistently observed to have a larger diameter than unmyelinated axons (Duncan, 1934; Matthews and Duncan, 1971; Hoffman et al., 1984; de Waegh et al., 1992). Myelination increases the cross sectional area of the myelinated axonal segment up to 45% more than the unmyelinated segment of the same axon (Monsma et al., 2014). Structurally, medium and heavy neurofilaments (NF-M and NF-H) principally determine the caliber of the axon, and a defective NF phenotype presents with abnormal axonal caliber (Hoffman et al., 1987; Cleveland et al., 1991; Muma and Cork, 1993). Therefore, NF-M and NF-H could be among the putative molecules responding to glial-derived, radial axon growth signals. In SC-DRG co-culture experiments, myelinated axonal segments were observed to contain nearly 42% more NF-M as compared to unmyelinated segments of the same axon (Monsma et al., 2014). SC-DRG co-culture experiments have suggested that the factor responsible for an enlarged diameter of myelinated axons was not secreted but rather a cell-to-cell contact molecule (Windebank et al., 1985). So far, myelin-associated molecules such as MAG, PLP, PMP22, MBP, and sulfated and non-sulfated galactolipids (sulfatide/GalC) have been suggested to play a role in axon-glial communication and adhesion, to modulate the radial growth of axons (de Waegh and Brady, 1990; Smith et al., 2013). MAG is a component of non-compact PNS myelin in the periaxonal membrane (Trapp and Quarles, 1984; Patzig et al., 2011). Mice having a complete absence of MAG, and rats having a mutation in MBP [LES (long Evans shaker)], show: (1) decreased NF density in sciatic nerve axons; (2) decreased NF phosphorylation; (3) decreased axonal caliber and, (4) progressive axonal loss by Wallerian degeneration (Yin et al., 1998; Smith et al., 2013). Addition of soluble MAG-Fc to DRG neuronal cultures was found to increase the phosphorylation of NF-H, and negatively regulate axonal degeneration induced by vincristine, thus supporting the role of MAG in modulating axonal caliber (Nguyen et al., 2009). However, so far very few studies have addressed the mechanistic pathway connecting MAG or other myelin-associated proteins to the radial growth of axons. Growth cone collapse and the mechanisms thereof have received a higher emphasis to date. MAG and OMgp also cause longitudinal growth inhibition of axons by synergizing with another MAI, Nogo-A, which binds to NgR1 to bring about growth cone collapse (Cafferty et al., 2010). Lipid-sulfatides are a novel class of MAIs that presumably work through the canonical MAI pathway leading to growth cone collapse in a Rho/ROCK dependent manner (Winzeler et al., 2011).

Unraveling the mechanisms involved in the post-translational modulation of NFs from glial derived signals remains an ongoing pursuit. Initial thinking was that in the myelinated segments of axons, heavily phosphorylated NF tail domain KSP motifs exert repulsive forces on neighboring NFs by long side-arms and hence spread apart. Whereas, in unmyelinated segments they are less phosphorylated and therefore tightly packed (Hisanaga and Hirokawa, 1988; Mukhopadhyay et al., 2004; Sihag et al., 2007). Previous work has unequivocally shown that the phosphorylation of NFs not only promotes NF-NF interactions, but also affects their ability to associate with molecular motors such as kinesin, dynein and myosinV (Yabe et al., 2000; Xia et al., 2003; Motil et al., 2006; Kushkuley et al., 2009). On the contrary, experiments conducted with a substitution of serine in the KSP motifs of NF-M with phosphorylation-incompetent amino acids demonstrated radial axonal growth to be independent of the NF-M-KSP phosphorylation motif, but dependent on the C terminal region of NF-M, thus prompting a relook at the prevailing model of radial axonal growth (Garcia et al., 2009). Recent evidence suggests that the C-terminal domains of NF-M and NF-H can play a role in the stabilization of NFs by modulating its proteolysis (Rao et al., 2012). Unmyelinated peripheral axons do express components of all three different forms of the proteolytic machinery (ubiquitin-proteasome system, lysosomes and autophagy), which can be regulated by neurotrophins (e.g., NGF; Frampton et al., 2012). Taken together, post-translational modification of NFs seems to play a significant role in modulating axonal caliber. However, mechanistic details of how MAG, or any other myelin-associated molecule, can modulate axonal caliber remains largely speculative. GSK3α/β, CDK5, MAPK, SAPK/JNK, CKI/II are some of the implicated

phosphokinases that can phosphorylate NFs, whereas PP1, PP2A and PTP1B are some of the phosphatases known to reverse NF phosphorylation (Shea et al., 2004; Snider and Omary, 2014). The proposed hypothesis is that MAG phosphorylates NFs by interacting with the low affinity neurotrophin receptor (p75NTR) to activate Raf/$MEKK_{1/2}$/$ERK_{1/2}$ via NRAGE. In addition, p75NTR/NRAGE can inhibit PKA, leading to increased Rho and inactivation of p35/Cdk5. This could produce the decreased Cdk5 phosphorylation of NF as well as decreased Cdk5-mediated inhibition of $ERK_{1/2}$. The signaling from MAG/p75NTR/NRAGE could then fine-tune the phosphorylation status of NF-M and NF-H (Garcia et al., 2003). However, unequivocal confirmation that MAG can recruit these signaling pathways remains to be shown. Recently, in a work identifying new players connected to NF phosphorylation, a study found that the loss of β1 integrin could lead to the phosphorylation of Tau at Ser262 (Lei et al., 2012). In a separate study, MARK4/3 (microtubule-associated regulating kinase) expression correlated with pTau (Ser262) levels in granulovacuolar degeneration bodies of the Alzheimer's diseased brain, which shows progressive defects in NF phosphorylation (Lund et al., 2014). Whether myelin-associated proteins could mediate trans-regulation of the axonal proteolytic system remains to be explored.

In summary, post-translational modifications of NF may have differential effects on its stability and its association with other NF molecules as well as transport motors, depending on the specific kinase/phosphatase/proteolytic machinery within the local environment. Specific glial and myelin-associated molecules that regulate axonal caliber and their partners, along with the related signaling network upon the axonal membrane, are a significant gap in our understanding of axon-glial signaling that needs further attention.

Myelination and Axonal Transport

The trembler mouse, which displays hypomyelination in the PNS, also shows an altered axonal transport (de Waegh and Brady, 1990). The trembler mouse has a point mutation in PMP22 that encodes a peripheral-myelin enriched protein (Suter et al., 1992). Decreased myelination of the sciatic nerve in the trembler mouse was associated with increased NF transport (1.73 mm/d in trembler vs. 1.56 mm/d in control; de Waegh and Brady, 1990). In contrast, a slower NF transport velocity of 0.16 mm/d occurred in DRG neuron axonal segments that were SC-myelinated, compared to 0.22 mm/day in the unmyelinated segments (Monsma et al., 2014). It is interesting to note that the NF transport rate has been reported to be quite heterogeneous, depending on the cell type and the analysis method employed (Shea and Chan, 2008). There is a considerable debate on the role of phosphorylation of the NF tail domain in restricting NF transport, since the evidence so far supports the role of NFs in the radial growth of axons (Yuan et al., 2006; Sihag et al., 2007; Shea and Chan, 2008).

Interestingly, addition of soluble MAG to the axonal compartment of rat cortical cultures promoted the accumulation of detyrosinated tubulin, and decreased tyrosinated tubulin in a NgR-independent, but GalNAc-T dependent manner (Nguyen et al., 2009). Though exciting, this finding needs to be reconfirmed since the antibody used by Nguyen et al. (2009) to detect de-tyrosinated tubulin (Chemicon: AB3210) was raised against β-tubulin and the antibody used to detect tyrosinated tubulin (Sigma: T9028) was raised against α-tubulin. Post-translational modification of tubulin-dimers (typically α-tubulin) by the addition or deletion of tyrosine is known to regulate its association with kinesins and many plus-end tracking proteins (+TIPS), including p150Glued, a member of dynein-dynactin retrograde motor complex, and CLIP-170, a CAP-Gly domain-containing protein implicated in injury signaling (Lomakin et al., 2009; Lloyd et al., 2012; Gumy et al., 2013; Prota et al., 2013; Schneider et al., 2015). In addition, other players can also be involved in modulating the axonal cytoskeleton. Work has pointed towards notch signaling involvement in the stabilization of microtubules through transcriptional regulation of the microtubule severing protein, spastin (Ferrari-Toninelli et al., 2008). Thus, post-translational modulation of axonal tubulin by OL/SC surface molecules opens a completely new chapter in glia-modulated structural and functional aspects of the axonal cytoskeleton.

The precise signaling mechanisms involved in the regulation of tubulin tyrosination remain unknown. Post-translationally (phosphorylation) controlled tubulin tyrosine ligase (TTL) activity regulates tubulin tyrosination (Idriss, 2000). Loss of TTL leads to severe defects in microtubule-dependent retrograde transport, specifically after axonal injury (Idriss, 2000; Song et al., 2015). Mechanistic details of TTL phosphorylation remain elusive, but MKP-1 can be one such putative candidate. A novel MKP-1 (Map Kinase Phosphatase-1; $MKP\text{-}1^{ASA}$) was constructed by Jeanneteau et al. (2010) by mutating its RRR motif in the MAPK binding domain, which eliminated the binding of MKP-1 to $ERK_{1/2}$ and p38 MAPK, but selectively retained the MKP-1/JNK-1 interaction (Jeanneteau et al., 2010). Ectopic expression of $MKP\text{-}1^{ASA}$ in the postnatal rat cortex decreased JNK-1 activity and increased tyrosinated-α-tubulin in both excitatory neurons and interneurons (Jeanneteau et al., 2010). Moreover, different isoforms of JNK along with their substrates, stathmins (e.g., SCG10, SCLIP), are known to be crucial for microtubule stability and axon growth (Tararuk et al., 2006; Barnat et al., 2010). Whether myelin-associated proteins selectively trans-activate the MKP-1/JNK pathway to regulate axonal TTL is an open question. A study suggested that retrogradely transported TrkB-pERK could regulate the transient expression of inducible MKP-1 (Jeanneteau et al., 2010). It is therefore tempting to speculate that regeneration-associated signaling and myelination could mutually influence each other through cytoskeletal components. Elucidating these mechanisms could provide novel avenues for regeneration research.

Adaptive Myelination

Neuronal activity influences oligodendrogenesis, OL differentiation, myelination, and myelin thickness (Gyllensten and Malmfors, 1963; Tauber et al., 1980; Barres and Raff, 1993; Stevens et al., 1998; Gibson et al., 2014). Decreasing the neuronal activity just prior to developmental myelination of the optic nerve, with the application of a sodium channel blocker

(tetrodotoxin), has been shown to produce a 60% decrease in the optic nerve myelination without affecting OPC numbers (Demerens et al., 1996). In contrast, exposure of developing zebrafish to the GABA antagonist pentylenetetrazole (PTZ), in the bathing medium, not only increased neuronal activity, but also led to a 40% increase in the number of myelin sheaths made by OLs upon reticulospinal axons of the ventral spinal cord, without any major changes in OL numbers (Mensch et al., 2015).

Emerging evidence suggests that OLs can sense neuronal activity and make decisions regarding the ensheathment of axons (Hines et al., 2015). In a study involving spinal cord samples of zebrafish embryos, individual OLs were seen to lay the myelin sheath within 5 h of contact with the target axons that were electrically active (Czopka et al., 2013). Blocking sodium channels in zebrafish using TTX, biased the stabilization, extension and maintenance of myelin sheaths away from highly active axons, without affecting OL differentiation. Further experiments highlighted that vesicle-associated membrane protein (VAMP2) dependent extra-synaptic axonal secretion regulated the maintenance of OL ensheathment (Wake et al., 2011; Hines et al., 2015). Inhibition of synaptic vesicle release in developing zebrafish could reduce the average number of myelin sheaths for OLs by 30%, OL numbers by 10% and the number of myelinated reticulospinal axons by 40% (Mensch et al., 2015). Optogenetic stimulation of the premotor cortex (M2) in a transgenic mice (heterozygous for Thy1::ChR2) led to similar findings of activity-dependent myelination with functional modulation (Gibson et al., 2014).

Extrasynaptic neurotransmitter release from neurons is known to modulate purinergic and glutamatergic signaling in glia (Stevens et al., 2002; Kukley et al., 2007; Ziskin et al., 2007; Thyssen et al., 2010; Wake et al., 2011). Extrasynaptic glutamate release from DRG neuronal cultures is vesicular, whereas ATP release appears to be non-vesicular (Wake et al., 2011). Sensory neuronal activity activates the PI3K/Akt/CREB pathway in SCs by an ATP-dependent mechanism, possibly involving P2Y purinergic receptors, which results in an inhibition of the proliferation and differentiation of SCs (Stevens and Fields, 2000). On the contrary, priming by adenosine, not ATP, promotes OPC differentiation and subsequent myelination of DRG in co-culture studies (Stevens et al., 2002). The pro-myelinating features of purinergic signaling have been found to operate predominantly through the OPC somata, which is not in direct contact with the axon underneath (Wake et al., 2011). Previous studies with neuron-OPC co-cultures hinted towards the existence of soluble signals from neurons that could affect the way OPCs regulate myelin biogenesis (Simons and Trajkovic, 2006). This work found that neuronal signals reversed the cholesterol-dependent endocytosis within OPCs and triggered the fusion of PLP/DM20 (compact intermodal myelin proteins) carrying late endosomes/lysosomes (Simons and Trajkovic, 2006). DRG:OPC co-culture studies revealed that glutamate release from axons forms the main hub for axon-glial signaling through its interaction with metabrotopic (mGluR) and ionotropic (NMDR) glutamate receptors on the OPC processes (Wake et al., 2011). More recent studies have highlighted the role that AMPA/Kainate glutamate receptors play in regulating OPC response to vesicular glutamate release from axons/*de novo* synapses (Fannon et al., 2015; Gautier et al., 2015). In DRG:OPC co-cultures, electrical stimulation of neurons produced an increased clustering of phosphorylated Fyn kinase at cholesterol-rich micro domains on OPCs, as visualized by the transferrin receptor (TfR; Wake et al., 2011). Phosphorylated Fyn kinase has been reported to, in turn, phosphorylate heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2), which is located within the OPC processes. Fyn phosphorylation of hnRNPA2 releases the repression on MBP mRNA, making it available for local translation (White et al., 2008). Indeed, neuronal activity enhances the local translation of MBP in the OPC processes (Wake et al., 2011).

There is emerging evidence that the activity-dependent regulation of OPC programming and their subsequent ability to myelinate axons might have many more players than previously thought. Intriguingly, in DRG-OPC co-cultures, NRG (NRG1β, soluble) and BDNF were able to switch the OPC myelination program from activity-independent to activity-dependent (Lundgaard et al., 2013). N-methyl-D-aspartate receptors (NMDA) on OPCs mediate NRG-induced OPC maturation and myelination (Lundgaard et al., 2013). The activity-dependent myelination program in OPCs, in the presence of NRG, was inhibited by an antibody that blocked β1 integrin (Lundgaard et al., 2013). Additionally, sodium-dependent glutamate transporters (excitatory amino acid transporters, EAAT1/2/3) can mediate glutamate signaling via CaMKIIβ, to modulate OPC morphology and myelin biogenesis (Martinez-Lozada et al., 2014). This highlights that adaptive myelination could be further subjected to *cis*-regulation on myelinating glia.

Studies have also suggested that activity-dependent axon-glial/neuron-glial communication could be mutual. Neuronal activity can promote Rab35-mediated exosome release from OPCs, in an AMPA/NMDA-dependent manner. Neurons internalize these exosomes by taking them up via axonal and somatodendritic compartments (Fruhbeis et al., 2013). The exosomes contain myelin component proteins (e.g., PLP) and heat shock proteins, which provide ER and starvation protection to neurons. Neuronal activity has also been identified to directly regulate axonal growth by modulating GAP-43 transcription and post-translational regulation, in addition to altering key signaling pathways (e.g., Notch) that might have a profound impact on regeneration and neuropathic pain post-injury (Cantallops and Routtenberg, 1999; Howe, 2003; Alberi et al., 2011). Neuronal activity regulates neuronal notch signaling in an Arc/Arg3.1-dependent manner (Sestan et al., 1999; Alberi et al., 2011). In adult $NG2^+$ OPCs, neuronal activity was also reported to promote ADAM10 dependent cleavage of NG2, one of the extrinsic inhibitory factors for axonal regeneration post-injury (Dou and Levine, 1994; Ughrin et al., 2003; Sakry et al., 2014).

Taken together, these findings suggest that axon-glia communication is dynamic, offering a potential untapped target for remyelination therapeutics. The molecular machinery in adaptive myelination includes those responding to both

paracrine (NRG1 and neurotransmitter) as well as local signaling (β1 integrins). Further experiments to understand the precise molecular nodes required for the provision of an on-demand stimulation of the myelination program remain to be undertaken.

SUMMARY

Nervous system injury, such as trauma to the spinal cord, poses a challenge to the intricately laid myelin structure surrounding axons. The loss of the myelinating glia or myelin damage leads to eventual myelin degradation and denuding of the axon, then axonal retraction of cut axons or conduction block within those spared, but demyelinated axons (Olby and Blakemore, 1996; Grossman et al., 2001; Guest et al., 2005; Hagg and Oudega, 2006; Lytle and Wrathall, 2007; Ek et al., 2012; Seidl, 2014). Axon-glial communications during the myelination process affect two critical decisions in axonal growth. The first of these are the signals for ensheathment and myelination of the axon that originate in axons themselves, while the second are signals that inhibit longitudinal growth to promote radial growth of the axon that originate from glia. Studies have highlighted the importance of PI3K/Akt in the myelination program and ERK/p38MAPK in axonal growth. However, accumulating evidence also suggests that outcomes of Akt/MAPK signaling cascades can be tremendously influenced by various *cis* and *trans* regulators on axon-glial surfaces, myelin components and co-activated signaling pathways. In addition, neuronal activity regulates the nature of axon-glial communication. Multiple molecules, canonically known to be important for myelination and axonal growth (e.g., notch, NRG, integrins and L1-CAM etc.) respond actively to neuronal activity. On certain occasions, surface ligands themselves can alter the downstream canonical signaling. In summary, various axon-glial communications regulate the myelination program and axonal cytoskeleton, by modulating the critical nodes of intracellular signaling pathways and the neuron-glia transcriptional network. This highlights a need for combinatory approaches that modulate both surface axon-glial communication and intracellular signaling to effectively control the process of axon growth and myelination.

There is an enormous need to revisit axon-glial signaling in clinically relevant nervous system injury models to generate an index of perturbed pathway components and axon-glial surface molecules. Specifically, a comprehensive understanding of the signaling pathways that are mediated through myelin components, both intact and debris, in the regulation of radial axon growth is needed. Further investigation into how these signals intersect with those pathways implicated in the initiation and elongation of axons during longitudinal growth, may offer unique opportunities for targeting the temporal regulation of endogenous and exogenous (implanted) glia interactions, as well as their communication with the injured axon to maximize axon growth to appropriate targets. Given the amount of glial cells the regenerating axons encounter in an injury scenario, it would therefore be appropriate to negatively target glial-derived axon growth inhibitory signals, and positively amplify longitudinal axon growth promoting signals for effective regeneration. Understanding this critical network holds the key to prime selectively the myelination program or to promote axonal growth.

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Flipping the transcriptional switch from myelin inhibition to axon growth in the CNS

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Poor regeneration of severed axons in the central nervous system (CNS) limits functional recovery. Regeneration failure involves interplay of inhibitory environmental elements and the growth state of the neuron. To find internal changes in gene expression that might overcome inhibitory environmental cues, we compared several paradigms that allow growth in the inhibitory environment. Conditions that allow axon growth by axotomized and cultured dorsal root ganglion (DRG) neurons on CNS myelin include immaturity (the first few postnatal days), high levels of cyclic adenosine mono phosphate (cAMP), and conditioning with a peripheral nerve lesion before explant. This shift from inhibition to growth depends on transcription. Seeking to understand the transcriptome changes that allow axon growth in the CNS, we collaborated with the Marie Filbin laboratory to identify several mRNAs that are functionally relevant, as determined by gain- and loss-of-function studies. In this Perspective, we review evidence from these experiments and discuss the merits of comparing multiple regenerative paradigms to identify a core transcriptional program for CNS axon regeneration.

Keywords: DRG, mRNA expression, axon growth, IL-6, metallothionein, SLPI

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Regenerative Paradigms to Identify Genes

To boost axon growth in the Central Nervous System (CNS), one strategy is to alter the CNS environment, to make it more conducive to growth by eliminating growth inhibitors or by adding a growth substrate, such as cells or material scaffolds. The other general approach is to increase the regenerative potential of neurons so that they may be able to grow in spite of the inhibitory environment. We considered the interplay of these two factors by asking which changes in gene expression could allow axonal regrowth in the inhibitory CNS environment. This Perspective is intended to review our approach and is not intended as a comprehensive review of regeneration-associated genes, which has been done by others, including in this issue (Ma and Willis).

One of the most actively investigated paradigms of regeneration is the conditioning lesion model. Axotomy of the peripheral dorsal root ganglion (DRG) axon normally results in regeneration while injury of the central axon does not. If the peripheral branch is injured first, however, and the central axon is injured 1 day to 2 weeks afterwards, the central axons regenerate to a much greater extent (e.g., McQuarrie and Grafstein, 1973; Richardson and Issa, 1984; Chong et al., 1999; Neumann and Woolf, 1999). The peripheral conditioning lesion induces changes in the axonal growth capacity of the injured neuron. The change in axonal growth state is likely due to induction of gene products associated with regenerative function,

often called regeneration-associated genes or RAGs (Lankford et al., 1998). Smith and Skene (1997) demonstrated that cultured DRGs, which normally extend short, highly branched axons, will extend long, sparsely branched axons after a peripheral lesion. The switch from "arborizing" to "elongating" growth requires transcription and is blocked by addition of the transcription factor IIH (TFIIH)-associated protein kinase inhibitor 5,6-Dichloro-1-β-D-ribofuronosylbenzimidozole (DRB; Yankulov et al., 1995; Smith and Skene, 1997).

The conditioning lesion paradigm was used as an early strategy to screen for candidate gene products associated with re-growth. A prototypical RAG is GAP43. GAP43 was originally identified in a two-dimensional protein electrophoresis comparing DRGs with and without sciatic nerve injury (Skene and Willard, 1981). GAP43 is primarily localized to the growth cone and is elevated in other growth states, such as during development (Skene, 1989; Benowitz and Routtenberg, 1997), and recovery of function after injury is correlated with induction of GAP43 (Skene and Willard, 1981; Skene, 1989; Gispen et al., 1991; Plunet et al., 2002). Furthermore, overexpression of GAP43 and cytoskeleton-associated protein-23 (CAP23, encoded by the LOC10910172 gene in rat) increased the regeneration of dorsal column neurons after spinal cord injury (Bomze et al., 2001).

Based on this early success, several groups screened neurons after peripheral nerve lesion to isolate additional, putative RAGs. Techniques to isolate these changes include differential display (Kiryu et al., 1995; Su et al., 1997; Kim et al., 2001; Schmitt et al., 2003), expressed-sequence-tag (Tanabe et al., 2000), subtractive hybridization (Mladinic et al., 2005), and microarray (Fan et al., 2001; Bonilla et al., 2002; Costigan et al., 2002). Modeling the utility of microarrays for gene discovery, Bonilla and colleagues compared gene expression of mouse DRGs with and without nerve injury and found several genes whose expression differed between the groups (Bonilla et al., 2002). This group then showed that one of the gene products, small proline-rich repeat protein 1A (SPRR1A), when overexpressed, co-localized with actin in the growth cone and augmented axonal growth, even on inhibitory substrates. Reduction of SPRR1A restricted neurite outgrowth *in vitro*. This work highlights the utility of this approach for finding novel RAGs.

Another paradigm used to explore CNS regeneration is development. While adult mammalian CNS axons demonstrate little to no regeneration after injury, axons of young neurons may regrow (Bates and Stelzner, 1993; Hasan et al., 1993). A key event in the developmental loss of regenerative capacity is the postnatal myelination of long tract fibers. Evidence for this hypothesis includes experiments showing that delaying onset of myelination through immunological means extends the permissive period for regeneration (Keirstead et al., 1992, 1997). However, the loss of regenerative potential is due not only to the presence of myelin but also to the responsiveness of neurons to myelin. In particular, many types of neurons change their response to the myelin component myelin-associated glycoprotein (MAG), switching from growth promotion to inhibition during development (McKerracher et al., 1994; Mukhopadhyay et al., 1994; DeBellard et al., 1996). In DRGs this switch from promotion to inhibition occurs in a short period of time at postnatal day 3–4 (Johnson et al., 1989; Mukhopadhyay et al., 1994; DeBellard et al., 1996). A similar rapid decline in growth is seen in DRGs plated on myelin, but not those plated on the permissive substrate polylysine, at P2–3 (Cai et al., 2001).

Filbin and colleagues have shown that cyclic adenosine mono phosphate (cAMP) levels increase with conditioning lesion (Qiu et al., 2002) and decrease during development (Cai et al., 2001), paralleling regenerative potential. Treatment with cAMP increases the ability of older neurons to grow on myelin (Cai et al., 2001). The strength of the *in vitro* effects are similar to that of a conditioning lesion (Qiu et al., 2002), and intraganglionic administration of cAMP can mimic the effect of the conditioning lesion on dorsal column axon growth (Neumann et al., 2002; Qiu et al., 2002). Administration of the protein kinase A (PKA) inhibitor H89 blocks the growth of previously lesioned neurons (Qiu et al., 2002) or postnatal day 1 (P1) neurons on myelin, and the PKA inhibitor KT5720 decreases the number P2–3 corticospinal tract axons that grow into an embryonic tissue graft (Cai et al., 2001). The Filbin lab also showed that the increased growth after administration of cAMP depends on transcription, and they implicate the gene arginase-1 as an indispensable RAG in this system (Cai et al., 2002). It is not known whether exogenous cAMP completely recapitulates the regenerative capacity of DRG neurons early in development or following conditioning lesion, so we investigated all three methods to find genes regulated in common in all three models.

Thus, these studies probed three robust paradigms for CNS regeneration: young developmental stage, conditioning lesion and cAMP administration. All depend on cAMP signaling (as evidenced by blocking the effect with PKA inhibition), and both conditioning lesion and direct application of cAMP require transcription to activate outgrowth. All three paradigms are carried out in rat DRGs, cells that survive axotomy and can be easily cultured (Coggeshall et al., 1997). We examined gene expression differences between neurons with high growth capacity and those with low capacity to grow in a CNS environment. We hypothesized that gene expression differences that are in common between each of these paradigms would represent common and important RAGs.

Genes associated with regeneration may function by changing their expression levels either up or down. However, most previously-defined RAGs have increased levels in high growth states (e.g., GAP43, SPRR1A, and tubulin isoforms). The approach we took to isolate common RAGs, therefore, was one comparing the genes that were increased with cAMP treatment and conditioning lesion and decreased during development. These changes correspond to the changes in cAMP levels noted by the Filbin group in each of these paradigms (Cai et al., 2001; Qiu et al., 2002). Therefore, we were most interested in the subsets of genes with increased expression in the cAMP and conditioning lesion paradigms or decreased expression during development. Results identified a large number of genes (223) that were altered in the predicted ways by one or more of

the regeneration paradigms. We were surprised, however, that there was little overlap in the candidate RAGs (7 total). This suggests the different paradigms that allow axon growth in the CNS environment may achieve regeneration through parallel mechanisms.

Candidate Regeneration-Associated Genes

To validate the candidate genes, we first focused on comparison of DRGs treated with cAMP (at 18 h) with untreated DRGs. We targeted the validation on genes common to the three regeneration paradigms. We also included a few genes whose expression was strikingly divergent between the paradigms. We compared changes in gene expression by DRGs with and without exposure to cAMP for 18 h using both microarrays and quantitative Polymerase Chain Reaction (qPCR), which has a greater dynamic range. Microarray design and methods were described previously (Carmel et al., 2004). Selected results are shown in **Figure 1**. The full results of the microarrays can be found at NIH GEO with accession numbers GSE69466 and GSE69467.

The most striking finding was a large increase in Interleukin-6 (IL-6) expression. At 18 h, IL-6 increased 15-fold in DRG neurons treated with cAMP compared with untreated DRG, as measured by qPCR. In addition, the levels of IL-6 mRNA dramatically increased (77-fold) two days after lesion compared to those without injury. While IL-6 had the greatest change of any transcript for both cAMP treatment and conditioning lesion, IL-6 mRNA levels did not change significantly during the first five postnatal days. Due to the magnitude of the gene expression changes with cAMP and conditioning lesion, we pursued IL-6 as a possible RAG.

Initially described for its role in inflammation and other immune functions, IL-6 is part of the neuropoietic family of cytokines. This family includes ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M, cardiotrophin-1, and interleukins 6 and 11. These molecules bind to specific cytokine type 1 receptors, but all members require the common receptor gp130 for signal transduction. To determine if the changes in IL-6 with cAMP and conditioning lesion were limited to IL-6 or may involve other members of the gp130 family, we assayed the levels of mRNA for the cytokines and their receptors (**Figure 2**). For both 18 h of cAMP treatment (**Figure 2A**), and for the conditioning lesion (shown at several time points in **Figure 2B**), the predominant change is in IL-6, with more modest effects in other gp130 family members.

Subsequent experiments carried out at the Filbin laboratory demonstrated that IL-6 is a functional RAG (Cao et al., 2006). In addition to mRNA induction, IL-6 protein is also

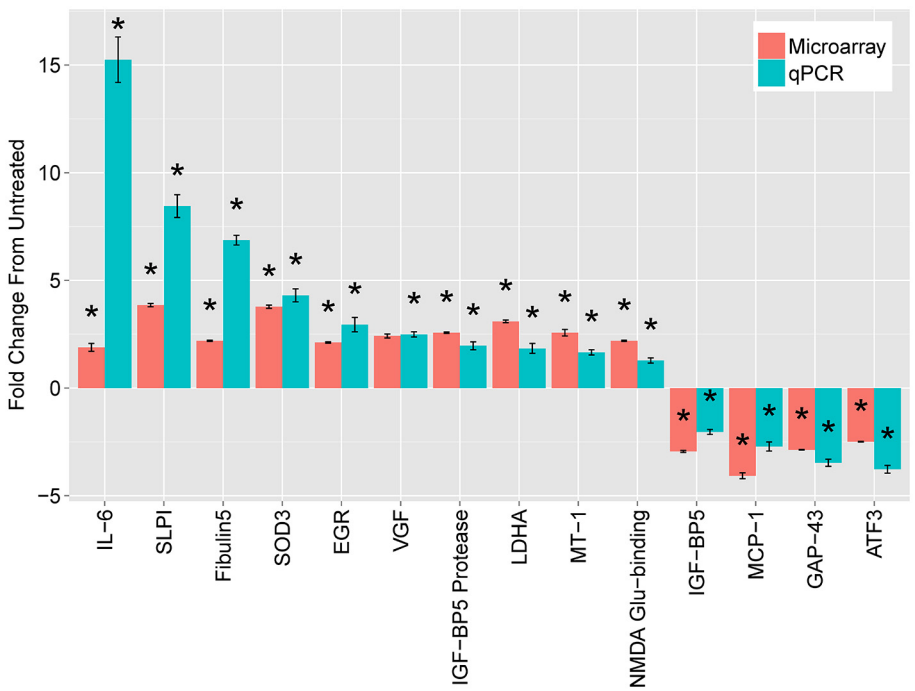

FIGURE 1 | Leading mRNA changes at 18 h following dbcAMP treatment. The top mRNAs identified by microarray (red bars), compared with quantitative real-time PCR (qPCR, blue bars). Cultured, dissociated cells from L4 and L5 rat DRGs were treated with or without 1.5 mM dbcAMP for 18 h, harvested, and used to extract total cellular RNA. For microarrays, treated samples were labeled with one dye (Cy5) and untreated samples with another (Cy3), which were mixed and hybridized to a spotted-oligo, glass-slide array (Carmel et al., 2004). The dynamic range of the two-color microarray technique is reduced compared with qPCR and more recent technologies such as RNA-seq. Results are mean fold-change ± SEM, $n = 3$, $^*p < 0.05$ Student's t-test.

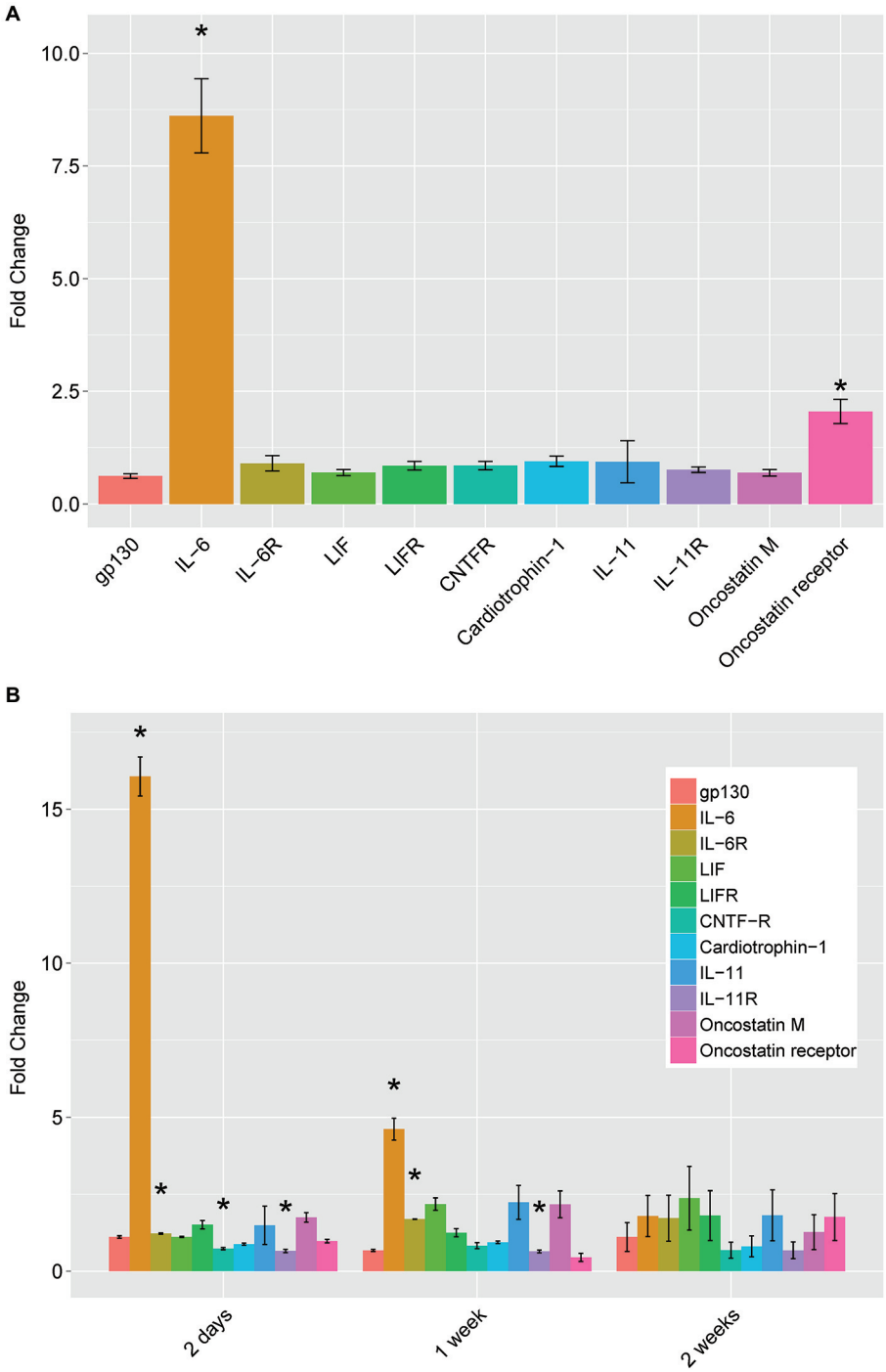

FIGURE 2 | mRNA levels of IL-6 family neuropoietic cytokines and their receptors by qPCR. (A) dbcAMP treatment for 18 h. Dorsal root ganglion (DRG) cultures were prepared and treated as described in **Figure 1** and total cellular RNA was prepared and assayed by qPCR. **(B)** Conditioning lesion at three time points. The sciatic nerve was surgically transected mid-thigh at postnatal day 20–24. After the times indicated, L4 and L5 DRG were removed and processed for total cellular RNA and assayed by qPCR. Results are mean fold-change SEM, $n = 3$, $^*p < 0.05$ Student's t-test.

induced in DRGs and hippocampal neurons grown on inhibitory substrates and treated with cAMP. Administration of IL-6 protein mimics the effect of cAMP, reversing the growth inhibition of both DRGs and hippocampal neurons grown on inhibitory substrates. Importantly, exogenous IL-6 did not cause increased neurite outgrowth of DRGs grown on the permissive

substrate poly-L-lysine. This suggests that IL-6 specifically reverses inhibition and is not due to a general trophic effect. Importantly, intrathecal delivery of IL-6 to DRG neurons blocks inhibition by myelin when they are explanted *in vitro* and *in vivo*, effectively mimicking the conditioning lesion.

However, the effects of cAMP and the conditioning lesion did not depend on IL-6. Blocking IL-6 signaling did not affect the ability of cAMP to overcome myelin inhibitors. In addition, IL-6-deficient mice respond to a conditioning lesion as effectively as wild-type (wt) mice. These data suggest that IL-6 can mimic both the cAMP effect and the conditioning lesion effect but is not an essential component of either response. IL-6 has also been found to promote the expression of other RAGs and to promote neurite outgrowth of cortical neurons (Yang et al., 2012). That IL-6 is sufficient but not necessary for axon regeneration fits with a larger point from these studies: the regeneration paradigms seem to act through parallel gene expression programs each of which is effective but not reliant on the others.

The second largest change in gene expression after cAMP administration was in secretory leukocyte protease inhibitor (SLPI), which was also identified in a screen of gene expression changes after spinal cord injury (Urso et al., 2007). SLPI is a serine protease inhibitor belonging to the family of whey acidic protein motif-containing proteins (Thompson and Ohlsson, 1986; Eisenberg et al., 1990). Gain- and loss-of-function studies in the Filbin lab show an essential role of SLPI in axon regeneration (Hannila et al., 2013). SLPI can overcome inhibition by CNS myelin and significantly enhance regeneration of transected retinal ganglion cell axons in rats. Furthermore, regeneration of dorsal column axons does not occur after a conditioning lesion in SLPI null mutant mice, indicating that expression of SLPI is required for the conditioning lesion effect. Thus, SLPI is both sufficient and necessary for axon regeneration in the damaged CNS.

The final putative RAG tested by the Filbin lab is metallothionein (MT). MTs are small cysteine-rich, zinc-binding proteins expressed throughout the CNS. Two closely related isoforms, MT-I and MT-II, when administered together, promote neurite outgrowth in adult DRG's in the presence of myelin inhibitors (Siddiq et al., 2015). Likewise, a single intravitreal injection of MT-I/II after optic nerve crush promotes axonal regeneration. In contrast, adult DRGs from MT-I/II-deficient mice extend significantly shorter processes on MAG compared to wt DRG neurons, and regeneration of dorsal column axons does not occur after a conditioning lesion in MT-I/II-deficient mice. These experiments suggest that MT, like SLPI, is both necessary and sufficient for axon regeneration in the CNS.

Additional Candidate Genes

Several additional transcripts were identified but we have not yet pursued their roles in regeneration. For example, VGF encodes a secretory-peptide precursor involved in plasticity and metabolism. VGF is modulated *in vivo* by paradigms which lead to neurotrophin induction, synaptic remodeling and axonal sprouting (Snyder et al., 1998a). VGF shows greatest expression during times of axonal outgrowth and synaptogenesis in the developing brain (Lombardo et al., 1995; Benson and Salton, 1996; Snyder et al., 1998b). Mouse VGF knockouts (KO) are small, hypermetabolic, and have reduced leptin levels, suggesting a prominent role in energy metabolism (Hahm et al., 1999). These findings provide evidence for a neurotrophic role for VGF, in addition to its function as neuroendocrine molecule.

The cAMP responsive event modulator (CREM) gene encodes both antagonists and activators of the cAMP-dependent transcriptional response by alternative splicing (for review see Della Fazia et al., 1997). CREM is inducible by activation of the cAMP signaling pathway with the kinetics of an early response gene. An alternatively splice repressor form, inducible cAMP early repressor (ICER), may be important for the transient nature of cAMP-induced gene expression. The role of this molecule in regenerative signaling remains to be determined.

We found two genes, ATF3 and GAP43, whose expression differed markedly between cAMP treatment and conditioning lesion. These differences suggest possible differences in mechanism between these pro-regenerative paradigms. ATF3 is part of the activating transcription factor/CREB family of transcription factors. The gene encodes a leucine zipper transcription factor (Hsu et al., 1991) that is increased by cellular stress (reviewed in Hai et al., 1999). ATF3 is strongly induced by sciatic nerve lesion in dorsal root ganglia and spinal motor neurons (Tsujino et al., 2000) and in the geniculate ganglion after chorda tympani injury (Tsuzuki et al., 2002). ATF3 represses transcription as a homodimer (Chen et al., 1994) and activates transcription as a heterodimer with cJun (Hai and Curran, 1991; Chu et al., 1994). Jun has been investigated as a RAG (Broude et al., 1997; Houle et al., 1998; Lerch et al., 2014), and the possibility of cJun and ATF3 acting as inducers of RAG expression has been validated in DRG (Seijffers et al., 2007). Whether ATF3 might be acting as a transcriptional activator or repressor in these models must be investigated further.

Surprisingly, GAP43 expression decreased in our cAMP model (**Figure 1**). GAP43 is the prototypical RAG and is often used as a marker for regenerating axons (Benowitz et al., 1990). Schreyer and colleagues have previously shown that GAP43 may be repressed by cAMP (Andersen et al., 2000a,b). In a search for the signal that causes DRG neurons to downregulate GAP43 after they reach their targets, this group found decreases on both the mRNA and protein levels in cAMP-exposed DRG neurons. Interestingly, exposure of rat cortical neurons to spinal cord extract, but not extract from cerebellum or muscle, was sufficient to decrease GAP43, and this repression was adenyl cyclase-dependent (Karimi-Abdolrezaee and Schreyer, 2002). The finding of increased neurite outgrowth associated with GAP43 repression challenged the widespread notion that regeneration is accompanied by GAP43 induction. This has subsequently been shown in other models of regeneration, such as following cJun activation of axon growth (Lerch et al., 2014).

Implications

These studies produced three surprising results. First, we found little overlap in the gene expression changes produced by the

three regeneration paradigms we studied. Although the neurons in each of these models all express high levels of cAMP in the regenerative state, the changes in transcription differ markedly. Rather than uncovering a core regeneration program that allows a switch from inhibition to growth in the CNS environment, we found different transcriptional changes, each leading to a similar regenerative phenotype. This suggests the existence of several transcriptional programs with overlapping function.

Second, we found that the first three transcripts studied are each sufficient to elicit regeneration *in vivo*. In addition, both SLPI and MT are necessary for the regeneration induced by cAMP or conditioning lesion. This remarkable "hit rate" could be serendipity, or it could tell us that regeneration may not require a coordinated program requiring multiple gene products. It could be that IL-6, SLPI, and MT all create a coordinated cellular response of several pathways. But it is heartening that regeneration can be produced with each of these varied approaches as monotherapy. Whether the effects of these therapies in combination would be more effective is a critical question for future study. In addition, in the animal studies done so far, no loss of function has been done in CNS resident neurons. But these experiments are more difficult since CNS neurons do not regeneration spontaneously, thus requiring the use of a regeneration competent background (e.g., PTEN deletion, as has been done for DLK with the optic nerve injury model; (Watkins et al., 2013). Finally, the function of the regenerated axons has not yet been tested by measures of physiology or behavior.

Finally, the gene for GAP43, critical to regeneration after conditioning lesion, is strongly downregulated by cAMP treatment. This evidence argues strongly that these different regenerative paradigms act through diverse transcriptional programs. Rather than a single path to successful regeneration, many possible transcriptional changes may lead to re-growth. Mapping these paths will be critical to flipping the transcriptional switch from inhibition to growth in the CNS.

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Looking downstream: the role of cyclic AMP-regulated genes in axonal regeneration

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Elevation of intracellular cyclic AMP (cAMP) levels has proven to be one of the most effective means of overcoming inhibition of axonal regeneration by myelin-associated inhibitors such as myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte myelin glycoprotein. Pharmacological manipulation of cAMP through the administration of dibutyryl cAMP or rolipram leads to enhanced axonal growth both *in vivo* and *in vitro*, and importantly, upregulation of cAMP within dorsal root ganglion neurons is responsible for the conditioning lesion effect, which indicates that cAMP plays a significant role in the endogenous mechanisms that promote axonal regeneration. The effects of cAMP are transcription-dependent and are mediated through the activation of protein kinase A (PKA) and the transcription factor cyclic AMP response element binding protein (CREB). This leads to the induction of a variety of genes, several of which have been shown to overcome myelin-mediated inhibition in their own right. In this review, we will highlight the pro-regenerative effects of arginase I (ArgI), interleukin (IL)-6, secretory leukocyte protease inhibitor (SLPI), and metallothionein (MT)-I/II, and discuss their potential for therapeutic use in spinal cord injury.

Keywords: cyclic AMP, transcription, arginase I, interleukin-6, SLPI, metallothionein, regeneration, spinal cord injury

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Cyclic AMP: Overcoming Inhibition by Inducing Transcription

The identification of myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte myelin glycoprotein as inhibitors of neurite outgrowth was a turning point in the study of axonal regeneration, providing compelling evidence that CNS myelin proteins contributed to regenerative failure after injury. Not surprisingly, their discovery sparked great interest in developing experimental approaches to overcome this inhibition, and one approach in particular has provided unique insight into the intrinsic mechanisms that regulate axonal regeneration. Soon after the conditioning lesion effect was described by Neumann and Woolf (1999), the laboratory of Dr. Marie T. Filbin demonstrated that elevation of intracellular cyclic AMP (cAMP) levels through the administration of dibutyryl cAMP (dbcAMP) reverses inhibition of neurite outgrowth by MAG and CNS myelin (Cai et al., 1999). In subsequent studies it was shown that elevation of cAMP underlies the conditioning lesion effect, as cAMP levels became elevated in dorsal root ganglia (DRG) in response to a sciatic nerve lesion, and injection of dbcAMP directly into DRG replicated the effects of a conditioning lesion on dorsal column axon regeneration (Neumann et al., 2002; Qiu et al., 2002). The most important implication of these findings was that cAMP

activates an intrinsic pro-regenerative program within the neuron that allows it to overcome MAG- and myelin-mediated inhibition at the molecular level.

Experiments aimed at characterizing this mechanism soon revealed that the effects of cAMP were dependent on the activation of protein kinase A (PKA), as administration of the PKA inhibitor KT5720 abolished the ability of dbcAMP to overcome inhibition by MAG (Qiu et al., 2002). One of the primary downstream targets of PKA is the transcription factor cyclic AMP response element binding protein (CREB), which is phosphorylated at Ser133 and recruits the co-activator CREB-binding protein (CBP)/p300, which in turn leads to initiation of transcription by RNA polymerase II (Mayr and Montminy, 2001). Interestingly, there is evidence that like CREB, CBP/p300 activity can be regulated by PKA-mediated phosphorylation (Vo and Goodman, 2001), and other kinases such as cyclin E/Cdk2, calcium/calmodulin kinase IV, and mitogen-activated protein kinase have also been shown to play a role in the regulation of CBP/p300 (Vo and Goodman, 2001). CBP also facilitates transcription through its histone acetyltransferase activity, and it has been shown that histone hyperacetylation mediated through inhibition of histone deacetylases enhances neurite outgrowth for cerebellar neurons plated on CNS myelin substrates (Gaub et al., 2010). This response is dependent on CPB/p300 activity, as siRNA knockdown of this protein abolished the ability of neurons to overcome inhibition by CNS myelin, and it was also shown that CPB/p300 expression is suppressed in the presence of myelin (Gaub et al., 2010). Lastly it was shown that CPB/p300, together with p300-CBP-associated factor (P/CAF), induces acetylation of p53 (Gaub et al., 2010), which has been shown to form a signaling complex with CPB and P/CAF and promote transcription of genes with known roles in promoting axonal growth (Di Giovanni et al., 2006; Tedeschi et al., 2009). The data presented in these studies indicated that CBP and CREB-mediated transcription play significant roles in overcoming inhibition by CNS myelin, and these findings supported those reported in an earlier study by Gao et al. (2004) which showed that induction of CREB-mediated transcription is required for cAMP to promote neurite outgrowth in the presence of MAG, and that expression of constitutively active CREB in DRG neurons is sufficient to enhance regeneration of transected dorsal column axons. Having shown that the effects of cAMP were transcription-dependent (Cai et al., 2002; Gao et al., 2004), the work of the Filbin lab logically progressed to the identification of the genes that were responsible for mediating axonal regeneration. In this review, we will highlight the four genes that have been studied to date: arginase I (ArgI), interleukin (IL)-6, secretory leukocyte protease inhibitor (SLPI), and metallothionein (MT), and discuss the collective role of these and other genes in enhancing axonal regeneration.

Arginase I

The first cAMP-regulated gene product to be investigated in the context of MAG and myelin-mediated inhibition of neurite outgrowth was ArgI, the rate-limiting enzyme of polyamine synthesis (Cai et al., 2002). ArgI catalyzes the hydrolysis of arginine to ornithine, which is converted to putrescine by ornithine decarboxylase, and putrescine in turn is hydrolyzed to spermidine and spermine (Munder, 2009). In cerebellar granule neurons (CGN) treated with dbcAMP, levels of ArgI mRNA and protein were visibly increased and this was accompanied by a corresponding increase in putrescine synthesis. To assess the importance of increased ArgI expression in enhancing neurite outgrowth, ArgI was overexpressed in CGN using adenoviruses and this allowed these neurons to extend neurites on monolayers of MAG-expressing Chinese hamster ovary (CHO) cells, which indicated that ArgI activity was sufficient to mediate this response. Similarly, the role of polyamine synthesis was investigated by administering two compounds that block the synthesis of these molecules: N (omega)-hydroxynor-L-arginine 5 (NOHA), an inhibitor of ArgI (Boucher et al., 1994), and DL-2-difluor-omethyl-ornithine (DFMO), which inhibits ornithine decarboxylase (Slotkin et al., 1982). Treatment with either NOHA or DFMO abolished the ability of dbcAMP to overcome inhibition by MAG and myelin, and this in turn could be reversed by the addition of putrescine to the cultured neurons, which suggested that polyamines were directly responsible for promoting neurite outgrowth in the presence of myelin-associated inhibitors. The first evidence to support this was provided by experiments showing that putrescine can overcome MAG inhibition either through direct addition or through priming, a procedure in which the neurons were treated overnight with putrescine prior to transferring them to CHO cell monolayers (Cai et al., 2002). It was subsequently shown that putrescine must be converted to spermidine to have this effect, as treatment with the spermidine synthase inhibitor bis-cyclohexylammonium sulfate (BCHS) blocked the ability of putrescine to enhance neurite outgrowth (Deng et al., 2009). Priming neurons with spermidine led to dose-dependent increases in neurite outgrowth in the presence of either MAG or myelin, and more importantly, regeneration of retinal ganglion cell axons in the injured optic nerve was significantly increased following a single intravitreal injection of 20 μM spermidine (Deng et al., 2009). This conclusively demonstrated that the products of ArgI activity can directly promote axonal regeneration *in vivo*.

Interleukin-6

Expression of the pleiotrophic cytokine IL-6 was found to be strongly induced by dbcAMP in DRG neurons in a microarray performed in collaboration with Jason Carmel, then a member of the laboratory of Dr. Ronald Hart (Cao et al., 2006). IL-6 is a member of the gp130 cytokine family, and other members of this family such as leukemia inhibitory factor and ciliary neurotrophic factor have been shown to have roles in promoting axonal regeneration (Cafferty et al., 2001; Müller et al., 2007). Delivery of IL-6 was found to promote both neurite outgrowth in the presence of myelin-associated inhibitors and *in vivo* axonal regeneration in the injured spinal cord (Cafferty et al., 2004; Cao et al., 2006; Leibinger et al., 2013). Retinal ganglion cells (RGCs)

can overcome inhibition by myelin following IL-6 treatment and IL-6 has the added benefit of being neuroprotective, which allowed intravitreal injection of IL-6 to promote axonal regeneration after optic nerve crush (Leibinger et al., 2013). Though this pro-regenerative function is an unexpected finding for a pro-inflammatory cytokine, it brings up the intriguing point that restricted inflammation maybe a positive factor in promoting axonal growth. Though little has been elucidated about IL-6's mechanism of action, intravitreally injected IL-6 was shown to elevate phosphorylation of signal transducer and activator of transcription (STAT)-3 in the ganglion cell layer (Leibinger et al., 2013). STAT-3 activation triggers neurite outgrowth in both PC-12 and Neuro2A cells (Ihara et al., 1997; Zorina et al., 2010) and overexpression of STAT-3 in RGCs promotes regeneration when an optic nerve crush is performed in combination with lens injury (Leibinger et al., 2013). The latter suggests that STAT-3 alone is not sufficient to promote axonal regeneration and that additional stimuli are required. Hence, further elucidation of the mechanisms and downstream pathways involved with IL-6 dependent axonal regeneration could lead us to potential targets that could promote regeneration without exacerbating the hyper-inflammatory response that is elicited after injury to the CNS.

Secretory Leukocyte Protease Inhibitor

The same microarray that identified IL-6 also showed that expression of SLPI was increased 3.9-fold in response to elevation of cAMP, and subsequent quantitative real-time PCR analysis of these samples revealed an 8.5-fold increase in SLPI mRNA levels (Hannila et al., 2013). These results were later confirmed in experiments that showed that SLPI mRNA levels were significantly increased following either exposure to dbcAMP or a peripheral conditioning lesion (Hannila et al., 2013). While SLPI was one of the most highly expressed genes identified in the microarray, its role promoting in neurite outgrowth was not immediately apparent, as SLPI is serine protease inhibitor best known for its anti-microbial, and anti-inflammatory functions. These include inhibiting the growth of bacteria, blocking HIV infection of monocytes/macrophages, and inhibiting the expression of the pro-inflammatory cytokines such as tumor necrosis factor α. In the CNS, elevated expression of SLPI has been observed in response to cerebral ischemia, and in a study by Wang et al. (2003), infarct volume was significantly reduced when SLPI was adenovirally overexpressed prior to middle cerebral artery occlusion. SLPI expression was also strongly upregulated following spinal cord contusion injury in mice, and administration of recombinant SLPI produced significant improvement in locomotor function, as well as increased tissue preservation and axonal density, in these animals (Ghasemlou et al., 2010). The findings of these studies have led to the hypothesis that SLPI is neuroprotective, and this has now been complemented by our work showing that SLPI has pro-regenerative effects as well.

In neurite outgrowth assays, neonatal DRG and cortical neurons treated with SLPI were able to overcome inhibition by MAG and myelin, and adult DRG neurons that received intrathecal delivery of SLPI showed enhanced neurite outgrowth in the presence of MAG compared to neurons that received infusions of sterile saline (Hannila et al., 2013). To provide definitive proof that SLPI could overcome inhibition by CNS myelin *in vivo*, a single intravitreal injection of SLPI (10 μg) was administered to adult rats immediately after optic nerve crush. When axonal regeneration was assessed 2 weeks later, there was a significant increase in axonal density distal to the lesion site in animals that received SLPI, which indicated that SLPI can promote axonal regeneration in the injured mammalian CNS.

The importance of SLPI in axonal regeneration is also demonstrated by its role in the conditioning lesion effect. When compared to wild type mice, SLPI null mutant mice displayed significantly less regeneration of dorsal column axons in response to a sciatic nerve lesion (Hannila et al., 2013). This indicated that SLPI is required for this response, and the underlying mechanism can be tied to the expression of Smad2, an intermediate in the transforming growth factor β signaling pathway that is essential for mediating inhibition by CNS myelin. Smad2 can be effectively knocked down using siRNA, leading to increased neurite outgrowth on myelin, and both dbcAMP and sciatic nerve lesions have similar effects, producing significant reductions in Smad2 levels within 18–24 h. In SLPI null mutant mice that received a sciatic nerve lesion, Smad2 expression was unaffected, which indicated that the ability of cAMP to reduce Smad2 is SLPI-dependent. It was also shown that SLPI is capable of rapidly localizing the nuclei of neurons and binding to the promoter for Smad2 with a high degree of specificity, which led us to conclude that cAMP induces expression of SLPI and that SLPI then facilitates axonal growth by blocking transcription of the Smad2 gene and reducing the amount of Smad2 available to mediate inhibition. It is therefore likely that both SLPI and Smad2 could prove to be viable targets for therapeutic intervention in spinal cord injury.

Metallothionein

MT were first identified nearly 60 years ago, are found in all cells of the body, and though they have been extensively studied, have a poorly defined physiological role. They are distinguished by their small size (approximately 6–7 kD) and abundance of cysteines (20 residues) which coordinate binding of up to seven divalent cationic metals, such as zinc, copper or cadmium (Chung et al., 2008). It is this metal binding ability that has often led to them being labeled as simple chelators of excessive non-protein bound zinc or copper. However, several reports have supported a more selective role for MT in that they could modulate the activity of zinc-dependent proteins, such as transcription factors, by either donating or removing the zinc moiety (Zeng et al., 1991). There are four isoforms of MT: MT-I, -II, -III and -IV; and of these isoforms only MT-IV is not found in the CNS. MT-I and -II are two very closely related isoforms and they are often co-expressed. It is the astrocytes that predominantly express MTs and have been shown to secrete MTs in response to injury (West et al., 2008; Miyazaki et al., 2011). Neurons also express MTs, but at levels that are 2–3 fold lower than glial cells. Extensive studies have been conducted in the

nervous system of MT-deficient mice, and these have shown that MT-deficient mice display extremely poor outcomes in models of stroke, seizures, and multiple sclerosis (Carrasco et al., 2000; Penkowa et al., 2001; Trendelenburg et al., 2002). It was through these studies that a neuro-protective role for MTs in the CNS was suggested, and it is believed that MT mediates this effect by scavenging reactive oxygen species (Penkowa et al., 2000; Kondoh et al., 2001).

In our microarray analysis, we observed a significant increase in expression of MT-I/II following either dbcAMP treatment in neonatal DRG neurons or a conditioning lesion in adult DRGs (Siddiq et al., 2015). Though MT-I/II levels have been shown to be elevated in response to divalent metals and glucocorticoids, it was intriguing to see that it was induced in response to elevated levels of cAMP. Though the promoter of MT-I/II lacks a clear cAMP response element, there are several IL-6 response elements. It is therefore possible that the cAMP-dependent increase in IL-6 levels subsequently leads to elevated expression of MT-I/II, and this is supported by the fact that IL-6-deficient mice display reduced expression of MT-I/II after injury to the CNS (Penkowa et al., 2000).

In our study, MT-I/II applied to primary neurons can overcome MAG- or myelin-mediated inhibition and MT-I/II-deficient mice have an attenuated response to conditioning lesion compared to wild-type littermates (Siddiq et al., 2015). Intravitreal injection of MT-I/II also promotes modest axonal regeneration following optic nerve crush, and this pro-regenerative effect is amplified with lens injury (Siddiq et al., 2015), which implies that a combination of MT-I/II and other regeneration-associated genes are required for robust axonal regeneration. Mechanistically, zinc chelation had no effect on myelin-mediated inhibition of neurite outgrowth, suggesting that this was not the source of MT-I/II's effects (Siddiq et al., 2015). However, MT-I/II directly inhibited the activity of α-secretase, and it also blocked MAG-induced phosphorylation of protein kinase C and activation of the small GTPase RhoA (Siddiq et al., 2015), which suggests that MT-I/II enhances axonal regeneration by interfering with the downstream signaling pathways activated by myelin-associated inhibitors.

Summary

It has become increasingly clear that there is no single pathway, protein, or drug that can promote robust axonal regeneration in the injured CNS, but our microarray analysis of the conditioning lesion effect has provided us with valuable insight into the spectrum of genes that are modulated to produce axonal regeneration. In the case of ArgI, IL-6, SLPI, and MT-I/II, each protein can overcome inhibition by myelin and promote modest axonal regeneration, but all four have uniquely different mechanisms for overcoming the inhibitory environment of the CNS (**Figure 1**). Another gene that is likely contributing to the conditioning lesion effect is brain-derived neurotrophic factor (BDNF). Like the genes identified in the microarray, BDNF expression is regulated by cAMP and CREB (Finkbeiner et al., 1997), and BDNF has been shown to significantly enhance survival of axotomized neurons

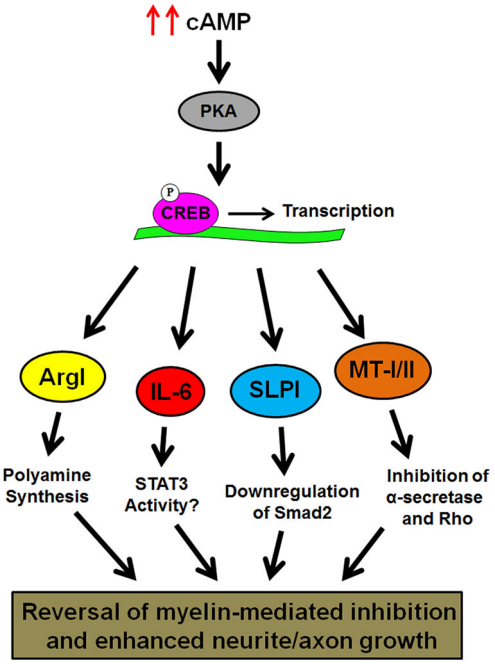

FIGURE 1 | Schematic representation of cAMP-induced gene expression that has been shown to overcome inhibition by CNS myelin. Elevation of intracellular cAMP leads to activation of protein kinase A (PKA), and transcription of the ArgI, IL-6, SLPI, and metallothioneins (MT)-I/II genes by phosphorylated CREB. The products of these genes then reverse myelin-induced inhibition of neurite outgrowth through their own distinct mechanisms.

following spinal cord injury, and promote axonal sprouting and regeneration (Kobayashi et al., 1997; Kwon et al., 2002). To develop effective treatments for spinal cord injury, it will be necessary to examine the combinatorial effects of IL-6, ArgI, SLPI and/or MT-I/II, as well as other growth-promoting agents such as BDNF, and optimize the dosages to achieve synergistic effects.

In the microarray, we also identified other genes that are upregulated in response to elevated cAMP, such as fibulin 5, superoxide dismutase 3, nerve growth factor inducible protein (VGF), and lactate dehydrogenase A (full microarray results available at http://genome.rutgers.edu/slpi/, Hannila et al., 2013), and their roles in axonal growth have not yet been explored. It should also be noted that numerous genes were significantly down-regulated, such as insulin-like growth factor-binding protein 5, monocyte chemoattractant protein-1, and intriguingly, MT-III (Hannila et al., 2013),[1] which has been suggested to be inhibitory to neurite outgrowth (Uchida et al., 1991). This suggests that cAMP may not only upregulate the expression of growth-promoting genes, but also limit the expression of genes that negatively impact axonal regeneration. By continuing to investigate the effects of these cAMP-regulated genes on axonal growth, we will advance our understanding of endogenous repair mechanisms, move

[1] http://genome.rutgers.edu/slpi/

closer to the ultimate goal of developing methods to enhance them and produce clinical benefit for patients with spinal cord injury.

Author Contributions

MS and SH conceived, wrote, and critically revised the manuscript.

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This review is dedicated to the memory of our mentor, Dr. Marie T. Filbin. Her work leaves a lasting legacy in the fields of spinal cord injury, CNS myelin biology, and axonal plasticity. We are privileged to have been members of her lab and we will never forget the many lessons she taught us.

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REVIEW
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What makes a RAG regeneration associated?

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Regenerative failure remains a significant barrier for functional recovery after central nervous system (CNS) injury. As such, understanding the physiological processes that regulate axon regeneration is a central focus of regenerative medicine. Studying the gene transcription responses to axon injury of regeneration competent neurons, such as those of the peripheral nervous system (PNS), has provided insight into the genes associated with regeneration. Though several individual "regeneration-associated genes" (RAGs) have been identified from these studies, the response to injury likely regulates the expression of functionally coordinated and complementary gene groups. For instance, successful regeneration would require the induction of genes that drive the intrinsic growth capacity of neurons, while simultaneously downregulating the genes that convey environmental inhibitory cues. Thus, this view emphasizes the transcriptional regulation of gene "programs" that contribute to the overall goal of axonal regeneration. Here, we review the known RAGs, focusing on how their transcriptional regulation can reveal the underlying gene programs that drive a regenerative phenotype. Finally, we will discuss paradigms under which we can determine whether these genes are injury-associated, or indeed necessary for regeneration.

Keywords: regeneration, regeneration associated genes, injury conditioning, cyclic AMP, transcription factors

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It likely comes as no surprise the striking dichotomy that exists between peripheral nervous system (PNS) and central nervous system (CNS) neurons following injury. While PNS neurons show robust regenerative capacity, CNS neurons exhibit negligible capacity. This difference has been known and intensively studied since the time of Ramón y Cajal, and in those years many reasons have been postulated for this fundamental difference (Ramón y Cajal, 1914). What then have we learned from nearly 100 years and hundreds of studies trying to unravel this mystery? Deservingly, the gene transcription response to axon injury has drawn considerable interest; however, its increasingly appreciated complexity poses a formidable challenge from a therapeutic perspective. Given this potential hurdle, ongoing work has given hope that if the appropriate manipulations of neuronal physiology are enacted, both the central and PNS could be efficiently repaired.

What Evidence Pointed to the Importance of Regeneration Associated Genes (RAGs)?

Regeneration of damaged axons is dependent on the neuron-intrinsic transcription of regeneration-associated genes (RAGs). Axotomy of PNS neurons induces broad and coordinated gene transcription, a response that is lacking following CNS injury

(Schreyer and Skene, 1993; Mason et al., 2002; Starkey et al., 2009; Ylera et al., 2009; Geeven et al., 2011). These differences in the ability to induce RAG expression, along with extracellular environmental factors, underlie the disparate ability of PNS and CNS axons to regenerate (Filbin, 2003).

Early observations that peripheral axon injury induced a "cell body" response that included increased neuronal mRNA and protein synthesis indicated an active process by which peripheral neurons *prepared* to regenerate axons (Lieberman, 1971; Grafstein, 1975). Along with findings that specific axonal proteins were upregulated following injury (i.e., GAP43), the idea that the expression of growth-related proteins promoted the regeneration of axons began to take hold (Skene and Willard, 1981; Skene, 1989; Tetzlaff et al., 1991). As a result of these early observations, the hypothesis formed that injury-induced gene transcription was required for axon regeneration, and importantly, raised the possibility that the expression of RAGs may confer regenerative capacity to CNS neurons.

This brought to question whether the primary driver of regenerative failure in the CNS was due to the inhibitory environment or the failure to appropriately upregulate RAGs. If the latter, it suggested that a reasonable course of action to confer regeneration capacity to the CNS was to identify and manipulate the RAGs responsible for the PNS response.

What Constitutes a RAG?

With the early evidence suggesting that the regenerative transcriptional response could be used to improve regeneration, both under permissive and non-permissive conditions, considerable effort has been directed at identifying the genes that are upregulated following injury and designing methods to modulate their expression to enhance regeneration in CNS neurons.

Several seminal observations supported the existence of neuron-intrinsic factors capable of promoting CNS regeneration. Though typically incapable of spontaneous regeneration, CNS neurons will regenerate damaged axons when provided a permissive environment. Indeed, some damaged spinal cord axons grow into transplanted peripheral nerve segments in the rat spinal cord, indicating that these CNS neurons retained the intrinsic capacity to regenerate given a permissive (or growth-stimulating) environment (David and Aguayo, 1981). Interestingly, though not all types of CNS neurons exhibit this behavior, those that could regenerate upregulate RAG expression in the presence of the graft (Anderson et al., 1998; Mason et al., 2002; Murray et al., 2011).

Manipulations that increase RAG expression in CNS can also promote regeneration of "resistant" axons into these nerve grafts. For instance, treatment with BDNF of rubrospinal neurons induces RAG expression and growth into peripheral nerve grafts, while upregulating cyclic adenosine monophosphate (cAMP) levels can increase RAG expression and allow modest CNS axon regeneration in *in vivo* CNS injury models (Kobayashi et al., 1997; Ye and Houle, 1997; Neumann et al., 2002; Qiu et al., 2002; Li et al., 2003; Storer et al., 2003; Jin et al., 2009). Indeed, cAMP is one of the few manipulations that has repeatedly been shown to drive axon regeneration in a variety of CNS injury models performed by numerous research groups.

Dorsal root ganglia (DRG) neurons have provided an important *in vivo* platform to test whether RAG induction allows regeneration of CNS axons. These sensory neurons possess pseudounipolar axons that extend in the periphery and into the spinal cord; a subset of these axons ascend the dorsal column of the spinal cord (Bradbury et al., 2000). Peripheral nerve injury (transection or crush) induces the expression of RAGs, whereas injury to the central projecting branch does not (Schreyer and Skene, 1993; Smith and Skene, 1997; Mason et al., 2002; Hanz et al., 2003; Seijffers et al., 2006; Ylera et al., 2009; Geeven et al., 2011). Intriguingly, a peripheral *conditioning* lesion enhances regeneration of proximally reinjured peripheral axons, and allows regeneration of a subsequently injured central branch (McQuarrie and Grafstein, 1973; McQuarrie et al., 1977; Oblinger and Lasek, 1984; Neumann and Woolf, 1999). These observations have led to substantial research efforts aimed at understanding this mechanism. This conditioning lesion effect was shown to be transcription dependent, indicating that the physiological induction of RAG transcription confers regenerative capacity to an otherwise regeneration-deficient axon (Smith and Skene, 1997). Together, these observations indicate that the expression of genes induced by peripheral axon injury are necessary for spontaneous regeneration and the forced expression of these (by peripheral injury) can be sufficient to drive regeneration of CNS axons.

Though many candidate RAGs have been identified, genome-wide profiling studies have provided a comprehensive view of the transcriptional changes that result from peripheral axon injury (Zigmond et al., 1998; Costigan et al., 2002; Xiao et al., 2002; Schmitt et al., 2003; Tanabe et al., 2003; Küry et al., 2004; Di Giovanni et al., 2005; Bosse et al., 2006; Stam et al., 2007; Szpara et al., 2007; Moore et al., 2009; Zou et al., 2009; Michaelevski et al., 2010; Geeven et al., 2011; Ma et al., 2011; Blesch et al., 2012). From these, it is apparent that the expression levels of thousands of genes are changed by injury. Elucidating the identity and function of intrinsic contributors to regeneration has been aided by numerous studies performed in lower vertebrates and invertebrates, which offer advantages of forward genetic screens and *in vivo* imaging, and complement those studies performed in mammals. They offer insights into how injury-induced gene expression both recapitulates and differs from pathways involved during development. For example, screening 654 conserved genes in an axotomy model of mechanosensory neurons in *C. elegans* identified clusters of genes that promote or repress axon growth. Many of these are components of pathways critical for neuronal plasticity of both development and regeneration; however, some clusters are only required for regeneration (Chen et al., 2011). This highlights how the repair process utilizes plasticity mechanisms important for neuronal development, but also has members unique to the repair process. As with development and many other biological processes, these genes function in regulatory networks and are interconnected by their interactions with other genes (**Figure 1**). Genes in a network

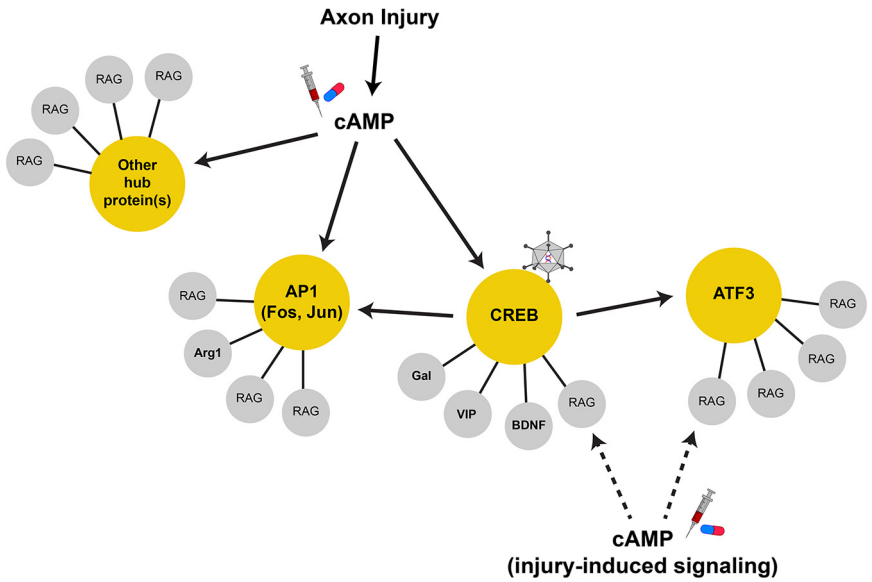

FIGURE 1 | Regeneration-associated gene networks. Axonal injury activates many signal transduction pathways that can lead to gene transcription. The upregulation of cAMP levels after injury is important for RAG expression, serving to activate CREB, AP1, and possibly other transcription factors in parallel. These transcription factors can serve as hub proteins (in yellow circles) to control the transcription of terminal RAGs (in gray circles) that may serve related physiological functions. Some hub proteins, such as CREB, drive the transcription of other hub proteins. In this case, AP1 subunits and ATF3 are direct CREB target genes. As such, CREB is a highly connected node of the RAG transcription network and serves to coordinate the transcription of many terminal RAGs through their proximal hub proteins. These highly connected nodes are attractive therapeutic targets that can recapitulate more of the RAG response and can be targeted by viral-mediated gene delivery (i.e., constitutive-active CREB, virus cartoon). Additionally, injury-induced signals may also work locally and interact with the protein products of the transcribed RAGs to augment axon growth. Thus, strategies that increase/induce RAG expression along with activation of injury signals (i.e., cAMP, syringe and pill cartoon) may show synergy in promoting axon regeneration.

can be "connected" in various ways, including by experimental evidence for physical interaction of the gene products (proteins), common gene function, or predicted regulation by signaling pathways or transcription factors. Because the gene network induced by injury is large and may contain redundant and/or complimentary genes, manipulating singe (or small subsets) of terminal genes is unlikely to recapitulate the effect of the entire regenerative "program." Equally unlikely is our ability to experimentally recapitulate the entirety of the injury response.

Genes that have many connections act as "hubs" that coordinate the expression or activity of the connected genes (Batada et al., 2006; Van Kesteren et al., 2011; Song and Singh, 2013). Transcription factors serve as hubs in gene regulatory networks, and as such, are attractive targets for the manipulation of many genes that may sub serve common functions (**Figure 1**). Transcription-associated networks can be built by connecting genes based on the presence of promoter transcription factor binding sites *in silico* (Michaelevski et al., 2010; Geeven et al., 2011). Along with profiling of the injury-induced phosphoproteome to determine the associated signaling pathways activated, one study has distilled the RAG network to ~40 transcription factors downstream of multiple parallel signaling pathways (Michaelevski et al., 2010). Other independently identified transcription factors include ATF3, c-Jun, C/EBPβ, CREB, KLF family members, p53, SMAD1, SOX11, and STAT3 among others (Jenkins and Hunt, 1991; Herdegen et al., 1997; Schwaiger et al., 2000; Tsujino et al., 2000; Schweizer et al., 2002; Gao et al., 2004; Nadeau et al., 2005; Jankowski et al., 2006; Okuyama et al., 2007; Moore et al., 2009; Zou et al., 2009). It should be noted that the expression levels of some transcription factors might not be upregulated by injury (i.e., CREB and p53); however, their activity is induced and contribute to the overall RAG response (Gao et al., 2004; Tedeschi et al., 2009). While this number of pathways/transcription factors remains daunting, it brings to question whether the entire injury response is necessary to facilitate regeneration, and whether all injury-induced genes are necessarily RAGs (**Table 1**).

Individually identified (not via microarray) non-transcription factor "terminal" RAGs span many functional categories, including genes that encode adhesion/guidance molecules (i.e., integrin subunits, CD44; Kloss et al., 1999; Jones et al., 2000), neuropeptides (i.e., VIP, Gal, CGRP, NPY, PACAP, etc.; Mohney et al., 1994; Holmes et al., 2000; Suarez et al., 2006; Sachs et al., 2007; Armstrong et al., 2008; Toth et al., 2009), structural and cytoskeletal-associated proteins (i.e., GAP43, CAP23, SCG10, CRMP2, Sprr1a; Skene and Willard, 1981; Bomze et al., 2001; Bonilla et al., 2002; Iwata et al., 2002; Mason et al., 2002; Suzuki et al., 2003), and metabolic enzymes (i.e., arginase 1; Cai et al., 2002). The contribution of specific RAGs to axon regeneration/growth (either in the PNS or CNS neurons) has been assessed both *in vitro* and *in vivo* by evaluating their sufficiency or necessity for the regenerative response. Necessity

TABLE 1 | Regeneration-associated transcription: RAGs and genetic manipulations that alter axon regeneration after injury *in vivo*.

Gene (s)	Regeneration phenotype after injury	Reference
Terminal RAGs		
α7 integrin	Knockout delayed facial nerve regeneration	Werner et al. (2000)
BDNF	Conditional knockout decreased sciatic nerve axon growth into peripheral nerve graft; adenoviral overexpression in sensorimotor cortex neurons increased CST axon sprouting/midline crossing	Zhou and Shine (2003) and English et al. (2013)
β2-microglobin	Knockout decreased sciatic nerve motor axon regeneration	Oliveira et al. (2004)
Cdc42	Delivery of constitutive-active protein by hydrogel increased CST tract axon growth through spinal cord lesion	Jain et al. (2011)
CGRP	Local siRNA against CGRP at site of axon injury reduced regeneration of sciatic nerve	Toth et al. (2009)
CRMP2	Adenoviral overexpression increased hypoglossal motor axon regeneration	Suzuki et al. (2003)
Galanin	Knockout decreased sciatic nerve regeneration	Holmes et al. (2000)
Galectin-1	Knockout delayed functional recovery of whisker movement after facial nerve crush; allograft treated with recombinant-oxidized galecitin-1 increased axon regeneration into sciatic nerve	Fukaya et al. (2003) and McGraw et al. (2004)
GAP43 + CAP23	Double transgenic overexpression increased central sensory axon regeneration into a peripheral nerve graft in the spinal cord	Bomze et al. (2001)
GDNF	Transplanted fibroblasts expressing GDNF at spinal cord transection sites increased spinal cord axon regeneration	Blesch and Tuszynski (2003)
Hsp27	Transgenic overexpression increased sciatic nerve motor and sensory axon growth rate and functional recovery	Ma et al. (2011)
IL6	Knockout delayed sciatic nerve regeneration; intrathecal injection of IL6 increased dorsal column sensory axon regeneration	Zhong et al. (1999) and Cao et al. (2006)
$p21^{cip1/WAF1}$	Knockout delayed sciatic nerve motor axon regeneration and functional recovery	Tomita et al. (2006)
PACAP	Knockout decreased facial nerve regeneration; PACAP delivery by hydrogel increased regenerating axons in contusion model of SCI	Armstrong et al. (2008) and Tsuchida et al. (2014)
Rac1	Delivery of constitutive-active protein by hydrogel increased CST axon growth through spinal cord lesion	Jain et al. (2011)
Regeneration-associated transcription factors		
ATF3	Overexpression increased sciatic nerve regeneration	Seijffers et al. (2007)
C/EBPdelta	Knockout delayed sciatic nerve regeneration	Lopez De Heredia and Magoulas (2013)
c-Jun	Knockout reduced facial nerve reinnervation and delayed functional recovery	Raivich et al. (2004)
CREB	Adenovirus overexpression of constitutive-active CREB in DRG neurons increased dorsal column sensory axon regeneration	Gao et al. (2004)
KLF7	AAV overexpression of constitutive-active KLF7 of sensorimotor cortex neurons increased CST tract axon regeneration	Blackmore et al. (2012)
p53	Knockout decreased number of regenerating facial nerve axons	Tedeschi et al. (2009)
SMAD1	Increasing SMAD1 activity with BMP4 in DRG neurons increased dorsal column axon regeneration	Parikh et al. (2011)
SnoN	AAV overexpression of degradation-resistant SnoN in DRG neurons increased dorsal column axon regeneration	Do et al. (2013)
SOX11	Knockdown with membrane-permeable siRNA decreased sciatic nerve regeneration; HSV overexpression accelerated saphenous nerve regeneration	Jankowski et al. (2009) and Jing et al. (2012)
STAT3	Knockout in DRG neurons decreased the initiation of regenerating peripheral axons while AAV overexpression increases terminal sprouting of dorsal column axons; AAV overexpression in motor cortex increased CST axon sprouting	Bareyre et al. (2011) and Lang et al. (2013)

AAV, adeno-associated virus; CST, cortical spinal tract; HSV, herpes simplex virus; SCI, spinal cord injury, siRNA, small-interfering RNA.

is addressed by either pharmacological inhibition or genetic attenuation/deletion of the candidate RAG. These studies often show that knockdown of individual downstream RAGs results in small decreases, or delays, in peripheral regeneration. By contrast, deletion of possible hub proteins (transcription factors or kinases) leads to more dramatic effects *in vivo*. For example, genetic knockout of c-Jun, the first identified transcription factor RAG, reduced re-innervation of peripheral targets by 4-fold after facial nerve axotomy and was associated with decreased induction of its downstream target RAGs α7 integrin, CD44, and galanin (Herdegen et al., 1991; Jenkins and Hunt, 1991; Raivich et al., 2004). By contrast, deletion of α7 integrin or galanin only delayed target re-innervation and functional recovery (Holmes et al., 2000; Werner et al., 2000). This dichotomy also holds true for the above-mentioned terminal RAGs and hub transcription factors. Taken together, these studies suggest that the modulation of any single downstream RAG is unlikely to have robust effects on overall regeneration.

Similarly, sufficiency experiments have yielded mixed effects as the overexpression of terminal RAGs or transcription factors can recapitulate some aspects of peripheral nerve injury, but none capture the phenotypic entirety of the regenerative "program."

For instance, though there was much interest in GAP43 as a RAG, its overexpression failed to promote regeneration in many CNS injury models, though it induced significant sprouting (Buffo et al., 1997; Harding et al., 1999; Mason et al., 2000). Instead, the co-expression of both GAP43 and CAP23, two related growth cone proteins, was required to drive regeneration into a peripheral nerve graft in the spinal cord (Bomze et al., 2001). While the overexpression of some hub transcription factors facilitated *in vivo* regeneration, these effects were also mixed. One example was ATF3, whose overexpression afforded only increased PNS (but not CNS) axon regeneration *in vivo*. Further, ATF3 overexpression did not allow axon growth on inhibitory substrates *in vitro*, suggesting that ATF3 increases the intrinsic growth of axons, but does not alter its response to the inhibitory environment of the CNS (Seijffers et al., 2007). By contrast, STAT3 overexpression seems to allow the initiation of CNS axon growth, but does not sustain its elongation (Bareyre et al., 2011). Taken together, the disparate effects of targeting transcription factors also suggests that each may play specific roles in the regeneration process, and that several hubs may need to be manipulated for a sufficient response (**Figure 1**). **Table 1** lists manipulations of terminal RAGs and regeneration-associated transcription factors that have been shown to impact axon growth *in vivo*.

Interestingly, some injury-induced genes may actually oppose axon growth. For instance, though both SOCS3, which suppresses cytokine signaling, and NFIL3, a transcription factor, are strongly induced by peripheral injury, their deletion or attenuation promotes axon growth (Miao et al., 2006; MacGillavry et al., 2009; Smith et al., 2009). Overexpression of SOCS3 further decreased axon growth, supporting that not all injury-induced genes are necessarily RAGs, and that attenuating negative regulators of axon growth may be beneficial (Miao et al., 2006). This further underscores the notion that not every component of the injury response is contributing to regeneration.

Sufficiency for Therapeutics?

As the changes in gene transcription following axon injury are broad, how do we assess whether they are truly regeneration-associated? As discussed, though many necessity studies show the contribution of candidate injury-induced RAGs to the regenerative response, the sufficiency of their expression in driving regeneration is typically modest. Therapeutic approaches must leverage the sufficiency of genes and transcriptional pathways, organized into larger hubs or programs, to drive axon growth and regeneration. For this purpose, genes and pathways that may not be *normally* recruited by injury could also be considered.

Given that many of the pro-regenerative pathways identified are involved in the neuronal plasticity during development, one major effort has been to reconstitute certain aspects of a "younger" developmental state in which CNS neurons are axon growth competent. These studies sought specific genes, transcription factors, or signaling pathway components that were changed between developmental epochs where CNS neurons lose their regenerative capacity. For example, the activation of Raf-MEK-ERK pathway that is a dominant driver of developmental axon growth downstream of growth factor signaling can also drive robust CNS axon growth in adult neurons (Hollis et al., 2009; O'Donovan et al., 2014). Other developmentally-regulated targets that, when modulated, increase CNS axon growth include transcription factors (KLF family, SnoN; Moore et al., 2009; Blackmore et al., 2012; Do et al., 2013), transcriptional/epigenetic regulators (Set-B and P300; Tedeschi et al., 2009; Trakhtenberg et al., 2014), and others (let-7 microRNA; Zou et al., 2013). It should be noted that some negative regulators of axon regeneration, such as KLF4, are upregulated during development in CNS neurons; attenuation of these genes, as with injury-induced inhibitors of axon growth, may be significant adjuncts to driving growth-associated targets (Moore et al., 2009).

Another fruitful strategy has been to increase the "metabolic growth state" of CNS neurons, focusing on upregulating anabolic processes such as protein translation through mTOR activation [i.e., Phosphatase and tensin homolog (PTEN) deletion or Rheb activation] or transcriptional regulation of anabolic processes (i.e., c-myc overexpression), all of which increase CNS axon growth after injury (Park et al., 2008; Liu et al., 2010; Kim et al., 2011; Belin et al., 2015). While these processes may decline with development in CNS neurons, augmenting these pathways also counteracts some injury-induced deficits such as mTOR and c-myc activity reduction, and is thought to provide the metabolic and energetic substrates required for axon growth (Park et al., 2008; Belin et al., 2015). These studies have also led to combinatorial approaches that pair increased metabolic state (PTEN deletion) with other manipulations that increase axon growth. Indeed, the co-deletion of PTEN and SOCS3, co-deletion of PTEN and SOCS3 plus c-myc overexpression, and deletion of PTEN plus BRaf activation have yielded highly robust long distance axon growth of injured optic nerves, with evidence for synergistic interaction of each manipulation (Sun et al., 2011; O'Donovan et al., 2014; Belin et al., 2015).

Though not necessarily induced by peripheral axon injury, the action of epigenetic modifiers has gained significant interest in facilitating RAG transcription. This is especially true given the difficulty in directly upregulating gene expression *in vivo*, which likely limits the therapeutic potential of these types of approaches. Epigenetic modification of DNA or DNA/protein complexes of chromatin can dictate the transcriptional activity of specific regions of DNA. The most studied of these in regeneration is the acetylation of histone lysine residues. Histones are aceytylated by histone acetyltransferases (HATs), which "opens" the chromatin to allow access to the associated genes for transcription. By contrast, histones are deacetylated by histone deacetylases (HDACs), which "closes" the chromatin and is typically repressive (Kouzarides, 2007). In this way, modulating histone acetylation can control the expression of many genes. Interestingly, peripheral axon injury, which drives regenerative gene transcription, increases acetylation of histones at the promoters of specific genes (some of which are RAGs), whereas this acetylation is not evident after central axon injury (Finelli et al., 2013; Puttagunta et al., 2014). In peripheral neurons, this is the "routine" injury response and is mediated by induced

PCAF activity, a HAT protein (Puttagunta et al., 2014). This injury-induced increase in acetylation is associated with nuclear export of HDAC5, which may serve to decrease the activity of other HDAC isoforms (Cho et al., 2013). As such, augmenting histone acetylation may foster the transcription of relevant RAGs in CNS neurons. Indeed, overexpressing the HATs PCAF and p300 leads to histone acetylation at the promoters of specific genes and increased optic nerve and spinal cord axon regeneration following injury (Tedeschi et al., 2009; Puttagunta et al., 2014). Moreover, pharmacological inhibition of HDAC1 can also increase acetylation, drive RAG expression, and allows central axon regeneration (Finelli et al., 2013). Interestingly, some of the gene changes induced by these epigenetic modifiers are discordant with the standard peripheral injury response, indicating that some induced RAGs may be peripheral to the regenerative response (Finelli et al., 2013; Puttagunta et al., 2014). Both HATs and HDACs can affect the acetylation state of non-histone proteins both in the nucleus and cytosol of neurons. These modifications also play important roles in both transcriptional (i.e., p53 regulation) and non-transcriptional (i.e., microtubule dynamics) aspects of axon growth (Rivieccio et al., 2009; Tedeschi et al., 2009; Cho and Cavalli, 2012). While targeting protein acetylation with small molecules is promising, systemic administration of these drugs may be problematic as acetylation/deacetylation is important for general cellular physiology (Kouzarides, 2007). As such, there remains the need for isoform-specific modulators and more tissue/cell selective modes of drug delivery.

The reduced neuron-intrinsic injury-induced RAG response is an important reason for the failure of CNS regeneration. An interesting point to consider is why CNS neurons fail to upregulate these RAGs in response to injury. What inhibitory mechanisms prevent this response, and can we use this information to inform approaches to enhance repair? As we have discussed, the RAG program is regulated by transcription factors and epigenetic modifications. In addition to these regulatory mechanisms, post-transcriptional regulation of gene expression likely plays a critical role in regulating this program as well. The discovery of micro RNAs (miRNAs) and their role in RNA interference have added greatly to our understanding of regulation of gene expression. Based on bioinformatic predictions, miRNAs likely regulate >30% of all mammalian protein coding genes (Filipowicz et al., 2008). This mechanism appears to be important in axonal regeneration, since deletion of dicer has been shown to impair nerve regeneration in a mouse model of peripheral nerve injury (Wu et al., 2012). In addition, miRNA microarrays have identified a group of miRNAs that are expressed following injury in regenerating sciatic nerves (Strickland et al., 2011). Following spinal cord injury, more than 50 miRNAs show significant changes in expression levels. Among these is miR-145, which inhibits neurite outgrowth *in vitro* by targeting robo2 and srGAP2 (Zhang et al., 2011). In addition, both miR-133 and miR-124 are downregulated following CNS injury. Both of these are implicated in axonal regeneration, with miR-133 known to target the growth inhibitor RhoA. Dysregulation of target mRNA expression by alteration of miRNA levels may result in a failure to sustain the regenerative response, leading to failed CNS regeneration. The possibility of using miRNAs as therapeutic targets, either by anti-miRNA molecules or miRNA mimetics, offers a highly attractive ability to modulate RAG gene expression.

Intrinsic Signals: cAMP and Conditioning Lesion Effect—What is the Role of cAMP-Mediated Transcription?

The induction of RAG expression by axon injury indicates an active process by which injured neurons sense axonal damage to activate an adaptive response. This can be achieved by the disruption of the retrograde flow of target-derived trophic signals (i.e., loss of NGF; Raivich et al., 1991; Gold et al., 1993), activation of existing or newly synthesized local (axonal) factors that are retrogradely transported (i.e., DLK, JNK, STAT3, CREB; Hanz et al., 2003; Cavalli et al., 2005; Cox et al., 2008; Ben-Yaakov et al., 2012; Shin et al., 2012), and depolarization of the axon due to the disruption of the plasma membrane (i.e., Ca^{2+} influx leading to cAMP elevation; Ghosh-Roy et al., 2010; Cho et al., 2013). Ultimately, the arrival of these signals to the cell body drives the transcriptional changes that initiate regenerative response, including the activation of transcription factors.

Interested in the molecular determinants underlying the developmental loss of regenerative capacity, the Filbin lab discovered a direct correlation between neuronal cAMP levels and the ability of neurons to regenerate. For instance, developmentally "younger" CNS neurons (i.e., early embryonic) contained high levels of cAMP and retained the ability to regenerate and overcome extrinsic axon growth inhibitors. This is in contrast to mature neurons (i.e., adult), which are incapable of regeneration and have markedly lower cAMP. In these studies, the regenerative competence of embryonic neurons *in vivo* was attenuated by pharmacological inhibition of PKA, an effector of cAMP signaling, indicating the importance of cAMP in axonal regeneration (Cai et al., 2001).

The importance of cAMP for driving axonal regeneration is not restricted to CNS neurons. Using the peripheral conditioning lesion model, the Filbin group showed that peripheral axon injury induced cAMP levels in the soma of peripheral neurons and that the *in vitro* effect of the conditioning lesion are indeed dependent on PKA (Qiu et al., 2002). Importantly, intraganglionic injection of db-cAMP, a cell membrane permeable cAMP analog, could recapitulate the conditioning lesion effect for neurons in both *in vitro* assays and *in vivo* models of dorsal column injury (Neumann et al., 2002; Qiu et al., 2002). Of note, the *in vivo* injection of db-cAMP allowed DRG neurons to overcome myelin-associated inhibitors *in vitro* in a biphasic manner. Acutely this effect was PKA-dependent (after 1 day); however, this was PKA-independent if the neurons were harvested 6 days after injection. These results indicated that the transient elevation of cAMP set forth longer-lived changes in neuronal function, which persist past the point of elevated cAMP levels. These findings were seminal as they identified a relevant, injury-induced second messenger that could initiate the regenerative process (Qiu et al., 2002).

How then does cAMP exert its benefit on axon regeneration? In mammalian cells, cAMP is generated from ATP by a family of either plasma membrane-bound or soluble adenylate cyclase (AC) enzymes. Membrane-bound ACs are regulated by the canonical heterotrimeric G-proteins, whereas soluble isoforms are activated by bicarbonate. Both soluble- and some isoforms of membrane-bound AC are also activated/regulated by Ca^{2+} (Kamenetsky et al., 2006). Axonal damage causes the depolarization of the neuron, and initiates a back-propagating Ca^{2+} transient (Ghosh-Roy et al., 2010; Cho et al., 2013); the generation of cAMP likely results from this elevation of intracellular Ca^{2+}. Indeed, electrical stimulation of the peripheral nerves is sufficient to increase neuronal cAMP levels in the cell body and can mimic some of the physiological effects of the conditioning lesion (Udina et al., 2008). Recent reports suggest a prominent role for soluble AC for axon growth, as attenuating soluble AC activity, either pharmacologically or genetically, decreases PKA-dependent basal axon growth in RGCs, and prevents neurotrophin-mediated priming for axon growth on myelin-associated inhibitory substrates *in vitro*. Moreover, this inhibition of membrane-bound AC did not recapitulate these effects, indicating that soluble AC was the source of cAMP (Martinez et al., 2014; Stiles et al., 2014).

The above studies serve to link the injury event to elevated cAMP levels via a Ca^{2+}-dependent regulation of AC activity. cAMP likely exerts its effects locally, affecting nearby target proteins. As such cAMP can function in both the axon and cell body compartments of neurons to regulate axon growth. In the axon/growth cone, cAMP regulates axonal guidance through the PKA-mediated inactivation of RhoA signaling and by the phosphorylation of other targets, which allows the cytoskeletal rearrangement that supports growth cone motility (Song et al., 1997; Ming et al., 2001; Kao et al., 2002; Murray et al., 2009; Cheng et al., 2011; Nicol et al., 2011; Forbes et al., 2012). Additionally, cAMP can also signal through EPAC to mediate axon guidance (Murray et al., 2009). In the cell body, cAMP may facilitate axon regeneration by inducing RAG transcription. In this way, cAMP-activated PKA can directly activate CREB-mediated gene transcription, or drive other signaling cascades that can lead to gene transcription changes (Lonze and Ginty, 2002; Gao et al., 2004; Blesch et al., 2012; Ma et al., 2014). Indeed, CREB has been linked to RAG transcription induced by the conditioning lesion, and the expression of a constitutive-active CREB protein can promote the regeneration of dorsal column sensory axons *in vivo* (Gao et al., 2004). Both cAMP and CREB are also necessary for synaptic plasticity (Kandel, 2012), highlighting that the structural plasticity of regeneration and synaptic plasticity of memory and learning utilize some of the same transcriptional substrates.

Is cAMP Enough?

While cAMP-mediated gene transcription is an important contributor to the injury-induced RAG response, the upregulation of cAMP alone may not harness the robustness of the conditioning lesion. In studies that have directly compared axon growth following the conditioning lesion with other stimuli that increase cAMP to the same extent, conditioning lesions shows superior efficacy for axonal regeneration (Udina et al., 2008; Blesch et al., 2012). Indeed, lesion of the peripheral axon recruits more injury-induced gene transcription than increasing cAMP alone by the pharmacological inhibition of its degrading enzyme, PDE4 (Blesch et al., 2012). In light of the at least 40 transcription factors predicted to contribute to injury-induced regeneration-associated transcription, these observations suggest that cAMP-mediated transcription is a contributor to the overall response, and may control specific aspects of axon regeneration (Michaelevski et al., 2010). Accordingly, it is possible that in the absence of axonal injury, the amount of cAMP accumulated by inhibiting its degradation or by electrical stimulation is insufficient to recruit the gene transcription necessary for axonal growth comparable to the conditioning lesion. A recent study published by the Filbin group showed that overexpression of soluble AC in RGCs increased regeneration of crushed optic nerve axons. Though cAMP levels were not measured in this study, the data suggest that ongoing cAMP production, which is likely to be *supra* physiological, may increase its regeneration-promoting actions. Further, soluble AC is found in both the cell body and axon, indicating that the cAMP produced may simultaneously stimulate both compartments (Martinez et al., 2014).

RAG expression occurs on the backdrop of the activated injury-induced signaling cascades that may also have non-transcriptional effectors. As such, the expression (or overexpression) of RAGs on their own may not be optimal for promoting regeneration. For instance, accumulating data suggests that peripheral injury triggers concerted responses that *prime* the neuron to mount an effective transcriptional response. In this case, the calcium transient triggered by axotomy stimulates the epigenetic modifications necessary to facilitate transcription activated by retrograde signals (Cho et al., 2013). In addition to cAMP-PKA, other signaling molecules can act in transcription-independent manners. For instance, though JNK-mediated phosphorylation of c-Jun is necessary for its transcriptional activity, JNK isoforms also regulate axon growth through controlling cytoskeletal organization (Barnat et al., 2010; Ruff et al., 2012). Similarly, local translation initiation of β-actin, which is required for robust sensory axon regeneration, requires the Src-mediated phosphorylation of the RNA binding protein ZBP1 (Huttelmaier et al., 2005; Donnelly et al., 2011). Together, these effects likely cooperate with RAG transcription to drive axon regeneration. This makes a case for combining RAG transcription with the activation of second messengers like cAMP or Ca^{2+}, in order to more closely recapitulate the injury conditioning response and drive regeneration.

Previous studies have suggested that cAMP can play a facilitative role in mediating CNS axon regeneration. In the optic nerve crush model, RGC axonal regeneration is stimulated by the release of oncomodulin by macrophages that are recruited by lens injury. cAMP is required for oncomodulin's actions on axon growth, likely through facilitation of receptor binding (Yin et al., 2006). Given the importance of injury-induced macrophage

recruitment to the DRG for injury conditioning, these factors may cooperate with cAMP to drive RAG transcription (Kwon et al., 2013; Niemi et al., 2013). Further, these manipulations seem to function in parallel to other targets for regeneration, as combining zymozan, which attracts macrophages that release oncomodulin, with cAMP and PTEN deletion synergize to allow some RGC axons to regenerate into the brain and partially restores some visual function (de Lima et al., 2012). Additionally, cAMP has been used to enhance the actions of neurotrophic factors including CNTF in the eye, and NT-3 and BDNF in the spinal cord to promote axon regeneration in systems where cAMP alone is insufficient (Cui et al., 2003; Lu et al., 2004, 2012).

Are "Nested" Transcription Factor Networks the Key?

Though cAMP is sufficient to promote regeneration in some instances, we wanted to better understand the transcriptional determinants of cAMP's actions. Our initial focus was CREB, as it is a well characterized transcriptional mediator of cAMP (Lonze and Ginty, 2002). Using a constitutive-active version of CREB (VP16CREB; CREB-CA) we found that either driving CREB activity in DRG neurons or application of db-cAMP alone increased axon growth on inhibitory and permissive substrates to a similar degree. Surprisingly, axon growth was significantly increased when CREB-CA-expressing neuron were treated with db-cAMP, which was paralleled by an increase in transcription of candidate RAGs. This suggested that levels of RAG transcription stimulated by CREB or cAMP alone were insufficient for the maximum observed axon growth in our paradigm. Interestingly, blocking CREB by expressing a dominant-negative variant (ACREB; CREB-DN) did not change cAMP-induced axon growth, indicating that CREB and cAMP can act synergistically, but also stimulate parallel pathways. This put forth the notion that CREB may serve as an important hub for regeneration (**Table 1**), but that physiological activation of CREB after injury (likely through cAMP) is insufficient to recruit the network needed to promote robust regeneration (Ma et al., 2014). This is supported by work in *C. elegans*, whose neurons exhibit robust regenerative capacity. In these neurons, cAMP upregulation elicited by axotomy-induced Ca2+ influx results in the increase of several basic leucine zipper domain transcription factors (Ghosh-Roy et al., 2010). Null mutation in the *C. elegans* homolog of CREB, crh-1, did not affect regrowth; however, it did decrease formation of ventral branches. Mutations of jun-1, however, did result in reduced regeneration. Taken together, these results suggest that CREB may not be the primary driver of cAMP-induced regeneration and that additional transcription factors may be recruited.

As CREB activity was unnecessary for cAMP's actions, we sought the necessary transcription factor(s) downstream of CREB and cAMP that mediated axon growth. Using arginase 1 (Arg1) as a model RAG, we identified an AP1 site in the proximal promoter region. Blocking AP1 activity with a dominant-negative Fos protein (which inhibits AP1 binding to DNA) blocked both CREB-CA and cAMP-mediated axon growth; which was mirrored by Arg1 expression levels. Importantly, a constitutive-active Fos showed only modest increases in axon growth, suggesting that the AP1-controlled genes cooperate with other CREB targets to stimulate regeneration (Ma et al., 2014).

The strong induction of RAG transcription by cAMP + CREB-CA may induce *supra* physiological expression of injury-induced RAGs. Additionally, this artificial activation of CREB may recruit genes that are not physiologically induced by peripheral axon injury. For instance, CREB-CA induces high and persistent levels of c-Fos expression, which is not observed after peripheral axon injury (Herdegen et al., 1992; Haas et al., 1993). As AP1 heterodimers containing c-Fos and c-Jun have higher transcriptional activity than homodimers of c-Jun, this could further drive the transcription of AP1-dependent RAGs (Angel and Karin, 1991); however, driving AP1 activity + cAMP alone did not recapitulate the effects of CREB-CA + cAMP, reinforcing the notion that the successful axon regeneration requires a concerted and broad transcriptional response. Indeed, the strong activation of CREB recruited other previously identified hub proteins, such as ATF3, suggesting that these responses may contain functional "modules" that mediate specific aspects of axon growth (Ma et al., 2014); however, the breadth of the overall injury response may indicate redundancy at higher organizational nodes of the network. This CREB-activated, AP1-dependent gene *module* may provide significant insight to the programs necessary and sufficient to drive axon growth; its analysis by RNAseq is ongoing. As with other genome-wide techniques, many genes will be identified, though only a few will be true RAGs. Understanding the differences between these two populations may be critical for identifying the "optimal" approach for driving regeneration. It is highly probable that other *nested* transcription factor networks exist; the evaluation of these should occur in a context where changes in gene transcription can be correlated to axon growth/regeneration in order to fully elucidate their contribution to the phenotype.

Where Do We Go From Here?

With each study of regeneration-associated transcription, we come closer to elucidating the critical programs required for axon regeneration. It is evident that the entire RAG response is difficult to recapitulate in CNS neurons due to its breadth. As such, therapeutic strategies must target more manageable critical hubs of RAG transcription or engage pathways far enough upstream to recruit the genes necessary to drive axon growth. Future strategies should combine RAG expression with non-injury induced means of increasing axon growth. This could pair genetic activation of potential upstream hubs (i.e., CREB) with the pharmacological upregulation of relevant signaling pathways (i.e., cAMP) to activate regeneration-associated transcription (**Figure 1**). Additionally, combinatorial strategies that increase metabolic state, recapture developmental axon growth potential, and favorably modify epigenetic state of RAGs could function in synergy with RAG expression to

further promote axon growth following injury. With the proper experimental design and the explosion of "big-data" analysis of transcriptomics data, we may now be poised identify the "regeneration-associated hubs" to target, and make good on the promise of Filbin's seminal observations on cAMP. We may find that it isn't necessary to completely recapitulate the peripheral-injury response in order to effect significant regeneration.

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Sialic Acid Is Required for Neuronal Inhibition by Soluble MAG but not for Membrane Bound MAG

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Myelin-Associated Glycoprotein (MAG), a major inhibitor of axonal growth, is a member of the immunoglobulin (Ig) super-family. Importantly, MAG (also known as Siglec-4) is a member of the Siglec family of proteins (sialic acid-binding, immunoglobulin-like lectins), MAG binds to complex gangliosides, specifically GD1a and/or GT1b. Therefore, it has been proposed as neuronal receptors for MAG inhibitory effect of axonal growth. Previously, we showed that MAG binds sialic acid through domain 1 at Arg118 and is able to inhibit axonal growth through domain 5. We developed a neurite outgrowth (NOG) assay, in which both wild type MAG and mutated MAG (MAG Arg118) are expressed on cells. In addition we also developed a soluble form NOG in which we utilized soluble MAG-Fc and mutated MAG (Arg118-Fc). Only MAG-Fc is able to inhibit NOG, but not mutated MAG (Arg118)-Fc that has been mutated at its sialic acid binding site. However, both forms of membrane bound MAG- and MAG (Arg118)- expressing cells still inhibit NOG. Here, we review various results from different groups regarding MAG's inhibition of axonal growth. Also, we propose a model in which the sialic acid binding is not necessary for the inhibition induced by the membrane form of MAG, but it is necessary for the soluble form of MAG. This finding highlights the importance of understanding the different mechanisms by which MAG inhibits NOG in both the soluble fragmented form and the membrane-bound form in myelin debris following CNS damage.

Keywords: myelin inhibition, MAG, gangliosides, sialic acid, neurite outgrowth

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INTRODUCTION

Myelin-associated glycoprotein (MAG), a member of the immunoglobulin (Ig) super-family, contains five Ig-like domains in its extracellular sequences, a single transmembrane domain, and a short cytoplasmic domain (**Figure 1A**; Lai et al., 1987a,b; Salzer et al., 1987, 1990). Since its recognition as a potent inhibitor of central nervous system (CNS) axon regeneration (McKerracher et al., 1994; Mukhopadhyay et al., 1994), enormous efforts have been directed at identifying its receptors. MAG was first reported as a sialic acid binding protein that interacts with the complex gangliosides GT1b and GD1a (Vinson et al., 2001). The search for receptors that transduce its inhibitory effect resulted in the discovery of NgR1, NgR2, and PirB as functional MAG receptors (Domeniconi et al., 2002; Liu et al., 2002; Venkatesh et al., 2005; Atwal et al., 2008). MAG was also shown to bind and signal through β-1 integrin (Goh et al., 2008). Recently, the inhibition of neurite outgrowth (NOG) of cortical neurons plated on MAG-expressing Chinese hamster ovary (MAG-CHO)

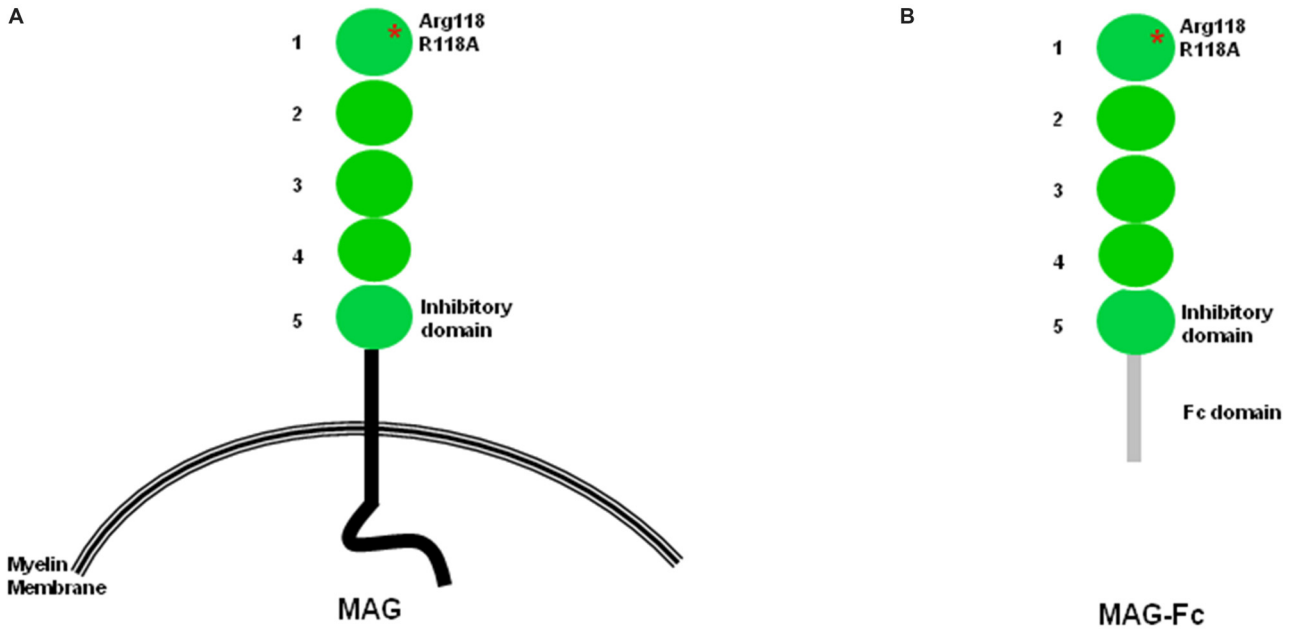

FIGURE 1 | Structural features of myelin-associated glycoprotein (MAG). (A) Structural features of MAG. Arginine 118, the sialic acid binding site and also part of the RGD site, is located within domain 1. The inhibition site is located within domain 5. **(B)** In soluble MAG-Fc, the protein portion after domain 5 is replaced by the Fc domain.

cells was found to be independent of NgRs and gangliosides. Rather, PTEN, a lipid phosphatase that activates AKT, was found to mediate the MAG inhibitory signal, suggesting the existence of other, as yet unknown, receptors (Perdigoto et al., 2011).

Discovering MAG receptors is critical for identifying therapeutic targets for promoting axonal regeneration, and it is also important to clarify the role of gangliosides in MAG-induced inhibition of NOG. Here, we focus on the relationship between MAG and sialic acid in gangliosides, please see a recent extensive review of sialic acids (Schnaar et al., 2014).

MAG AS A MEMBER OF THE SIGLEC PROTEIN FAMILY

The Siglec protein family is a subgroup of the Ig super-family that has similar amino acid sequences in the first four Ig-like domains and shows sialic acid-dependent binding to cells (Kelm et al., 1998). Members include sialoadhesin (Siglec-1), CD22 (Siglec-2), CD33 (Siglec-3), and MAG (Siglec-4; DeBellard et al., 1996; Tang et al., 1997a; Kelm et al., 1998). Each member of the Siglec family binds with different specificity. MAG preferentially binds α2,3-linked sialic acid residues attached to O-linked glycoconjugates (Kelm et al., 1994), CD22 binds to α2,6-linked sialic acid attached to N-linked glycoconjugates, and sialoadhesin and CD33 recognize α2,3-linked sialic acid attached to O- or N-linked glycoconjugates (Kelm et al., 1994; Cornish et al., 1998).

MAG is expressed early in development (for a thorough description of MAG in normal tissue, see Baldwin and Giger, 2015) exclusively at the interface between myelinated axons and periaxonal myelin membrane (Trapp et al., 1989). MAG plays a role in axon-glial interactions (Schachner and Bartsch, 2000) and influences myelin formation. Genetic depletion of MAG results in altered myelination, reduced axon caliber, reduced neurofilament spacing and phosphorylation, and progressive axonal degeneration (Li et al., 1994; Montag et al., 1994; Fruttiger et al., 1995; Yin et al., 1998).

Early postnatal MAG-deficient mice exhibit delays in the formation of compact myelin in the optic nerves and reduced density of retinal ganglion cell axons surrounded by compact myelin (Montag et al., 1994). Although the ultrastructure of compact myelin is unaffected, MAG-deficient mice exhibit a dilated periaxonal space and abnormal formation of the periaxonal cytoplasmic collar (Li et al., 1994; Montag et al., 1994). Some axons in MAG-deficient mice are surrounded by more than one myelin sheath (Bartsch, 1996). Moreover, in adult MAG-deficient mice, oligodendrocytes show degeneration of distal processes in the periaxonal space or within compact myelin, which is also observed in immune-mediated demyelinating diseases including multiple sclerosis (Rodriguez-Peña et al., 1993; Lassmann et al., 1997). Thus, in the CNS, MAG is involved in the initiation of myelination, formation of myelin sheaths, and long-term maintenance of oligodendrocyte structure and myelin integrity. In the peripheral nervous system (PNS), MAG seems to be involved only in the formation of intact myelin and long-term maintenance of myelin structure but not in the initiation of myelination.

Collectively, therefore, *in vitro* experiments and studies using MAG-deficient mice show that MAG is a cell adhesion molecule, a receptor that transduces signals into the interior of myelin-forming glial cells, and a contributor to cross-talk between myelin-forming glial cells and axons.

INHIBITORY SITE ON MAG

Using several chimeric constructs in which domains 4 and 5 of MAG are exchanged with the corresponding domains of sialoadhesin, the Filbin lab showed that the MAG inhibition site is on domain Ig-5 (**Figure 1**) and is distinct from the sialic acid binding site on domain Ig-1 (Cao et al., 2007). Importantly, several chimeric molecules containing the sialic acid binding site sialoadhesin do not inhibit NOG, reinforcing the notion that the sialic acid binding domain is not necessary for neuronal inhibition (Cao et al., 2007). Another group arrived at a similar conclusion using a different set of molecular tools focusing on domain Ig-4 of neural cell adhesion molecule (N-CAM) and domain Ig-5 of MOG (Wörter et al., 2009).

SIALIC ACID AS COMPONENT OF GANGLIOSIDES

Gangliosides are glycosphingolipids containing one or more sialic acid residues in their oligosaccharide structure (Sonnino et al., 2007). They are components of all animal cell membranes and are particularly abundant in the plasma membranes of neurons. Gangliosides are complex lipids with a strong, amphiphilic, big saccharide head-group and a double-tailed hydrophobic moiety. The lipid moiety of gangliosides, shared across sphingolipids, is called a ceramide and is constituted by a long-chain amino alcohol termed sphingosine (Karlsson, 1970), connected to fatty acids by an amide linkage. Sialic acid is a sugar that differentiates gangliosides from neutral glycosphingolipids and sulfatides and defines all derivatives of 5-amino-3,5-dideoxy-d-glycero-d-galacto-non-2-ulopyranosonic acid or neuraminic acid (Schauer, 1982). Gangliosides are positioned to interact with other molecules in their own membrane and molecules on opposing cell membranes (Lopez and Schnaar, 2009). Gangliosides are typically anchored in the outer leaflet of the plasma membrane, where they are driven by ceramide to partition laterally into lipid rafts, which are membrane micro-domains containing other sphingolipids, cholesterol, and signaling molecules. This lateral interaction in the membrane normally results in ganglioside-mediated regulation of membrane proteins, such as receptor kinases. Ganglioside glycans also extend outward from the cell surface, where their sialoglycans participate in intermolecular interactions. This interaction with proteins on opposing membranes results in ganglioside-mediated cell-cell recognition, such as myelin-axon interaction.

Ceramide is the common precursor of glycosphingolipids and sphingomyelin and is transported from the endoplasmic reticulum to the Golgi apparatus by unknown mechanisms. Glycosphingolipids are synthesized by the stepwise addition of monosaccharide sugars to ceramide and the growing sugar. Subsequent addition of galactose, sialic acid, and N-acetylgalactosamine from their nucleotide sugar donors to the growing saccharide chain generates penta, hexa, and hepta saccharide glycans (Kolter et al., 2002). The ganglioside biosynthetic pathway (**Figure 2**) involves a sequential process of glycosylation via two main pathways: the "a" series (GM2, GM1a, GD1a) and "b" series (GD2, GD1b, GT1b; van Echten and Sandhoff, 1993). Each ganglioside is structurally more complex than its precursor molecule, and the stepwise addition of monosaccharide or sialic acid residues by specific membrane-bound glycosyltransferases in the Golgi apparatus is catalyzed by the same glycosyltransferases in both pathways. In an investigation of the differential distribution of GM1, GD1a, GD1b, and GT1b in the adult mouse CNS (Vajn et al., 2013), GD1b and GT1b was expressed in gray and white matter, GM1 was expressed in white matter, and GD1a was expressed in specific nuclei/tracts. This differential expression of gangliosides could explain why MAG appears to use different receptors on different neurons to inhibit NOG (Mehta et al., 2007; Venkatesh et al., 2007).

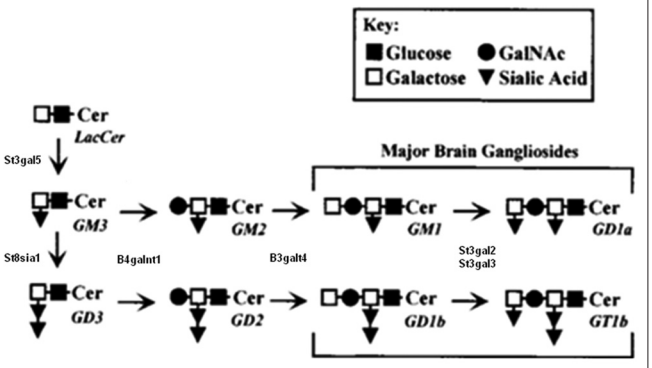

FIGURE 2 | Partial biosynthesis pathway for major brain gangliosides. Schematic biosynthetic relationship between major brain gangliosides and their precursors. Disruption of the *B4galnt1* gene blocks the synthesis of gangliosides, including GT1b and GD1a, to which MAG binds. *B4galnt1*-deficient mice lack all complex gangliosides but express higher levels of simple gangliosides GM3 and GD3. Modified from Sheikh et al. (1999), reproduced with permission.

B4GALNT1-DEFICIENT MICE

Among many glycosyltransferases, β1, 4 GalNAc-transferase (β1, 4 GalNAc-T; GM2/GD2 synthase; EC2.4.1.92), coded by the gene B4GALNT1, plays an important role in the biosynthesis of almost all complex gangliosides (**Figure 2**). Two different groups independently disrupted the B4GALNT1 gene to generate *B4galnt1*-deficient mice. Takamiya et al. (1996) disrupted exon 4 of the B4GALNT1 gene by inserting a neomycin-resistant plasmid and found that *B4galnt1*-deficient mice express no complex gangliosides but higher concentrations of the simple gangliosides GD3 and GM3. By 12 weeks of age, *B4galnt1*-deficient mice show decreased central conduction velocity but normal brain weight and shape, myelination of white matter, and myelinated fiber and synapse structure, suggesting that

complex gangliosides are required for neuronal function but not brain organogenesis. This lack of complex gangliosides might be compensated by higher levels of GM3 and GD3 in these mice. A later study reports that deficits exhibited by these *B4galnt1*-deficient mice can be rescued by tissue-specific GalNacT constructs. Using the neurofilament light promoter (restricted to neurons) and proteolipid protein promoter (restricted to myelinating glia, including oligodendrocytes and Schwann cells), the restoration of complex gangliosides in neurons but not glia was found to be critical for maintaining CNS integrity (Yao et al., 2014).

Sheikh et al. (1999) generated a different strain of *B4galnt1*-deficient mice using a vector, in which exon 6 and 7 and part of exon 8 are deleted and replaced with a MC1NeoPolyA selection cassette (Sheikh et al., 1999). These mice have a normal life span but show decreased central myelination, central and peripheral axonal degeneration, and increased levels of GD3 and GM3. Similar to MAG-deficient mice, *B4galnt1*-deficient mice show abnormalities, such as doubly myelinated axons with cytoplasm between the two myelin sheaths. Whereas young *B4galnt1*-deficient mice express normal levels of MAG, adult *B4galnt1*-deficient mice show a ∼30% decline in MAG levels in the CNS and PNS and develop Wallerian degeneration (Sheikh et al., 1999). Sheikh et al. (1999) attribute the differences between their findings and the findings of Takamiya et al. (1996) to the fact that the first group's animals were examined at a young age, whereas the effects of *B4galnt1* deficiency become more prominent with increasing age. They argue that because conduction velocity depends on myelination and axon diameter, the decreased conduction velocity observed by the first group could be attributed to myelination defects and axonal atrophy. Indeed, in a later study, MAG protein but not mRNA was found to decrease substantially by 12 months of age (Sun et al., 2004).

The more recently generated double-heterozygous mice carry null mutations in B4GALNT1 (called *Galgt1*) and ST3GAL5 (called *Siat9*, which encodes GM3 synthase [CMP-NeuAc:lactosylceramide α-2,3-sialytransferase; EC 2.4.99.9]; Yamashita et al., 2005). Although the mice are viable, they exhibit neurodegeneration with severe pathology in white matter and CNS axons. In addition, the mice were generated with mutated ST3GAL2 and ST3GAL3, which are sialyltrasferase genes responsible for terminal sialylation of gangliosides and biosynthesis of GD1a and GT1b (**Figure 2**). These mice exhibit dysmyelination marked by substantial reductions in major proteins, myelinated axons, myelin thickness, and MAG expression, as well as molecular defects in the Nodes of Ranvier (Yoo et al., 2015), indicating that terminal sialylation is required for optimal brain structure and function.

ROLE OF GANGLIOSIDES IN MAG-MEDIATED INHIBITION OF NOG

In the NOG assay developed by the Filbin lab, primary neurons are grown on a monolayer of control CHO or MAG-CHO cells, and the growth of neurites is quantified 24 h later (Mukhopadhyay et al., 1994). In this assay, MAG inhibits the growth of postnatal neurons (Mukhopadhyay et al., 1994; DeBellard et al., 1996). Also, removal of neuronal sialic acid residues by sialidase treatment does not alter the inhibitory effect of MAG on NOG (Tang et al., 1997a). The sialic acid binding site on MAG was mapped at Arginine (Arg) 118, a highly conserved residue in many Siglecs, which is part of a RGD site that binds and signals through β-1 integrin (Goh et al., 2008). Arg 118 is located in the first Ig-like domain. Surprisingly, mutations of this amino acid to either Alanine or Aspartate do not affect the inhibitory effect of MAG (Tang et al., 1997a), indicating that MAG-sialic acid interactions might not be required for MAG inhibition.

The Filbin lab also used purified soluble forms of the extracellular domain of MAG fused to the Fc portion (MAG-Fc; **Figure 1B**) of human IgG1 (Kelm et al., 1994; Tang et al., 1997b). In this modified, soluble-NOG assay, neurons are incubated with MAG-Fc and then plated on L1-Fc substrate in wells coated with anti-Fc antibodies. Although MAG-Fc inhibits the outgrowth of neurites, no inhibition occurs when mutated Fc constructs (MAG (R118A)-Fc or MAG (R118D)-Fc) are used (Tang et al., 1997a), suggesting that MAG-sialic acid interactions are necessary only when MAG is in soluble form. In addition, a truncated form of MAG containing the first three Ig-like MAG domains (MAG d1–3-Fc), which binds neurons in a sialic acid-dependent manner, does not inhibit neurite growth in the soluble-NOG assay (Tang et al., 1997a).

Similarly, Vinson et al. (2001) report that mutation of the R118 residue in the first Ig-like domain reduces the potency of MAG inhibition in the soluble-NOG assay. MAG specifically binds both GT1b and GD1a, which are expressed at the surface of MAG-responsive neurons. Pre-incubation of hippocampal neurons (HNs) with increasing concentrations of alpha methyl sialic acid 3 sialyllactose before addition of MAG-Fc blocks MAG-Fc inhibition of NOG. Also, pre-incubation of HNs with purified GT1b and GD1a blocks MAG- and MAG (R118A)-Fc inhibition, indicating that MAG-GT1b interactions on the cell surface may be a mechanism of MAG-induced inhibition of NOG (Vinson et al., 2001).

Vyas et al. (2002) report that GD1a and GT1b are functional nerve cell ligands for MAG. Treatment of cerebellar granular neurons (CGNs) with *V. cholerae* neuraminidase, which removes sialic acid from cell surfaces, reverses MAG inhibition. Under the same conditions, depletion of gangliosides using the glycosylceramide synthase inhibitor P4 reverses MAG inhibition of NOG. Also, CGNs from mice lacking the complex gangliosides GD1a and GT1b (i.e., *B4galnt1*-deficient mice) are less vulnerable to MAG inhibition. Furthermore, experiments where CGNs are incubated on detergent-extracted myelin or membrane purified from MAG-CHO cells provide additional evidence of the importance of complex gangliosides when MAG is expressed in soluble form.

The Filbin lab also showed that soluble MAG released from damaged white matter, consisting of a proteolytic MAG fragment containing the entire extracellular domain (d1–5), inhibits axonal regeneration (Tang et al., 2001). This finding highlights the importance of understanding the different mechanisms by which MAG inhibits NOG in both the soluble fragmented form and the membrane-bound form in myelin debris following CNS damage.

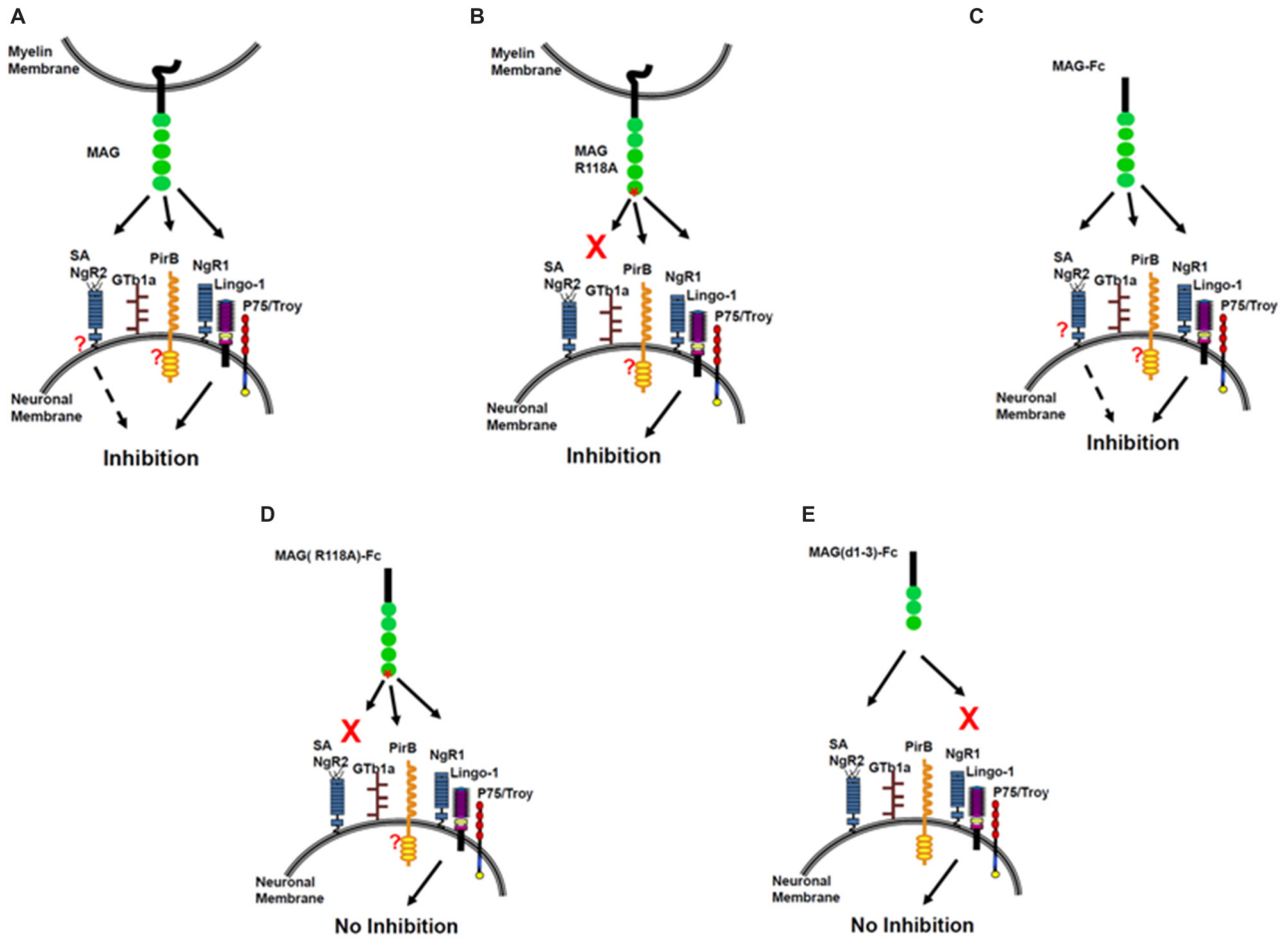

FIGURE 3 | The sialic acid binding on MAG R118 is critical for inhibition of axonal regeneration by soluble MAG-Fc but not by MAG expressed by Chinese hamster ovary (CHO) cells. Inhibition of NOG by MAG-CHO **(A)**, mutated MAG (R118A)-CHO **(B)** and soluble MAG-Fc **(C)**. However, mutated soluble MAG (R118A)-Fc does not inhibit NOG **(D)** nor, a truncated form of soluble MAG (d1–3)-Fc, that binds to sialic acid but lacks the inhibitory domain of MAG **(E)**.

An interesting experiment by Vinson et al. (2001) sheds light on the function of sialic acid. They demonstrated that aggregating sialic acid, achieved by adding IgM generated from anti-GT1b antibodies, reduces NOG in dose-dependent manner (Vinson et al., 2001), mimicking the inhibitory effect of MAG. Also, pre-incubation of GT1b antibody with purified GT1b, but not with other purified gangliosides, blocks inhibition of NOG by anti-GT1b antibodies. Finally, a Rho kinase inhibitor blocks inhibition of NOG by MAG or anti-GT1b antibody in both HNs and CGNs (Vinson et al., 2001). By contrast, Vyas and colleagues show that highly multivalent clustering of either anti-GT1b or anti-GD1a, achieved using IgG1 monoclonal antibody pre-incubated with secondary anti-Fc, mimics MAG-mediated inhibition of NOG (Vinson et al., 2001; Vyas et al., 2002). This inhibition cannot be due to gangliosides, which do not have transmembrane properties, but rather to another molecule that crosses the membrane and perhaps interacts with gangliosides.

Indeed, lipid rafts have been suggested as a possible transducer of MAG inhibitory signals (McKerracher, 2002). One study in particular indicates that myelin rafts may associate with neurons (Vinson et al., 2003). Specifically, MAG is found in a section of the membrane characterized by its solubility to Triton X-100 but not to Lubrol WX. MAG-Fc detected this section in lipid raft preparations from CGNs in a region containing GT1b, p75, Rho, NgR, and caveolin (Vinson et al., 2003). In another study, MAG-Fc, anti-GD1a, or anti-GT1b brings p75 (and also Flotilin-1, a marker of lipid rafts) to the Brij-58 insoluble lipid raft fraction isolated from mouse CGNs (Fujitani et al., 2005). Also, biochemical studies show the binding of GT1b and GM1 to NgR1 (Williams et al., 2008). Analytical ultracentrifugation analysis shows that GT1b and GM1, but not asialo-GM1, induce additional peaks in a concentration-dependent manner, with higher sedimentation coefficients than the coefficient of NgR1(310)-Fc. When the GT1b binding site on NgR1 is blocked with a cyclic peptide, GT1b antibody fails to inhibit NOG. Finally, GT1b antibody does not inhibit NOG in neurons from NgR1 knock-out mice (Williams et al., 2008). Together, these findings indicate that the effect of GT1b antibody requires NgR1.

CONCLUSIONS: A WORKING MODEL

In summary, MAG binds sialic acid at Arg118, which is located in domain 1 of MAG, distant from the inhibitory site in domain 5. Soluble MAG requires sialic acid binding for inhibition of NOG. When Arg118 is mutated, inhibition by soluble MAG is abolished. However, sialic acid binding is not required for MAG-expressing CHO cells to inhibit NOG. We conclude that there are at least two binding sites on MAG—the sialic acid binding site at Arg 118 and inhibitory site at domain 5. Therefore, we propose a two-site model for MAG inhibition (**Figure 3**). When CHO cells express MAG, both sites engage neurons, and NOG is inhibited (**Figure 3A**). When CHO cells express MAG that is mutated at its sialic acid binding site, NOG is still inhibited (**Figure 3B**). However, it is unclear whether another molecule helps achieve the correct orientation for inhibition to occur and/or allow membrane-associated MAG to achieve the correct conformation to interact with the receptor. When soluble MAG is added to neurons, both sites engage neurons, and NOG is inhibited (**Figure 3C**). By contrast, when soluble MAG is mutated at the sialic acid binding site, it cannot bind to neurons, and the inhibition site cannot be engaged, resulting in no inhibition of NOG (**Figure 3D**). When soluble MAG (d1–3) is added to neurons, it binds through its sialic acid binding site but does not inhibit axonal growth because the inhibition site is absent (**Figure 3E**). This model suggests that gangliosides are necessary in order to inhibit NOG, but only when soluble form of MAG is present; perhaps gangliosides help achieve correct alignment to NgR, but not when MAG is expressed by CHO cells.

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NA-B and WM wrote the manuscript, made direct contributions to the work, and approved it for publication. Work in the lab was supported by the Specialized Neuroscience Research Program (NINDS Grants 3U54NS041073 and RO1NS037060) and an infrastructure grant from the Research Centers in Minority Institutions Program (NCRR Grant RR003037) at Hunter College. This work is dedicated to the memory of Marie T. Filbin.

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