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Multipotent Neural Progenitor Cells Exhibit Enhanced Neurite Outgrowth on Adult Myelin: Implications for Neural Regeneration

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ABSTRACT OF THE THESIS

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Adult central nervous system (CNS) axons fail to regenerate after injury, and one of the hurdles limiting CNS regeneration is the presence of potent inhibitory molecules in adult white matter. However, multipotent neural progenitor cells exhibit remarkably extensive axonal elongation through adult white matter when grafted in vivo (Lu et al., 2012), suggesting either that early stage neurons

are not inhibited by, or are actually stimulated by, white matter. To address these possibilities, we used multipotent neural progenitor cells (NPCs) isolated from E12 mouse spinal cords for cell culture on adult CNS myelin in vitro and compared findings to cells cultured on non-myelin substrates.

Compared to cells cultured in the absence of myelin, adult DRG neurons exhibited a reduction in neurite outgrowth on myelin, whereas NPC cultures exhibited a significant *increase* in neurite length. While adult neurite growth inhibition is mediated in part by interactions with Nogo receptors and their ligands, Nogo, MAG, and OMgp, we found no change in neurite outgrowth when neural progenitor cells were plated on Nogo, MAG, or OMgp-deficient myelin compared to wild-type myelin. This suggests that classic adult myelin-related inhibitory mechanisms are not involved in facilitation of developing neuronal process outgrowth, and that other mechanisms are likely to be involved. Overall, these findings indicate that one of the mechanisms underlying the remarkable growth ability of early stage neurons in the injured adult CNS is a stimulatory influence of adult myelin.

Introduction

In the United States, there are approximately 12,000 cases of non-lethal spinal cord injuries (SCI) per year, with an estimated national incidence of 270,000 total cases in 2012 (NSCISC, 2012). Damage to the spinal cord results in debilitating consequences, including loss of normal motor, sensory, and autonomic function. Motor sensory loss is caused by either a partial or complete severing of axons along the length of the spinal column. As of 2012, estimated lifetime costs resulting from SCI range from 1 to 4.5 million dollars per individual (NSCISC, 2012). In an effort to improve the quality of life of those afflicted with functional loss, the spinal cord regeneration field aims to restore function of the damaged spinal cord.

Central vs Peripheral Nervous System Regeneration

Spinal cord regeneration has been canonically defined as the regrowth of a severed axon into and past the injury site, and the reinnervation of the normal target (Yoon and Tuszynski, 2012). Interestingly, the peripheral nervous system (PNS) demonstrates substantial regenerative capabilities: damaged peripheral neurons initiate molecular growth programs and are capable of reinnervating their original targets. On the contrary, axons in the central nervous system (CNS) are unresponsive following severance and fail to regenerate past the injury site. The PNS environment is so favorable for growth that PNS grafts into a CNS lesion promote growth of the injured central axons. However, the growing axons

fail to exit the graft, and ultimately do not reach their targets (Richardson and Issa, 1984). This suggests that the disparity between the regenerative potential of the PNS and the CNS can be attributed in part to differences in the extrinsic cellular environments (Huebner and Strittmatter, 2009).

Residing in the PNS are Schwann cells, whose physiological function is to secrete myelin, a structure that insulates neurons from charge dissipation. This function is halted shortly after injury to the PNS. Following axonal severance, the axon distal to the injury site undergoes Wallarian degeneration. This process entails the degradation of the distal axon and the removal of glial and neuronal debris by phagocytic Schwann cells and macrophages (Gaudet et al., 2011) Within the first 12 hours after injury, Schwann cells decrease lipid synthesis (White et al., 1989) and by 48 hours halt the expression of myelin proteins (Trapp et al., 1988). A Schwann cells' transcriptional profile changes to become phagocytic; and soon after clearance of debris, the Schwann cells proliferate to form the band of Bungner, a support structure for regenerating axons (Perry et al., 1995; Stoll et al., 1989). In small mammals, myelin debris is cleared within 7-14 days post injury in the PNS, making way for regenerating axons (George and Griffin, 1994; Griffin et al., 1992). In addition to creating a structurally permissive environment for regenerating axons, the surrounding Schwann cells' gene expression changes to create a chemically permissive environment for axonal regeneration. By 4 days post-injury, myelin associated glycoprotein (MAG), a molecule inhibitory to neuron regeneration, is downregulated to non-detectable

levels (LeBlanc and Poduslo, 1990), and the expression of growth stimulating molecules, such nerve growth factor (NGF), is upregulated (Anton et al., 1994).

In contrast to the PNS's robust regenerative response, the CNS poorly regenerates due to a variety of factors, including a non-permissive extrinsic environment, inactive growth programs, and a physical barrier. The first difference from the PNS is the types of glial cells residing in the CNS; instead of Schwann cells, the CNS contains oligodendrocytes, cells responsible for myelination (Wood, 1984) and astrocytes, cells that give metabolic and structural support to the neurons (Figley and Stroman, 2011). Unlike Schwann cells, oligodendrocytes have little or no capacity to remove myelin and axonal debris from the injury site (Ludwin, 1990). Although Wallerian degeneration also occurs in the CNS, myelin debris is removed over a much longer period of time compared to the PNS, resulting in the axonal and glial debris to linger in the lesion cavity (George and Griffin, 1994). Moreover, astrocytes invade the injury cavity as part of a wound response, eventually forming the glial scar, a physical barrier to axonal regeneration (Windle and Chambers, 1950).

Myelin-Associated Inhibitors

Damaged oligodendrocytes release myelin debris containing a class of molecules, known as myelin-associated inhibitors (MAI), that potently inhibit neurite outgrowth in vitro (reviewed in Lee and Zheng, 2012). Currently, there exist at least six recognized inhibitory proteins associated with oligodendrocytes. These include Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al.,

2000), myelin-associated glycoprotein (MAG) (Filbin, 1995; McKerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte-myelin glycoprotein (OMgp) (Wang et al., 2002a), netrin (Hedgecock et al., 1990), semaphorins (Kolodkin et al., 1992), and ephrins (Pasquale, 1997). The first three myelin associated inhibitors, Nogo, MAG, and OMgp bind to the Nogo receptor complex, which includes Nogo Receptor 1 (NgR1), p75, and LINGO-1 (Fournier et al., 2001; Liu et al., 2002a; Wang et al., 2002a).

A member of the immunoglobulin superfamily, MAG is a cell surface protein with five extracellular immunoglobulin-like domains, a transmembrane domain, and an alternatively spliced C-terminal tail (Lai et al., 1987). Expressed by both Schwann cells and oligodendrocytes, MAG is suggested to stabilize glial-axon interactions, as it is present in the periaxonal layer of myelin during the early phases of axonal myelin ensheathment (Bartsch et al., 1989; Trapp, 1990). MAG was the first myelin protein found to inhibit neurite outgrowth in vitro (McKerracher et al., 1994; Mukhopadhyay et al., 1994). In vitro, MAG binds to both the NgR1 complex and the PirB receptors, with its inhibitory effects reversed through immunodepletion in dorsal root ganglion cultures (Atwal et al., 2008; Liu et al., 2002a; Wang et al., 2012). Although MAG is expressed in both the PNS and the CNS, quick removal of myelin debris in the PNS contributes to regeneration. In the CNS, myelin debris, including MAG, accumulates and inhibits regenerating axons. Unlike the PNS, where MAG expression is down regulated upon injury, damaged oligodendrocytes cleave and release MAG's extracellular domain in a soluble form (Moller, 1996; Sato et al., 1984). This

soluble form of MAG inhibits neurite outgrowth of adult dorsal root ganglion cells (DRGs) in vitro (Tang et al., 2001; Tang et al., 1997). The immediate release of soluble MAG post-injury is thought to act as an inhibitor of regeneration prior to the formation of the glial scar.

Nogo, a member of the Reticulon family of proteins, is another potent inhibitor of regeneration. Physiologically, Nogo resides in the endoplasmic reticulum and helps maintain the ER's structure (Voeltz et al., 2006). Nogo is additionally found on the inner and outer membranes of the myelin sheath in the CNS and is expressed in three alternatively spliced isoforms (A, B, and C) (Buss and Schwab, 2003; Wang et al., 2002b). Nogo is found predominantly in oligodendrocytes (Huber et al., 2002), but is also expressed in a specific subset of mature neurons (Huber et al., 2002; Richard et al., 2005; Wang et al., 2002b). In particular, Nogo-A expression is present in pre and post-synaptic hippocampal neurons and localizes in the periaxonal sheath and synapse, suggesting that Nogo-A is involved in synapse formation (Lee et al., 2008; Wang et al., 2002b). Two domains on Nogo-A have been identified to inhibit neurite outgrowth, the N-terminus region, also known as amino-Nogo, and the 66 amino-acid transmembrane region (Nogo-66) (GrandPre et al., 2000). The N-terminus region of Nogo-A is reported to interfere with integrin binding (Hu and Strittmatter, 2008), and has recently been shown to bind to the sphingosine-1-phosphate receptor 2 (S1PR2), reducing neurite outgrowth (Kempf et al., 2014). S1PR2 activation is transduced through a G-protein coupled receptor, and downstream signaling is dependent on rhoGEF LARG and RhoA. Like MAG, the Nogo-66

domain binds to the NgR1 complex and PirB receptor to inhibit neurite outgrowth (Atwal et al., 2008; Fournier et al., 2001).

OMgp, the most recently discovered myelin-based inhibitor, is an extracellular membrane protein anchored to the cell membrane via glycophosphatidylinositol (GPI) intermediate. OMgp contains a highly conserved leucine rich repeat (LRR) domain that is responsible for neurite inhibition; upon deletion of the LRR domain, neurite inhibition is lost (Vourc'h et al., 2003). OMgp is expressed in a large population of neurons in the brains of post-natal rats, with expression levels peaking during myelination (Habib et al., 1998; Vourc'h and Andres, 2004). Large projection neurons, such as cortico-spinal and hippocampal neurons also express OMgp during development (Habib et al., 1998). OMgp has been hypothesized to inhibit sprouting at the nodes of Ranvier during development (Huang et al., 2005), although consensus on its function has yet to be reached.

Receptors for Myelin-Associated Inhibitors

Neurite inhibition is mediated by the NgR1 complex. NgR1, a CNS specific receptor, was first found to bind to Nogo-66 with high affinity and causes growth cone collapse in vitro (Fournier et al., 2001). Additionally, NgR1 has high affinity for MAG and OMgp (Liu et al., 2002a; Wang et al., 2002a), thus serving as a common receptor for all three major MAIs despite their lack of sequence homology. NgR1 expression is postnatal and localizes to axonal membranes (Wang et al., 2002b) and post-synaptic compartments (Lee et al., 2008; Wang et

al., 2002b). The localization of NgR1 seems to suggest a role in synaptic formation in addition to its function as a receptor for MAG, Nogo-66, and OMgp. The binding of Nogo-66 to NgR1 is dependent on the receptor's leucine-rich-repeat (LRR) region, located on the N and C termini flanking the 8 LRR cores (Barton et al., 2003), where a disruption to either of any of the LRR regions results in the inability to bind Nogo-66 (Fournier et al., 2002).

NgR1 is a GPI-anchored protein that clusters around lipid rafts and is capable of oligomerization (Barton et al., 2003; Fournier et al., 2002). Since NgR1 lacks a transmembrane domain, signal transduction is carried out through membrane-associated co-receptors including p75, a low affinity neurotrophin receptor, and LINGO-1, a nervous system specific LRR-Ig-containing protein. Studies in cerebellar granule neurons show NgR1 complexes with both p75 and LINGO-1, resulting in RhoA activation (Mi et al., 2004). The NgR1 does not exclusively complex with p75 and LINGO-1, but also complexes with TROY, a closely related TNF-alpha orphan receptor, leading to downstream RhoA activation (Park et al., 2005; Shao et al., 2005). Increased activation of the small GTPase Rho-A via NgR1 complex signaling (Alabed et al., 2006; Fournier et al., 2003) and its downstream effectors, Rho-associated kinase (ROCK), LIM Kinase 1 (LIMK1), and cofilin, results in growth cone collapse (Duffy et al., 2009; Fournier et al., 2003; Niederost et al., 2002).

Neutralization of NgR1 is unable to completely negate the MAI-based inhibition (Fournier et al., 2001), suggesting the potential for other receptors that contribute to neurite inhibition. In a screening for Nogo-66 receptors, Atwal and

colleagues identified Paired Immunoglobulin-like Receptor B (PirB) as an additional receptor to MAG, Nogo-66, and OMgp (Atwal et al., 2008). PirB was first described as a regulator of B-cell and myeloid cell function (Kubagawa et al., 1997). Genetic deletion of PirB in cortical granule neurons mitigates the inhibitory effects of recombinant Nogo-66 and myelin in vitro (Atwal et al., 2008); additionally, application of anti-PirB antibodies reduces the incidence of growth cone collapse (Atwal et al., 2008). Despite PirB's role in mediating neurite outgrowth inhibition in vitro, low amounts of PirB are detectable in CNS tissue (Lee et al., 2010); furthermore, no PirB is detected in the cortico-spinal tract (CST) via immunohistochemistry (Omoto et al., 2010). In vivo, deletion of PirB is not sufficient to enhance regeneration or functional recovery in a dorsal hemi section model (Nakamura et al., 2011). Despite this negative finding in vivo, PirB's potent effects in vitro suggest a role in myelin-associated inhibition.

Myelin inhibition In Vitro

Myelin-induced outgrowth inhibition seems to be dependent on the presence of growth stimulating molecules, a caveat that is easily overlooked. Neuronal cell lines such as the neuroblastoma NB-2A line used by Schwab and Caroni demonstrate outgrowth inhibition after the application of either dibutyryl-cyclic-AMP (cAMP) or glia-derived neurite-promoting factor (GDNF), which are required to induce differentiation and stimulate neurite formation (Braas et al., 1983; Schwab and Caroni, 1988; Zurn et al., 1988). Similar methods were used by McKerracher and colleagues, where the NG108-15 neuroblastoma cell line

was first primed with cAMP to stimulate neurite production, and then was treated with soluble MAG to show neurite inhibition (McKerracher et al., 1994). Further studies demonstrated that myelin inhibition could be reversed in a dose dependent manner with laminin (David et al., 1995). Both laminin, an extracellular matrix (ECM) protein, and cAMP promote axonal growth through the inhibition of the RhoA/Rock pathway (Heidemann et al., 1985; Leeuwen et al., 1997; Liu et al., 2002b; Oishi et al., 2012).

For primary neuron and neural explant cultures, growth-promoting ECM molecules or growth factors seem necessary in order to demonstrate myelin-induced outgrowth inhibition. In earlier experiments claiming neurite outgrowth inhibition of DRG neurons on CNS white matter tissue slices, neuronal growth factor (NGF) was frequently added in the culture media to stimulate growth of these cells (Carbonetto et al., 1987; Savio and Schwab, 1989). NGF, a TrkA agonist, is a common supplement for neuronal cultures that also stimulates neurite outgrowth through RhoA inhibition (Yamaguchi et al., 2001). Additionally, embryonic chick spinal cords cultured on myelin result in increased growth cone collapse; however, the cells were cultured on fibronectin, a growth stimulating substrate (Kuhn et al., 1999). More recently, two independent studies cultured adult DRGs on crude myelin extracted from mice deficient in MAG, Nogo, and OMgp. The triple knockout myelin was less inhibitory than the wild-type myelin, with the caveat that the neurons were cultured in the presence of laminin (Cafferty et al., 2010; Lee and Zheng, 2012).

In vitro myelin-mediated neurite outgrowth inhibition seems to be dependent on the competition of RhoA/ROCK signaling. Growth-promoting factors (e.g. laminin, fibronectin, NGF, cAMP, etc.) inhibit RhoA/ROCK activity, whereas MAIs (MAG, Nogo, OMgp) stimulate the RhoA/ROCK pathway. A study done by Hu and Strittmatter quantified the effect of purified Nogo on DRGs cultured on different stimulatory ECM substrates including laminin, fibronectin, collagen, vitronectin, and VCAM. The degree to which Nogo inhibition occurs is dependent on the ECM substrate (Hu and Strittmatter, 2008). It seems that myelin is able to negate the effects of these growth-stimulating molecules. A similar phenomenon was observed, where neurite inhibition by semaphorin3A (Sem3A), a growth cone collapsing substrate, is only seen upon stimulation of neurite outgrowth with NGF. With a high enough concentration of NGF, Sem3A inhibition is reversible (Kaselis et al., 2014). Niederost and colleagues observed neurite stimulation of embryonic cerebellar granule cells through RhoA/ROCK inhibition, only on growth inhibiting conditions (MAG expressing CHO cells and purified Nogo); whereas RhoA/ROCK inhibition on poly-L-lysine, a neutral growth substrate, resulted in no difference in outgrowth from the control (Niederost et al., 2002). These experiments suggest that outgrowth inhibition is contingent on the addition of growth stimulating molecules, such as laminin or NGF, to the culture. In order to correctly interpret myelin's impact on regeneration, it is important to understand the experimental nuances in previous myelin inhibition experiments.

Overcoming Myelin Inhibition in Vivo

Extensive efforts have been made to regenerate damaged CNS axons and to restore motor, sensory, and autonomic function. One approach has been to neutralize the components responsible for myelin-mediated inhibition. Schnell and Schwab demonstrated enhanced axon regeneration upon antibody neutralization of MAG in vivo. In the experiment, young rats underwent a CNS lesion and were treated with anti-MAG antibodies. Two-three weeks post-injury, axons regenerated up to 7mm past the lesion site in treated rats, whereas the axons in control animals rarely exceeded 1mm past the lesion site (Schnell and Schwab, 1990). Despite this increase in growth, the regenerating neurons fail to produce the long distance axonal regeneration necessary for functional recovery.

Numerous Nogo neutralization experiments have been conducted, however, due to conflicting results, it is hard to conclude whether the removal of Nogo results in enhanced regeneration. Zheng and colleagues generated Nogo-A/B/C deficient mice and observed a reversal of neurite outgrowth inhibition in P7 cerebellar granule neurons cultured on their Nogo-deficient myelin. Despite in vitro findings, in vivo experiments did not result in enhanced regeneration (Zheng et al., 2003). Lee and colleagues revisited this experimental paradigm and generated a separate Nogo-deficient mouse line. Again, in vitro experiments performed on their Nogo knockout mice did not result in increased regeneration (Lee et al., 2010). Contrary to Zheng and Lee's findings, other investigators observed enhanced growth of damaged corticospinal axons and improved behavioral function following an administration of anti-Nogo antibodies (Bregman

et al., 1995; Liebscher et al., 2005; Maier et al., 2009; Schnell and Schwab, 1990).

In addition to single knockout experiments, Lee and colleagues generated a triple knockout mouse to determine whether the combinatorial deletion of Nogo, MAG, and OMgp would enhance regeneration (Lee et al., 2010). Increased neurite outgrowth was observed in cortical granule neurons and adult dorsal root ganglion neurons cultured on the triple knock out myelin. In vivo, neither functional gains nor enhanced regeneration were observed in the triple knockout mice; however, Lee and colleagues detected increased collateral sprouting in all the single knockout mice, as well as the triple knockout mouse (Lee et al., 2010).

Instead of targeting myelin-associated ligands, another approach for promoting regeneration has been the neutralization of the NgR1 receptor complex. Experiments inhibiting or deleting NgR1, p75, and/or LINGO1 yielded conflicting results. It is not clear whether the neutralization of the NgR1 receptor complex improves either axonal regeneration or locomotor function. Two sources claim enhanced regeneration when NgR1 antagonist NEP1-40 was applied post-injury (GrandPre et al., 2002; Li and Strittmatter, 2003). On the contrary, two other groups replicated the experiment and both observed no regeneration after NEP1-40 treatment (Nakamura et al., 2011; Steward et al., 2008). In addition to transient neutralization, NgR1 and/or p75 knockout mice were generated, and enhance regeneration was not observed (Lee et al., 2010; Song et al., 2004; Zheng et al., 2005). Although suppression of MAIs or their receptors effectively

reverses myelin-induced inhibition in vitro, it is still unclear if this approach is sufficient to achieve regeneration in vivo.

Instead of overcoming inhibition through the modification of the CNS environment, the SCI field has started to make significant progress in the use of stem cells to restore functional recovery. Recently, Lu and colleagues transplanted multipotent neural progenitor cells (NPC) into a severe injury cavity, resulting in long distance axonal growth into host tissue and improved behavioral function. The transplanted embryonic spinal cord derived NPCs ideally serve as a relay, where injured host axons synapse onto the NPCs, and the NPCs synapse onto targets beyond the lesion site. There is precedence that adult CNS white matter is inhibitory to many types of neurons in vitro, yet surprisingly, it seems that the NPCs extend axons preferentially into the host white matter (Lu et al., 2012). Zhao and colleagues also show similar results when transplanting human primitive NPCs, with a majority of the axons extending into the host white matter (Zhao et al., 2013). Contrary to adult neurons that hardly regenerate past the injury site, NPCs possess the capability to grow into the host spinal cord, especially into the white matter.

Neuronal Cultures on CNS Tissue

In the past two and a half decades, precedence has been that neurons cultured on gray matter attach and robustly extend neurites; whereas neurons cultured on white matter poorly attach and hardly extend neurites. Both neonatal rat DRG neurons and neuroblastmas were cultured on adult CNS white matter

and gray matter tissue slices. Neurons cultured on the gray matter displayed robust neurite outgrowth; on the other hand, neurons cultured on white matter showed little to no neurites (Savio and Schwab, 1989). Embryonic chick sympathetic ganglion explants were also cultured on adult CNS tissue sections. Again, growth was observed on gray matter, but the white matter displayed poor neurite extension (Crutcher, 1989). Additionally, embryonic chick CNS and PNS neurons (neocortical and DRG cells respectively) were cultured on adult intact white matter and adult gray matter. Similar to previous results, gray matter proved to be a much more permissive substrate over white matter(Watanabe and Murakami, 1990). From these past experiments, it seems that both embryonic and post-natal neurons grow and attach preferentially on gray matter tissue over intact white matter tissue. Interestingly, embryonic chick DRG explants and dissociated neocortical cells are able to grow on lesioned adult CNS white matter (David et al., 1990; Watanabe and Murakami, 1990). Based on the preceding experiments, it seems unusual that transplanted NPCs do not grow robustly into the host gray matter.

Age and Cell Type Specific Effects of Myelin Proteins

Molecules that are inhibitory to neurite outgrowth in adult neurons may be stimulatory in immature neurons. Certain myelin associated proteins, such as MAG, OMgp, and Netrin, can either inhibit or promote growth, depending on the age of the neuron. For example, MAG inhibits neurite outgrowth of mature DRG cultures; however, in DRGs younger than post-natal day 3, MAG stimulates their

outgrowth (Mukhopadhyay et al., 1994). MAG is hypothesized to be involved in axon guidance, directing growth during development, and inhibiting sprouting in mature neurons. Turnley and Barlett observe embryonic mouse spinal cords are stimulated by MAG and OMgp, both of which are inhibitory in adult neurons (Turnley and Bartlett, 1998). This developmental switch of MAG and OMgp, from stimulatory to inhibitory, seems to play a key role in the NPCs' axonal extension into the host CNS white matter.

Another class of molecule that switches from axon attraction to repulsion is netrin, a diffusible signal that is present during neural development (Hedgecock et al., 1990). This bifunctional ligand acts as a guidance cue and can either attract or repel axons depending on the available axonal receptors. The netrin family secondary protein structure is highly conserved throughout all species. Given netrin's ability to attract axons, it is not surprising that it shares sequence homology with both laminin and epidermal growth factor (Barallobre et al., 2005; Brankatschk and Dickson, 2006). Binding of netrin to the Deleted in Colorectal Cancer (DCC) receptor results in axonal attraction; however, expression of UNC5 receptor, either alone or coexpressed with DCC, results in axonal repulsion (Killeen and Sybingco, 2008; Round and Stein, 2007). This switch from attraction and repulsion is executed through the silencing of DCC via the slit/Robo complex (Killeen and Sybingco, 2008).

White Matter Geometry

The physical, three-dimensional environment of white matter is another potential explanation of how grafted NPCs grow into the white matter instead of the gray matter. Embryonic sympathetic ganglion neurons cultured on corpus callosum tissue, where tissue sections were cut perpendicular to the axons, grew poorly; whereas neurons cultured on tissue sections cut parallel to the axon tracts grew six-fold in comparison (Pettigrew and Crutcher, 1999). These results suggest the possibility that the physical organization of the host's axon tracts may promote and guide graft derived axons into and through the white matter.

ERK Signaling

Neurite outgrowth signaling usually converges upon the mitogen-activate protein kinase (MAPK) pathway. MAPK belongs to the family of serine/threonine protein kinases that transduces extracellular signals into intracellular posttranslational responses (Lewis et al., 1998; Seger et al., 1995). Within this family is the extracellular signal-regulated protein kinase (ERK), which mediates peripheral retrograde injury signaling in DRG neurons (Obata and Noguchi, 2004), and neurotrophin-induced neurite growth in many types of neurons. Nerve growth factor (NGF), a potent inducer of neuronal survival and neurite outgrowth, is mediated in part through ERK signaling (Klesse and Parada, 1999; Miller and Kaplan, 2001). Additionally, cell adhesion molecules that stimulate outgrowth, such as laminin and N-cadherin, induce ERK activation (Perron and Bixby, 1999). ERK inhibition promotes self-renewal in pluripotent embryonic stem cells,

suggesting that ERK is necessary for differentiation (Burdon et al., 1999). With many distinct neurite outgrowth signaling pathways converging upon ERK signaling, it seems likely that adult white matter induces ERK activation in NPCs.

Conclusions and Experimental Proposal

Due to their recent availability, neural stem cells provide a new paradigm to potentially achieve functional repair in the damaged spinal cord. The use of neural stem cells provides several advantages over attempting to regenerate host axons. First, the stem cells' internal machinery is primed for growth and development; whereas injured adult neurons have to reactivate these growth and development pathways in order to regenerate. Stem cells are also able to survive and repopulate the injury site. In addition to generating neurons, these neural stem cells differentiate into necessary support cells such as oligodendrocytes and astrocytes (Lu et al., 2012).

Even with neural stem cell transplants becoming more accessible, little is understood about their interactions with the adult CNS environment. Previous data show other types of CNS and PNS neurons that grow well on gray matter, but poorly on white matter; however, recent in vivo experiments demonstrate robust growth of neural progenitor cells into adult white matter, rather than gray matter. Whereas adult injured axons fail to penetrate a severe SCI lesion site, grafts of multipotent NPCs are capable of filling the lesion cavity, and extending a dense number of axons, preferentially into the white matter.

Lee and colleagues provide a good example of how injured cortico-spinal and serotonergic neurons are unable to extend axons, either into or beyond the lesion site (Lee et al., 2010). In contrast, multipotent NPCs grafted into a severe injury site fill the lesion cavity, extend a dense number of axon fibers penetrating the lesion boundary, and grow for long distances into host white matter (Figure 1) (Lu et al., 2012). This ability of the NPCs to extend axons into an environment that is considered inhibitory suggests that the intrinsic state of NPCs, coupled with the environment of the graft, promotes growth.

From previous experiments, it is possible that distinct molecule(s) present in the adult CNS white matter may promotes growth specifically in NPCs, but not adult neurons. Additionally, the geometry of the axon tracts in white matter may play a role in providing a structurally permissive environment for axonal growth. **Based on the preceding observations, I hypothesize that adult CNS white matter, specifically myelin, promotes the growth of spinal cord derived NPCs.**

There are likely cues present in the adult white matter that promote growth of transplanted NPCs. By introducing NPCs into an in vitro environment, we can assay growth outside of the spinal cord three-dimensional environment in order to see the effects of adult CNS white matter as a substrate. We can also attempt to identify the underlying mechanisms mediating NPCs' growth on white matter. In this thesis, I will explore how adult CNS myelin affects neurite outgrowth in spinal cord derived multipotent neural progenitor cells

Materials and Methods

Animals

All procedures involving animals were carried out in strict adherence to guidelines provided by The Guide for the Care and Use of Laboratory Animals (The Institute of Laboratory Animal Resources, 2011), The Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH, 1986), The Animal Welfare Act/Regulations and subsequent amendments (PL 89-544), and The Veterans Health Administration Handbook 1200.07 "Use of Animals in Research" (2011); VA San Diego Healthcare System (VASDHS) Research Services Policy 01 section 151-04 (Institutional Animal Care and Use Committee, IACUC) and VASDHS IACUC Policy 03 (Pre and Post-procedural Care of Laboratory Rodents). The animal use protocol was approved by the VASDHS IACUC (Protocol number 11-010).

Myelin Extraction

Adult C57BL/6 (wt-mice) mice were euthanized; brains and spinal chords were removed, stripped of the dura, and collected in HBSS on ice. Myelin extraction was performed using a sucrose-gradient centrifugation as previously described (Larocca and Norton, 2007). Myelin total protein concentration was assessed using a Bradford assay (Bio-Rad, Hercules, CA).

Dorsal Root Ganglion Culture

Adult C57BL/6 (wt-mice) mice were euthanized and DRGs were removed, stripped of their roots and collected in HBSS on ice. DRGs were washed once with HBSS, digested with HBSS + 0.25% collagenase XI (Sigma-Aldrich) + 5 mg/ml Dispase (Worthington, Lakewood, NJ) for 30 min at 37°C, washed once with media (DMEM/F12 w/Glutamax, 1% penicillin and streptomycin (1000 U per 10 mg/ml final concentration), 10% FBS, 1x B27 (Invitrogen, Grand Island, NY)), triturated in media, counted and plated onto 48 well tissue culture treated plates (Thermo Scientific, Logan, UT) at 250 DRG neurons in 250 µl media per well. Plates were precoated with Poly-D-lysine hydrobromide (PDL, 20 µg/ml in water, Sigma-Aldrich) overnight at room temperature, washed 3 times with sterile water and air-dried. Some wells were coated with myelin (10ug/ml) overnight at 4°C, washed 3 times with PBS. Wells coated with laminin (1 µg/ml in PBS, Sigma-Aldrich) were incubated for 4 hours at room temperature, washed 3 times with media and not dried before plating. All DRG neurons were cultured at 37°C in 5% CO_2 for 48 hours. Neurons were fixed with 4% Formaldehyde in BBS for 20 min at 37°C, washed 3 times with TBS, permeabilized with TBST for 15 min at 37°C, washed 3 times with TBS, blocked in TBS + 10% donkey serum for one hour at 37°C, incubated with primary antibody mouse anti-ßIII-tubulin (1:2000, Promega, Fitchburg, WI) in TBS + 2% donkey serum overnight at 4°C, washed 3 times with TBS, incubated with secondary antibody Alexa-488 anti-mouse (1:1000) and DAPI (1 µg/ml) in TBS + 2% donkey serum for one hour at 37°C, washed 3 times with TBS and imaged with ImageXpress (Molecular Devices, Sunnyvale, CA).

For neurite outgrowth measurements, 3 biological samples were measured in triplicates.

Spinal Cord Derived Neural Stem Cell Culture

Embryonic day 12 (E12) spinal cords from C57BL/6 (wt-mice) mice were dissected in ice cold HBSS, digested in 0.25% Trypsin for 20 min at 37C, washed 3 times with 10% FBS in Neurobasal medium, triturated in 5 ml Neurobasal medium + B27, counted and plated onto 48 well tissue culture treated plates (Thermo Scientific, Logan, UT) at 10,000 cells in 250 µl media per well. Plates were precoated with Poly-D-lysine hydrobromide (PDL, 20 µg/ml in water, Sigma-Aldrich) overnight at room temperature, washed 3 times with sterile water and air-dried. Additionally, some wells were coated with myelin (10ug/ml) overnight at 4°C, washed 3 times with PBS. Wells coated with laminin (1 µg/ml in PBS, Sigma-Aldrich) were incubated for 4 hours at room temperature, washed 3 times with media and not dried before plating. All E12 spinal cord neurons were cultured at 37°C in 5% CO_2 for 48 hours. Neurons were fixed with 4% formaldehyde in BBS for 20 min at 37°C, washed 3 times with TBS, permeabilized with TBST for 15 min at 37°C, washed 3 times with TBS, blocked in TBS + 10% donkey serum for one hour at 37°C, incubated with primary antibody mouse anti-βIII-tubulin (1:2000, Promega, Fitchburg, WI) in TBS + 2% donkey serum overnight at 4°C, washed 3 times with TBS, incubated with secondary antibody Alexa-488 anti-mouse (1:1000) and DAPI (1 µg/ml) in TBS + 2% donkey serum for one hour at 37°C, washed 3 times with TBS and imaged

with ImageXpress (Molecular Devices, Sunnyvale, CA). For neurite outgrowth measurements, at least 3 biological samples were measured in triplicates.

ERK Enzyme Linked Immunosorbent Assay

Rat E14 spinal cord derived neural progenitor cells were plated in well tissue culture treated plates (Thermo Scientific, Logan, UT) at 500,000 cells in 2ml of media. Adult myelin was added (10ug/ml) to the media two hours post plating at a concentration. At 3, 6, 24, and 48 hours post treatment, media was removed and washed twice with PBS. Cell samples were then lysed and total ERK and Phospho-ERK were measured using ERK 1/2 Instant One Elisa Kit (Affymetrix, La Jolla, CA). Phospho-ERK and total ERK levels were visualized with a chemiluminescent substrate captured by a CCD camera. For 3 and 6-hour time points, one biological sample was obtained and for 24 and 48 hour time points, three biological samples were obtained. Quantities were measured in doublets.

MEK Inhibition Neurite Outgrowth Assay

MEK inhibitor PD98059 (Sigma, Milwaukee, WI) was added 2 hours post plating of E12 spinal cord derived NPCS. PD98059 was diluted in 0.01% DMSO at a final concentration of 10nM. 0.01% DMSO was used as a vehicle control. For neurite outgrowth measurements, two biological samples were measured in doublets.

Data and Statistical Analysis

Neurite outgrowth and branching was analyzed with MetaXpress (Molecular Devises). Results are represented as mean ± standard error of the mean (SEM) of at least three biological replicates, with at least three wells analyzed per replicate. Prism (graphPad, La Jolla, CA) was used to calculated the SEM and statistical significance of difference between groups, evaluated by one way ANOVA with Newman-Keuls' multiple comparison tests. In graphs *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Results

Adult CNS myelin reduces neurite outgrowth of adult dorsal root ganglion neurons only in the presence of laminin

In order to assess the properties of our myelin extract, we first performed a neurite outgrowth assay on adult DRG neurons. We cultured dissociated DRG neurons on the permissive substrate poly-D-lysine (PDL) or the stimulatory substrate laminin, either with or without myelin. We fixed the cells after 48 hours in vitro and assessed neurite morphology via immunostaining and imaging of the neurons specific microtubule βIII-tubulin via automated imaging and image analysis (ImageXpress, Molecular Devices).

DRGs cultured on laminin display over a 3-fold increase in neurite outgrowth ($p < 0.001$), and a 3-fold increase in neurite branching ($p < 0.001$) compared to our PDL control (Figure 2.2). DRGs plated on myelin in conjunction with laminin display an approximate 60% reduction of neurite outgrowth ($p < 0.001$) and branching ($p < 0.001$) when compared to DRGs plated on Laminin only. Unexpectedly, DRGs plated on Myelin/PDL show no difference in neurite outgrowth or branching compared to our PDL control (Figure 2.2), suggesting laminin is a necessary component in demonstrating myelin inhibition in adult DRG neurons. Additional measurements were quantified such as longest neurite and cell body processes and were found to have the same trend as neurite outgrowth and branching (Supplemental Figure 1). Morphologically, there is a large difference in neurite length and overall neurite density with the addition of

myelin to DRGs cultured with laminin (Figure 2.1). From this data, it seems that myelin can only inhibit DRGs receiving a stimulation signal, in this case, laminin.

Adult CNS Myelin stimulates neurite outgrowth of spinal cord derived multipotent neural progenitor cells

Based on NPCs' ability to extend axons long distances in dense numbers into host white matter, we investigated whether adult CNS myelin is a permissive substrate for spinal cord derived NPCs. To test the hypothesis that adult CNS myelin is a permissive substrate for neurite outgrowth, NPCs were grown on PDL or laminin, with or without myelin, for 48 hours. Neurite outgrowth was then assessed via βIII-tubulin immunostaining.

When plated on PDL, spinal cord derived NPCs display minimal growth (Figure 3.1). With the addition of laminin, neurite outgrowth increases nearly 2-fold ($p < 0.001$) and branching increases 3-fold ($p < 0.05$) (Figure 3.2). In both myelin conditions (PDL/myelin and laminin/myelin), neurite outgrowth increases over 3-fold compared to PDL alone ($p < 0.001$). Surprisingly, myelin substrates significantly enhance neurite outgrowth ($p < 0.001$). In addition, the myelin substrate alone results in greater neurite outgrowth than NPCs plated on both laminin and myelin ($p < 0.05$). Although myelin stimulates greater greater neurite outgrowth from NPCs, we find it unexpected that myelin in addition to laminin does not result in an additive effect. We hypothesize either there may be fractions of the myelin extract that interfere with the laminin stimulation pathway, resulting in a non-additive effect on outgrowth, or that the laminin coating might

mask the myelin coating and dampen the myelin's stimulatory effect. Similar results were also observed in E14 rat NPCs (Supplemental figure 2).

Lastly, we investigated whether adult CNS myelin influenced the proportion of neurons in the NPC culture. We compared βIII-tubulin positive cells against our overall cell count using DAPI, a nuclear marker, and normalized this ratio to PDL control (Supplemental figure 3). Neither laminin, myelin, nor the combination resulted in a change in population of βIII-tubulin positive cells.

Myelin deficient in Nogo, MAG, OMgp, or a combination does not influence growth of spinal cord derived NPCs compared to wild-type myelin

MAG and netrin's switch from stimulating to inhibiting neurite outgrowth during development (Mukhopadhyay et al., 1994; Turnley and Bartlett, 1998), raising the possibility that switches from inhibition to stimulation might mediate the growth-promoting effects of myelin observed on NPCs compared to adult neurons. Accordingly, we investigated whether Nogo, MAG, and OMgp affect neurite outgrowth of cultured NPCs. We assessed the effects of CNS myelin isolated from adult mice deficient in either Nogo, MAG, OMgp, both Nogo and MAG, and a triple knock outs (TKO) on NPCs; these materials were a gift of Binhai Zheng at UCSD. Adult DRGs grown on these Nogo knock out and TKO myelin were previously reported to significantly increase adult neurite outgrowth compared to wild-type myelin, indicating myelin's role as an adult neurite outgrowth inhibitor (Lee et al., 2010). In our NPC cultures we found that all forms of knockout myelin increase neurite outgrowth and branching ($p < 0.001$).

Despite the effect seen on adult DRGs, depletion of Nogo, MAG, OMpg, or a combination thereof, did not impair the stimulatory effect of the adult myelin extract on neurite outgrowth (Figure 4). Plating on laminin yielded similar results, with no change in either neurite outgrowth or branching among knockout myelin conditions (Supplemental figure 5).

Myelin signaling leads to transient ERK activation in NPCs

With many distinct neurite outgrowth signaling pathways converging upon ERK signaling, ERK is a candidate molecular mechanism for activation during NPC outgrowth on myelin. To test this hypothesis, we compared levels of phosphorylated ERK (p-ERK) in samples treated with myelin at various time points using an ELISA. Consistent with adult DRGs that have elevated ERK activation upon dissociation and plating (Agthong et al., 2006), we observe greater amounts of p-ERK in our control groups during earlier time points (3 and 6 hour) compared to our later time points, 24 and 48 hour (Figure 5.1). With the addition of myelin, ERK activation is saturated at 3 and 6 hour time points; however this activation falls by 24 hours. Our findings of early ERK activation combined with our data neurite outgrowth data, suggests that myelin-induced ERK activation is a mechanism for neurite outgrowth and is consistent with other molecules that stimulate neurite outgrowth such as NGF and laminin (Klesse and Parada, 1999).

MEK inhibition does not affect neurite outgrowth or branching of NPCs

To test if ERK activation is responsible for increased neurite outgrowth, we used PD98058, a well-documented MEK inhibitor, to determine whether outgrowth was inhibited. (Althini et al., 2004; Poplawski et al., 2012; Zhai et al., 2005). ERK activation is induced through phosphorylation via the MAPK-kinase, MEK. We therefore hypothesized that an inhibition of MEK would lead to a reduction of ERK mediated neurite outgrowth. Despite our findings of elevated levels of p-ERK at 3 and 6 hour time points (Figure 5.1), addition of PD98058 did not reduce neurite outgrowth or branching (Figure 5.2). To account for these findings, we speculate that the myelin-induced activation of ERK was fully saturated prior to the addition of PD98058 to the culture, and that the growth signal had already been propagated. Another possibility is that ERK becomes activated on myelin by a MEK-independent mechanism. Some studies have identified potential alternate signaling pathways, including ERK activation via phosphotidyl inositol-3 kinase – AKT (Aksamitiene et al., 2010; Grammer and Blenis, 1997), and future studies can investigate these in our culture system.

Discussion

Neural stem cells are an increasingly available resource for potentially treating spinal cord injury, and as such, their behavior in the adult CNS environment are yet to be fully understood. Both murine and human neural progenitor cells are capable of extending axons in dense numbers for long distances, particularly through the white matter (Lu et al., 2012; Zhao et al., 2013). Considering in vitro studies demonstrating gray matter being more permissive than white matter for adult axon regeneration, we found it surprising that the NPCs extended axons preferentially into the white matter. In this thesis, we investigated whether or not CNS myelin is a permissive substrate to spinal cord derived multipotent NPCs.

In developing our assay, we used adult DRGs as our control to reproduce the inhibitory effect of our crude CNS myelin. Consistent with previous myelin inhibition studies (Cafferty et al., 2010; Lee et al., 2010) neurite outgrowth was reduced by myelin, but only in the presence of laminin. When DRGs were plated on myelin without laminin, there was no reduction of growth compared to controls (Figure 2.2). Our findings are consistent with that of published data, where myelin inhibition occurs in the presence of a growth promoting substrate/growth factor (Carbonetto et al., 1987; Kuhn et al., 1999; Schwab and Caroni, 1988; Zurn et al., 1988). David and colleagues demonstrate that laminin dependent myelin inhibition can more precisely be described as competition between laminin and myelin (David et al., 1995). With this caveat, we decided to apply the same four

substrate conditions (PDL, laminin, laminin/myelin, and myelin) used in our adult DRG experiments.

The main finding of this thesis is that spinal cord derived NPCs exhibit increased neurite outgrowth on adult CNS myelin. Where previous studies demonstrate how poorly neurons, both embryonic and post-natal, grow on adult white matter (Crutcher, 1989; Savio and Schwab, 1989; Watanabe and Murakami, 1990), we observe increased neurite outgrowth (Figure 3.2). Surprisingly, we observed that myelin alone is much more stimulatory than laminin, whereas the opposite holds true for adult DRGs (Figures 2.2, 3.2). The NPCs' increased outgrowth was observed on both PDL and laminin substrates, where NPCs plated on PDL/myelin exhibited greater neurite outgrowth than our myelin/laminin condition. Initially, we expected there to be an additive effect of myelin and laminin stimulation, however this was not the case. As we observed, myelin inhibition in adult DRGs is only seen in the presence of laminin. In our NPC culture, there may have been fractions of the myelin which inhibited the laminin stimulation which resulted in a non-additive stimulation. Despite this, the stimulatory fraction of the myelin was more potent and stimulated neurite outgrowth of NPCs.

Our next step was to determine the mechanism responsible for neurite outgrowth stimulation in these cells. Because molecules such as netrin and MAG exhibit transient periods of neurite stimulation followed by inhibition during development (Brankatschk and Dickson, 2006; Mukhopadhyay et al., 1994), we first examined whether the myelin-induced neurite outgrowth is mediated by

either MAG, Nogo, OMgp, or a combination. We performed neurite outgrowth assays of NPCs plated on myelin deficient in MAG, Nogo, and OMgp, and observed neither a stimulation nor inhibition of outgrowth compared to our wild-type myelin control. Although Turnely and Bartlett report that MAG promotes growth in E17 spinal cord derived neurons, we do not observe a reduction of neurite outgrowth of our NPCs cultured on MAG deficient myelin (Turnley and Bartlett, 1998). This could most likely be attributed to the difference in experimental paradigms, where Turnley and Bartlett used MAG expressing CHO cells as their plating substrate (Turnley and Bartlett, 1998). In contrast to CHO cells expressing a single recombinant protein, our myelin extract contains a complex composition of molecules, where each individual molecule could be either stimulatory or inhibitory to outgrowth. The composition of molecules present in our whole myelin extract makes the direct comparison between our myelin and individual proteins difficult to assess. Additionally, Lee and colleagues report Nogo deficient myelin recovers neurite outgrowth in adult DRGs and post-natal cortical neurons (Lee et al., 2010), indicating the potency of Nogo mediated inhibition. In our cultures, Nogo deficient myelin had no difference from our wild type myelin (Figure 4), suggesting that Nogo is neither inhibitory nor stimulatory to NPCs. From this experiment, we conclude that there is a different molecular signaling mechanism responsible for myelin-induced neurite outgrowth.

In addition to looking for an external mechanism, we examined whether the myelin-induced growth response in NPCs follows canonical growth response mechanisms, specifically ERK activation. Many different stimuli for neurite

outgrowth, including laminin, NGF, and FGF (Liu et al., 2002b), converge upon ERK activation, so we hypothesized that myelin-induced growth activates the Raf-MEK-ERK pathway. At 3 and 6 hours after adding myelin to the cultures, we observe a complete activation of ERK, however, p-ERK levels return to baseline by 24 hours (Figure 5.1). The myelin produces a prolonged, transient activation of ERK that is observed in NGF-induced outgrowth of a variety of neurons (Agthong et al., 2006). Our results of transient ERK activation correlate with our neurite outgrowth data on NPCs, suggesting that ERK is in part, responsible for transducing myelin's growth signal.

Based on the fact that ERK phosphorylation is mediated by its upstream activator MEK, it seemed plausible to test the hypothesis that MEK inhibition will reduce ERK phosphorylation and thereby myelin mediated outgrowth stimulation. To do this, we applied PD 98059, a well-documented, small molecule MEK inhibitor, and quantified neurite outgrowth. Surprisingly, myelin-stimulated neurite outgrowth of the NPCs was not abolished after application of PD 98059. Although we have yet to confirm that either MEK or ERK activation is inhibited, there are several possible explanations for our observations. The most probable explanation is that upon plating the NPCs on the myelin, the ERK signal is propagated prior to adding PD 98058, which was added two hours post-plating of the NPCs; therefore ERK was already phosphorylated before the inhibitor was added. Although less probable, it is possible that ERK activation is mediated independently of MEK. Additionally, ERK activation may not stimulate neurite outgrowth in this cell type. BDNF induced neurite outgrowth in neonatal cochlear

spiral ganglion explants was shown to be regulated by Ras/p38 and PI3K/Akt pathways instead of MEK/ERK signaling (Mullen et al., 2012). We intend to follow up with experiments to elucidate ERK's involvement in myelin signaling.

Neural precursor cells have only recently been successfully transplanted into injured spinal cords, displaying dense axon growth into the white matter and growing for long distances (Lu et al., 2012; Zhao et al., 2013). It is clear through these experiments that CNS adult myelin promotes the growth of murine spinal cord derived multipotent NPCs, at least, in vitro. We predict our observations would hold true in human NPCs, making this type of cell a candidate for translational medicine. In vivo, NPC axons encounter intact myelin after penetrating the injury site, as they continue to grow into the host cord. The in vivo conditions add additional levels of complexity that make translating our in vitro findings difficult. Although previous experiments have proven MAG and OMgp to be stimulatory in E17 NPCs (Turnley and Bartlett, 1998), our experiments determined that neither MAG, Nogo, nor OMgp in myelinis responsible for in stimulating neurite outgrowth of the NPCs. Finally, we know that myelin stimulates ERK activity at early time points; however, we still lack evidence as that addresses whether increased ERK activity is directly responsible for increased growth.

In order to therapeutically utilize neural stem cells, it is optimal to elucidate and understand the mechanisms through which myelin promotes neurite outgrowth. In doing so, we can potentially translate the mechanisms of myelin-

induced axon growth of NPCs into injured adult neurons to promote regeneration through the injury site and into the white matter to innervate the original targets.

Figures

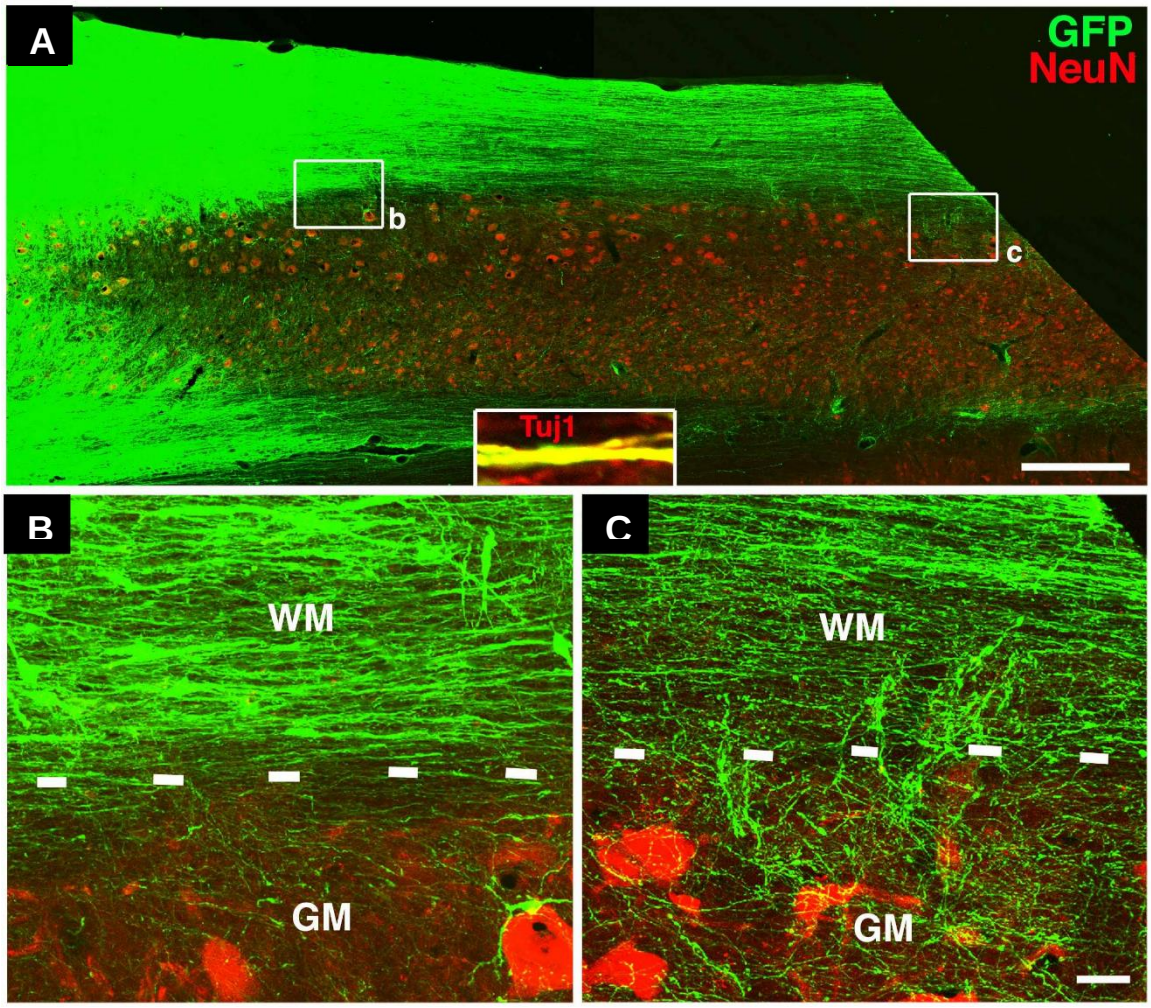


Figure 1: Long distance axonal outgrowth from multipotent neural progenitor cell graft. (**A**) GFP and NeuN immunolabeling reveals that GFP-expressing NPC grafts robustly extend axons into the host spinal cord caudal to the C5 hemisection at two months. Higher magnification displaying the white/gray matter interface (**B**) proximal and (**C**) distal to the lesion (Lu et al., 2012).

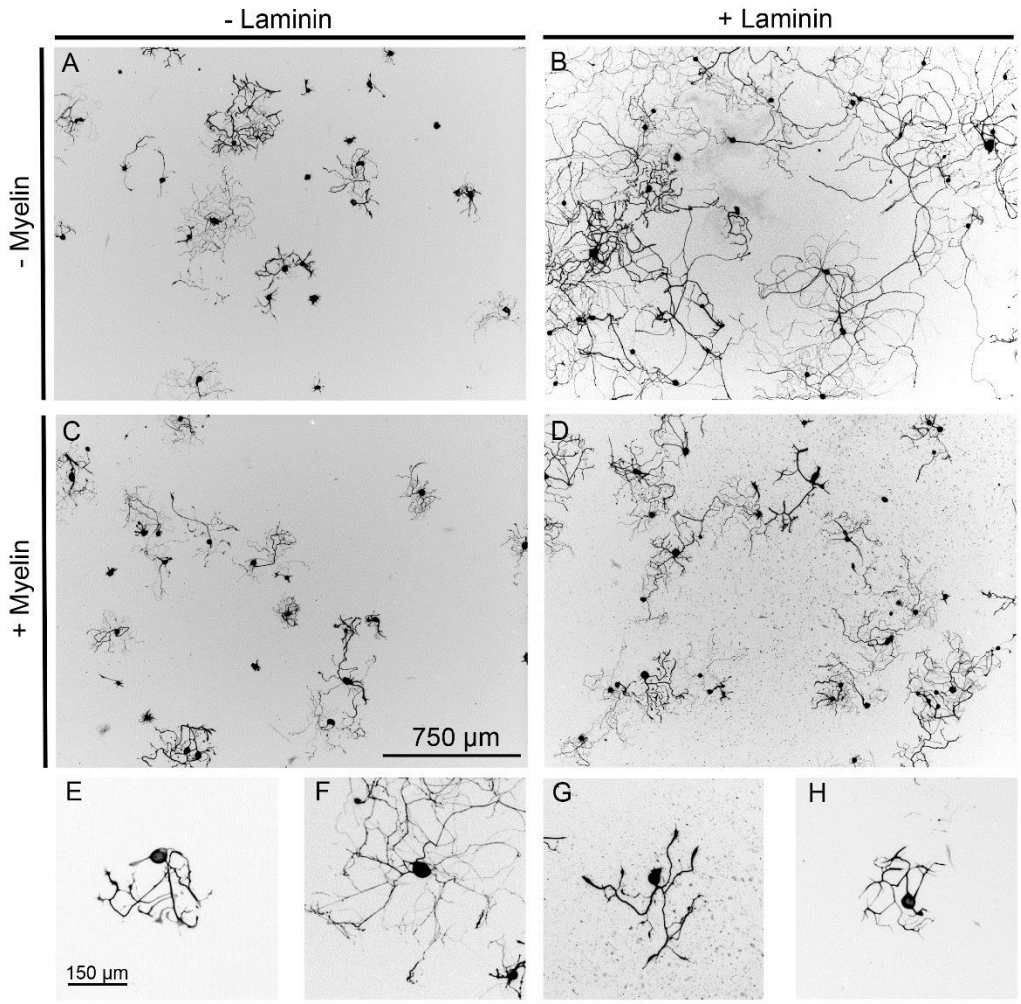


Figure 2.1: Representative images of adult mouse dorsal root ganglion neurons grown in vitro for 48 hours. Cells were grown in DMEM +F12 medium supplemented with B27. Substrates added include: (**A, E**) no substrate, (**B, F**) Laminin (1 µg/ml), (**C, G**) Myelin (10 µg/ml), and (**D, H**) Laminin (1 µg/ml) + Myelin (10 µg/ml). All conditions include a poly-D-lysine coating (20 µg/ml).

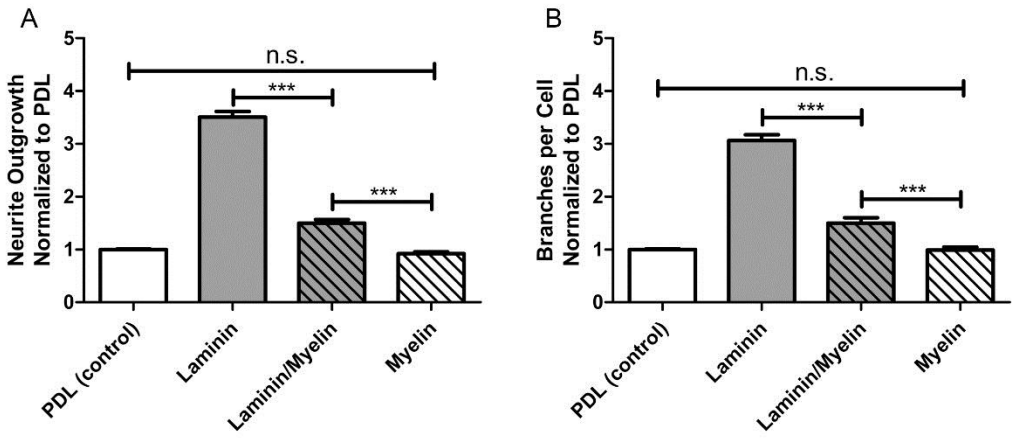


Figure 2.2: Myelin reduces neurite outgrowth and branching in adult DRGs plated on laminin. Quantification of (**A**) neurite outgrowth and (**B**) branching of adult DRGs plated on either laminin (1µg/ml), myelin (10µg/ml), or both substrates after 48 hours. All values were normalized to the PDL condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here ***$p < 0.001$; n.s. = not significant

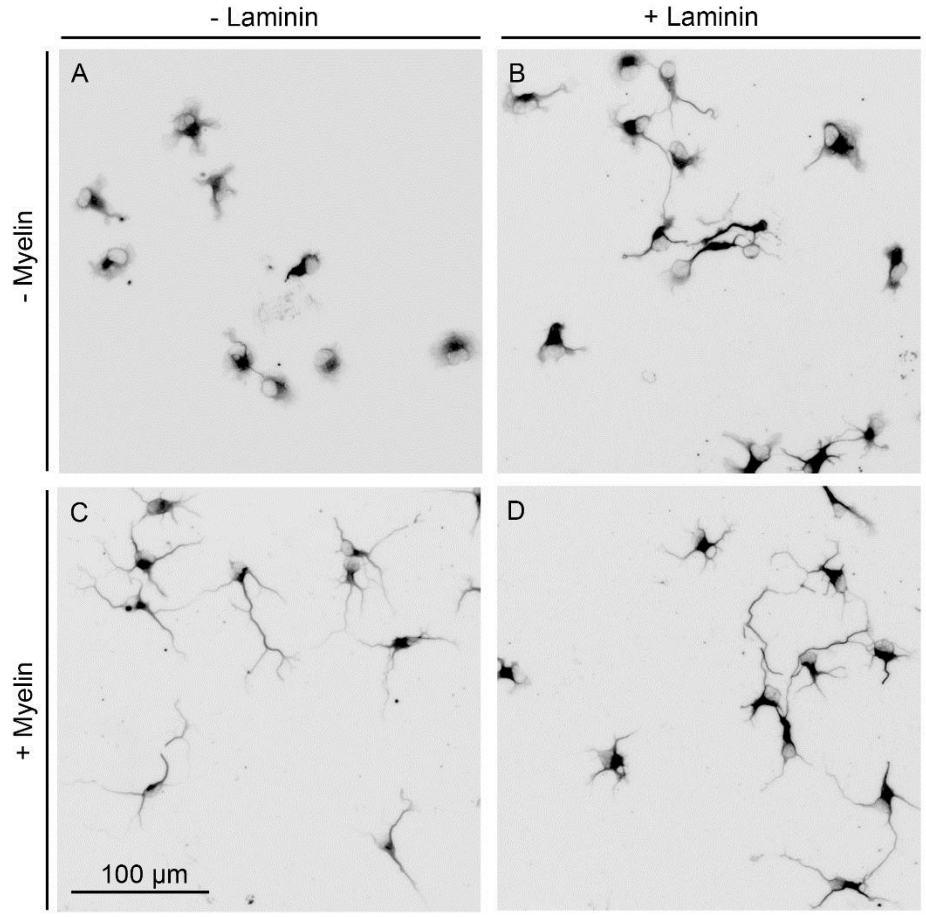


Figure 3.1: Representative images of mouse embryonic (E12) spinal cord derived neural progenitor cells grown in vitro for 48 hrs. Cells were grown in Neurobasal medium supplemented with B27. Substrates added include: (**A**) no substrate, (**B**) Laminin (1 µg/ml), (**C**) Myelin (10 µg/ml), and (**D**) Laminin (1 µg/ml) + Myelin (10 µg/ml). All conditions include a poly-D-lysine coating (20 µg/ml).

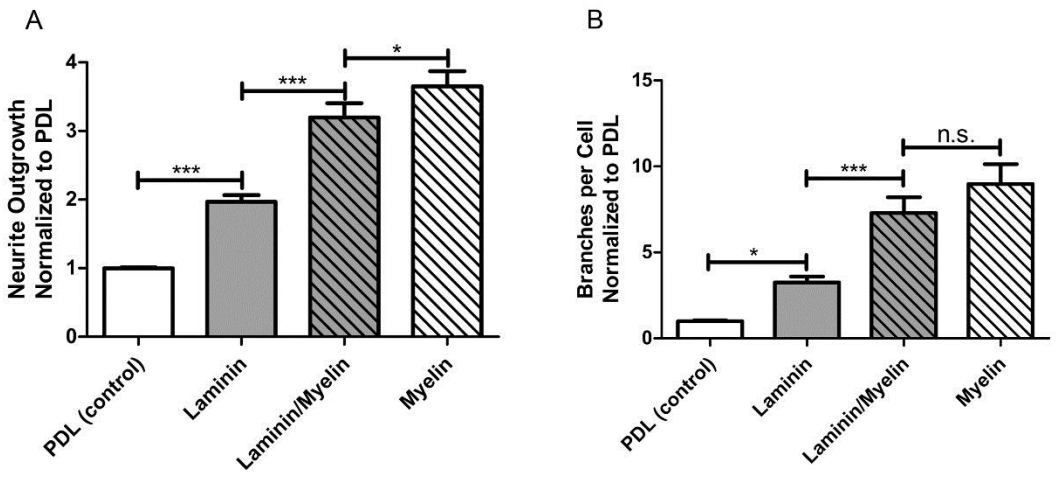


Figure 3.2 Adult CNS myelin promotes neurite outgrowth of mouse E12 spinal cord derived NPCs. Quantification of (**A**) neurite outgrowth and (**B**) branching of E12 Mouse NPCs plated on either laminin (1µg/ml), myelin (10µg/ml), or both substrates after 48 hours. All values were normalized to the PDL condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here *$p < 0.05$; ***$p < .001$; n.s. = not significant

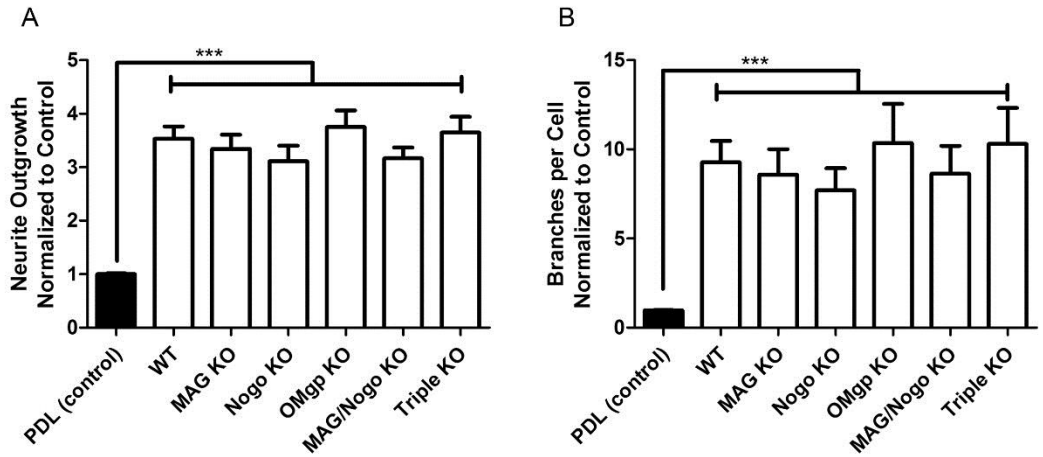


Figure 4: Influence of adult CNS myelin on mouse E12 NPCs cultured on adult CNS myelin deficient in either MAG, Nogo, OMgp, or a combination. Quantities measured were (**A**) neurite outgrowth and (**B**) branches per cell. All values were normalized to the PDL condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here ***$p < .001$

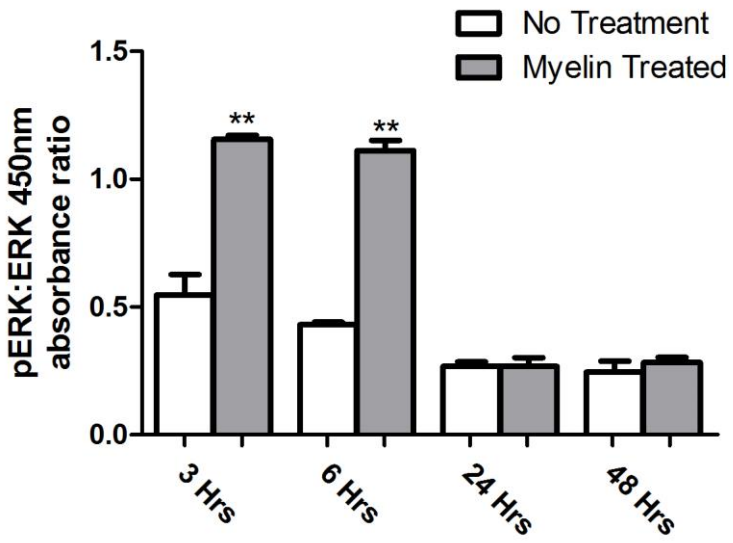


Figure 5.1: Myelin treatment in NPCs increases ERK activity at early timepoints. NPCs received either myelin treatment (gray bars) or no treatment two hours post plating. Lysates were collected at multiple timepoints (3, 6, 24, and 48 hours) and p-ERK and total ERK levels were quantified using ELISA. Values observed are the p-ERK:total ERK absorbance ratio at 450 nm. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here ** $p < 0.01$

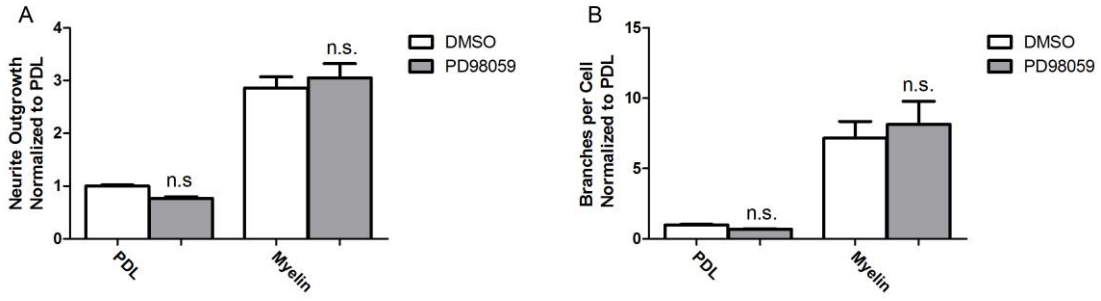
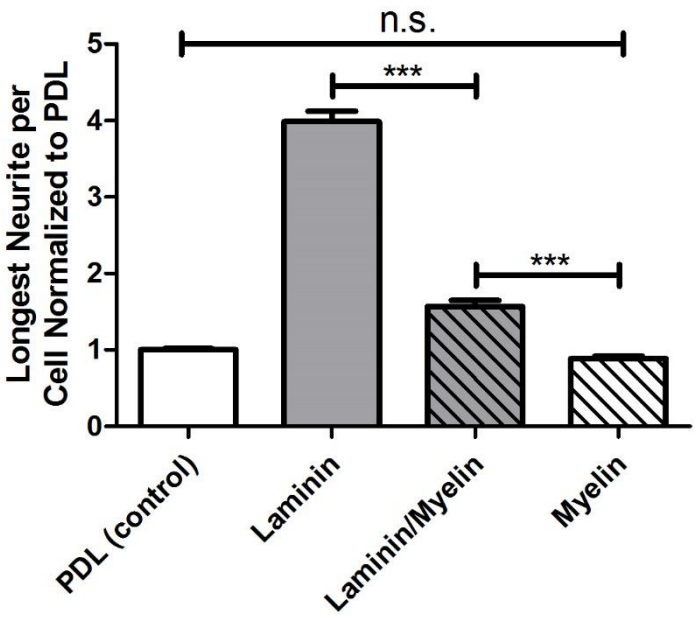
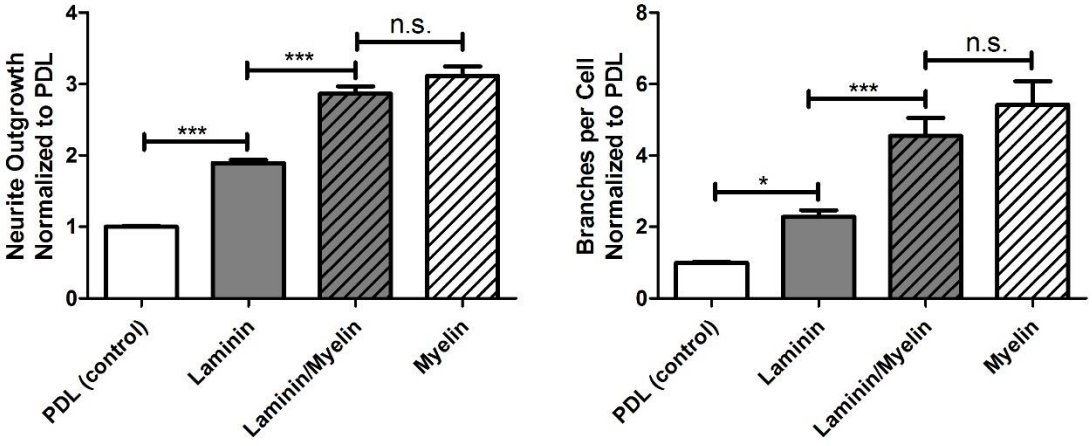


Figure 5.2: Application of MEK inhibitor PD98059 does not reduce neurite outgrowth or branching in NPCs. Quantities measured were (**A**) neurite outgrowth and (**B**) branches per cell. All values were normalized to the PDL condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here n.s. = not significant

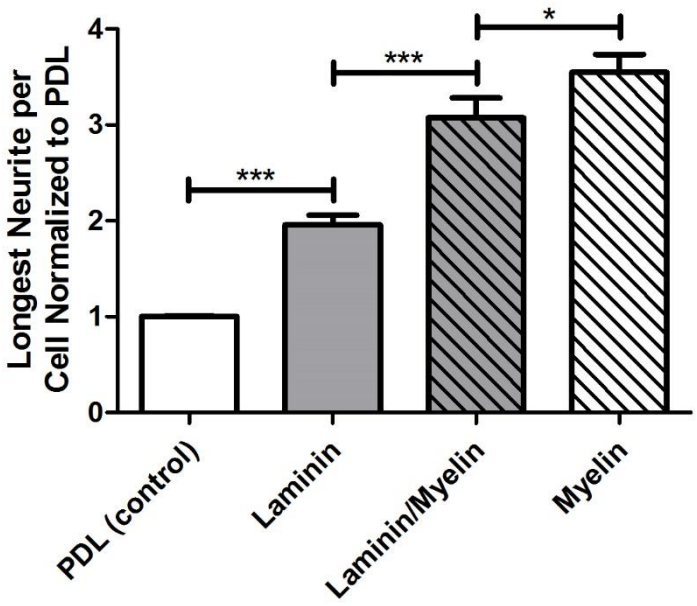
Supplemental Figures



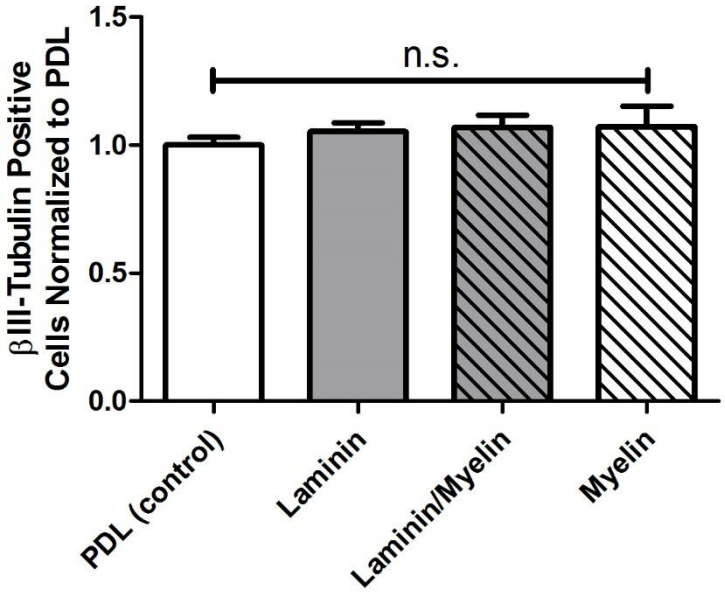
Supplemental Figure 1: Myelin reduces neurite longest neurite in adult DRGs plated on laminin. Quantification of longest neurite per cell of adult DRGs plated on either laminin (1µg/ml), myelin (10µg/ml), or both substrates after 48 hours. All values were normalized to the PDL condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here ***$p < 0.001$; n.s. = not significant



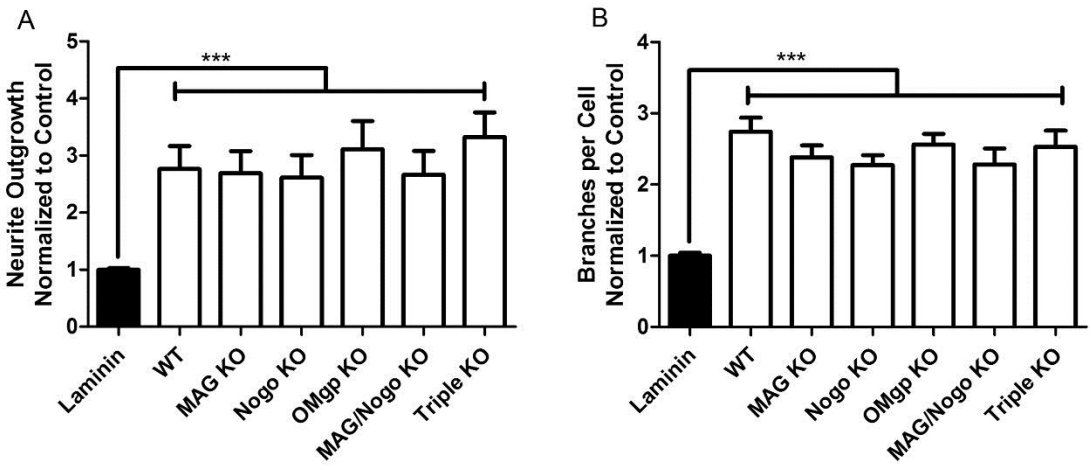
Supplemental Figure 2: Adult CNS myelin promotes neurite outgrowth of rat E14 spinal cord derived NPCs. Quantification of (**A**) neurite outgrowth and (**B**) branching of E14 Rat NPCs plated on either laminin (1µg/ml), myelin (25µg/ml), or both substrates after 48 hours. All values were normalized to the PDL condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here *$p < 0.05$; ***$p < .001$; n.s. = not significant



Supplemental Figure 3: Adult CNS myelin increases longest neurite of mouse E12 spinal cord derived NPCs. Quantification longest neurite per cell of E12 Mouse NPCs plated on either laminin (1µg/ml), myelin (10µg/ml), or both substrates after 48 hours. All values were normalized to the PDL condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here *$p < 0.05$; ***$p < .001$; n.s. = not significant



Supplemental Figure 4: Adult CNS myelin does not induce a greater βIII-tubulin positive population in E12 NPCs in vitro. Quantification βIII-tubulin positive cells plated on either laminin (1µg/ml), myelin (10µg/ml), or both substrates after 48 hours. All values were compared to overall cell count using DAPI, and normalized to the PDL condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here n.s. = not significant



Supplemental Figure 5: Influence of adult CNS myelin on E12 NSCs cultured on Laminin and adult CNS myelin deficient in either MAG, Nogo, OMgp, or a combination. Quantities measured were (**A**) neurite outgrowth and (**B**) branches per cell. All values were normalized to the Laminin condition for each individual experiment. Results represent the mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison test was used to determine significance of differences. Here ***$p < .001$

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