Molecular Mechanisms of Myelin-Associated Glycoprotein (MAG)- and Nogo-Induced Smad2 Phosphorylation

by

Justine Cadieux

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Department of Human Anatomy & Cell Science
University of Manitoba
Winnipeg

Abstract

The myelin-associated inhibitors (MAIs) Nogo-A and myelin-associated glycoprotein (MAG) are potent inhibitors of regeneration in the central nervous system (CNS). They effect inhibition through signaling pathways initiated by activation of the Nogo-66 receptor 1 (NgR1) complex, low-density lipoprotein (LDL) receptor-related protein-1 (LRP1), or paired immunoglobulin-like receptor B (PirB). The Smad2 protein, which is phosphorylated in response to transforming growth factor-β (TGFβ) receptor activation, has been shown to have a role in myelin-mediated inhibition of neurite outgrowth in cerebellar granule neurons (CGNs). We demonstrate that MAG and Nogo strongly induce Smad2 phosphorylation and that inhibiting TGFβ receptor activation abolishes this response in CGNs treated with MAG or Nogo. We have hypothesized that this receptor is being transactivated by another receptor such as NgR1, LRP1, or PirB. siRNA knockdown of NgR1 or LRP1 in CGNs did not result in significant reduction of Smad2 phosphorylation in response to MAG or Nogo. Similarly, CGNs from *PirB*$^{-/-}$ mice displayed no significant reduction in levels of phosphorylated Smad2 following treatment with MAG or Nogo. TGFβ receptor activation by MAIs is thus mediated through an unidentified receptor, and discovering this receptor may provide a novel target for pharmacological intervention as a means of promoting regeneration in the CNS following injury.

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List of Acronyms & Abbreviations

AIS	Axon Initial Segment
AP	Action Potential
BDNF	Brain-Derived Neurotrophic Factor
BMP	Bone Morphogenetic Protein
cAMP	Cyclic Adenosine Monophosphate
CGN	Cerebellar Granule Neuron
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
CREB	cAMP Response Element Binding Protein
CSPG	Chondroitin Sulfate Proteoglycan
CST	Corticospinal Tract
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DRG	Dorsal Root Ganglion
DREZ	Dorsal Root Entry Zone
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EPSP	Excitatory Post-Synaptic Potential
GPI	Glycosylphosphatidylinositol
HRP	Horseradish Peroxidase
LRP1	LDL Receptor-Related Protein 1

LRR	Leucine-Rich Repeat
MAI	Myelin-Associated Inhibitor
MAG	Myelin-Associated Glycoprotein
MBP	Myelin Basic Protein
NgR1	Nogo-66 Receptor 1
NT	Neurotrophin
OMgp	Oligodendrocyte-Myelin Glycoprotein
PirB	Paired Immunoglobulin-Like Receptor B
PNS	Peripheral Nervous System
PLP	Proteolipid Protein
PKA	Protein Kinase A
pSmad2	Phosphorylated Smad2
RAG	Regeneration-Associated Gene
RGC	Retinal Ganglion Cell
ROCK	Rho-Associated Protein Kinase
SARA	Smad Anchor for Receptor Activation
siRNA	Small Interference RNA
TGFβ	Transforming Growth Factor-β
TGFβR	Transforming Growth Factor-β Receptor
TNF	Tumor Necrosis Factor
Trk	Tyrosine Kinase

Chapter 1

Literature Review

1.1 Axonal neurobiology & regeneration

Axons are elongated neuronal processes responsible for the transmission of information between a neuron's soma and its synaptic bouton. Somatodendritic inputs are summated at the axon initial segment (AIS) located near the soma where suprathreshold depolarization of the cell initiates the propagation of an action potential (AP) (Clark et al., 2009). The AP is generated as a result of excitatory post-synaptic potentials (EPSPs) activating the large number of resident voltage-gated Na^+ channels in the AIS and thereby allowing positively charged Na^+ ions to enter the cell (Kole et al., 2008). Because neurons possess a negative resting membrane potential, sodium-induced depolarization past the cell's threshold will trigger an AP. The wave amplitude of this AP is maintained down the length of myelinated axons by virtue of saltatory conduction, which allows the current to "jump" between Nodes of Ranvier. These nodes are discrete segments of unmyelinated axolemma containing tightly packed clusters of voltage-gated Na^+ channels (~1000/μm^2) (Ritchie & Rogart, 1997). As Na^+ ions enter the axon, a chain reaction is induced. Myelinated axon segments lack ionotropic channels and therefore present an area of high electrical resistance and low capitance, preventing leakage of ions from the cell and allowing the current to travel along the membrane towards the Na^+ channels in the next Node of Ranvier (Hartline & Colman, 2007; Simons & Nave, 2015). Once the AP reaches the synaptic terminal, ionotropic Ca^{2+} channels are opened which induces the fusion of neurotransmitter-containing vesicles with the pre-synaptic membrane (Sudhöf, 2012). The neurotransmitters are released into the synaptic cleft where they are free to bind to receptors on the post-synaptic neuron. Although an AP will

propagate along an unmyelinated axon by sequential activation of Na^+ channels spanning the length of the membrane, myelin internodes prove advantageous by increasing the net speed of electrical transmission (Waxman & Bennett, 1972; Nave & Werner, 2014).

1.2 Schwann cells

Myelinating glia form internodes by enwrapping segments of axons numerous times in alternating electron-dense and -light layers of compacted myelin to provide insulation and metabolic support (Saab et al., 2013; Simons & Nave, 2015), as well as increase the conduction velocity of APs (Waxman & Bennett, 1972; Nave & Werner, 2014). Schwann cells are the myelinating glial cells of the PNS, differing functionally and morphologically from oligodendrocytes. Each segment of myelin along a peripheral axon is produced and maintained by a single Schwann cell, however not all Schwann cells are myelinating; small caliber axons are unmyelinated and grouped together by Schwann cells to form Remak bundles (Voyvodic, 1989). Apart from myelinating axons to increase the conduction velocity of APs, Schwann cells also provide trophic support to developing axons (Riethmacher et al., 1997) and regulate the formation of the perineurium and Nodes of Ranvier (Corfas et al., 2004). The integrity of the perineurium was compromised in mice lacking expression of Schwann cell-derived Desert hedgehog (Dhh) and gave rise to the proposition that Dhh is required for fibroblasts to form the perineurium via mesenchymal-to-epithelial transition (Parmantier et al., 1999), whereas Node of Ranvier deformation was observed in mice whose Schwann cells lack dystroglycan expression (Saito et al., 2003). Unlike oligodendrocytes, they are capable of dedifferentiation and redifferentiation depending on axon diameter (Voyvodic, 1989). Whereas developing Schwann cells depend on neurons to ensure their survival, mature Schwann

cells utilize autocrine survival circuits to survive independently of axonal contact which enables Schwann cells to contribute to PNS axon regeneration (Meier et al., 1999).

1.2.1 Regeneration in the peripheral nervous system

Following axotomy in the PNS, the severed distal segment is deprived of nutrients from the soma and begins to undergo Wallerian degeneration. During this highly regulated process, pro- and anti-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) are secreted by activated Schwann cells and hematogenous macrophages (Fenrich & Gordon, 2004; Dubový et al., 2014). By 48 hours post-injury, Schwann cells have ceased the production of myelin proteins, causing myelin to degrade (Trapp et al., 1988). The damaged myelin is phagocytosed by macrophages, leaving healthy myelin intact (Beuche & Friede, 1984; Avellino et al., 1995; Mueller et al., 2003). The rapid clearance of myelin debris relative to the CNS limits the exposure of the injured axon to myelin-associated inhibitory proteins and may contribute to the ability of PNS axons to regenerate (Fenrich & Gordon, 2004).

Upon loss of axonal contact, Schwann cells dedifferentiate into a growth-supportive phenotype resembling immature, developing Schwann cells (Chen et al., 2007; Arthur-Farraj et al., 2012) and go on to proliferate, migrate, and differentiate into myelinating or non-myelinating phenotypes (Hall, 1999; Bhatheja & Field, 2006; Mirsky et al., 2008). These changes coincide with the downregulation of myelin-associated proteins such as P0 and MAG within Schwann cells and the upregulation of neurotrophins such as brain-derived neurotrophic factor (BDNF) and extracellular matrix (ECM) molecules such as laminin and tenascin, both of which are believed to promote neurite regrowth (Martini, 1994; Campbell, 2008; Sulaiman & Gordon, 2013). This process is aided by the degradation of the ECM and cell adhesion molecules via secreted proteases

such as plasminogen activators (PAs) (Siconolfi & Seeds, 2001). The entire process of Wallerian degeneration in the PNS of mammals is relatively rapid, taking approximately 1-2 weeks (George & Griffin, 1994; Vargas & Barres, 2007) and permits regeneration of peripheral axons, which is achieved at a rate of approximately 1-3 mm/day depending on axon diameter and the nature of the injury (Sunderland, 1952; Wujek & Lasek 1983).

1.3 Oligodendrocytes

Oligodendrocytes are responsible for myelination in the CNS and selectively ensheathe axons with a diameter exceeding 0.2 μm (Simons & Trajkovic, 2006). A single oligodendrocyte typically generates 20-60 internodes (Simons & Nave, 2015) that insulate the axon to accelerate the propagation of APs and provide essential metabolic support (Bercury & Macklin, 2015). Oligodendrocyte-derived lactate, for instance, is metabolized within myelinated axons in order to contribute to mitochondrial ATP production (Fünfschilling et al., 2012). Oligodendrocytes are also required for maintaining the integrity and survival of axons, as mice lacking oligodendroglial proteins 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and proteolipid protein (PLP) developed widespread axonal swelling and degeneration (Griffiths et al., 1998; Lappe-Siefke et al., 2003). Oligodendrocytes thus have a variety of roles in the CNS and disruption of their functions can invoke a range of demyelinating (e.g., multiple sclerosis) or dysmyelinating (e.g., leukodystrophies) neuropathies (Suter & Scherer, 2003).

1.3.1 Regeneration in the central nervous system

Transected axons of the PNS readily regenerate and restore function due to upregulation of neurotrophins and ECM molecules, absence of astrocytic scarring, and rapid clearance and

enzymatic degradation of myelin debris. The swift phagocytosis of myelin debris from the site of injury may render the PNS environment more amenable to regenerating neurites (Fenrich & Gordon, 2004). This is not the case in the CNS, where myelin debris is removed from the injury site relatively slowly (George & Griffin, 1994) and can remain several years after CNS axon degeneration in humans (Buss et al., 2004). A combination of several inhibitory factors in the CNS prevents these axons from regenerating to any considerable degree following injury, a phenomenon documented as long ago as 1928 (Cajal, 1928).

The immature mammalian CNS is initially permissive to regrowth and abruptly reverses this response at developmental timepoints dependent on species and neuronal subtype (Mukhopadhyay et al., 1994; DeBellard et al., 1996; Turnley & Bartlett, 1998). Introducing fetal spinal cord tissue into the injured CNS of mature organisms illustrates this point as the fetal grafts permit growth of corticospinal tract (CST) axons (Bregman et al., 1989). The failure of mature CNS axons to regenerate is not only an instrinsic neuronal property but is also the consequence of a non-permissive environment. This was first demonstrated by David & Aguayo (1981) when they used autologous sciatic nerve grafts to bridge two sections of the spinal cord. Twenty-six and thirty weeks following grafting, retrograde and anterograde labelling with horseradish peroxidase (HRP) was used to visualize axons within the graft. Many CNS axons were able to grow in excess of 30 mm into the peripheral nerve bridge, however it could not be determined whether these axons had regenerated from injured neurons or sprouted from uninjured neurons proximal to the graft endings (David & Aguayo, 1981).

1.3.1.1 The conditioning lesion effect

The conditioning lesion effect proves useful in understanding intrinsic mechanisms that can enhance axonal regeneration in the CNS. This phenomenon can be described as increased regeneration in an injured nerve that is observed after the same nerve was previously "conditioned" by a lesion. This effect was first reported in 1973 when increased regeneration of the crushed sciatic nerve was observed following injury to the nerve two weeks prior (McQuarrie & Grafstein, 1973). DRG neurons are particularly useful to study the conditioning lesion effect as they are pseudo-unipolar neurons that have both a central and peripheral process. The lesioned peripheral DRG processes of adult mammals regenerate rapidly (4-7 mm/day) whereas transected central processes reliably fail to traverse the dorsal root entry zone (DREZ) (Ramer et al., 2001).

Retrograde tracing with HRP two to three months following intraspinal implantation of a peripheral nerve graft revealed that transection of L4/L5 DRG peripheral processes one week prior to axotomy of their central processes renders these axons one hundred times more likely to regenerate into the grafts (Richardson & Issa, 1984). A fivefold increase in the number of axons that had regenerated into a fresh graft was observed when a conditioning lesion was performed on the same day as grafting. If the lesion preceded the grafting by one week, however, a sevenfold increase was observed, with 10% of axons regenerating to the rostral end of the graft (Oudega et al., 1994). Conversely, lesioning the DRG central process between the cell body and the DREZ did not enhance regeneration of either the central or peripheral process (Oblinger & Lasek, 1984). Together, these findings highlight that environmental differences between the CNS and PNS play a substantial role in the ability of axons to regenerate.

Knowing that injury to DRG peripheral processes alters the intrinsic growth state of DRG neurons (Schreyer & Skene, 1991), one research group explored whether timing of such a lesion

could affect CNS regeneration. In rats that received a bilateral dorsal column transection, regenerating axons failed to enter the lesion site when visualized with HRP conjugated to cholera toxin subunit B two months or one year post-injury (Neumann & Woolf, 1999). When the sciatic nerves were lesioned concomitant with the dorsal column transection, however, a significant number of axons were found to have penetrated the lesion site (Neumann & Woolf, 1999). More extensive regeneration was observed when the sciatic nerve lesion preceded the dorsal column transection by one or two weeks, whereas no substantial regeneration was observed when sciatic nerve lesioning succeeded the dorsal column transection by two weeks (Neumann & Woolf, 1999). These experiments were the first to demonstrate that altering the intrinsic growth state of DRG neurons permits regeneration in a non-permissive environment and therefore that the inability of CNS axons to regenerate is a result of both intrinsic and extrinsic factors (Neumann & Woolf, 1999).

The molecular events underlying the conditioning lesion effect are partly explained by the upregulation of regeneration-associated genes (RAGs) including neuropeptide Y (Finelli et al., 2013), growth-associated protein-43 (GAP-43) (Schreyer & Skene, 1993), CAP-23 (Bomze et al., 2001), c-Jun (Raivich et al., 2004), SRY-box containing gene 11 (Sox11) (Jankowski et al., 2009), small proline-repeat protein 1A (Sprr1a) (Bonilla et al., 2002), and activating transcription factor-3 (ATF-3) (Seijffers et al., 2006). Another important transcription-mediated event promoting regeneration of DRG central processes is the upregulation of cyclic adenosine monophosphate (cAMP) in DRG neurons following a conditioning lesion. Intracellular levels of cAMP were shown to triple one day following lesioning of DRG peripheral processes (Qiu et al., 2002). As will be discussed shortly, myelin restricts axonal growth in the CNS (Schwab & Caroni, 1988), and endogenous cAMP levels drop rapidly in postnatal day (P)3-4 DRG neurons, which coincides

with the onset of myelin-mediated inhibition in these neurons (Cai et al., 2001). Injecting dibutyryl (db)-cAMP, a membrane-permeable, non-hydrolyzable cAMP analogue, into DRGs one, two, or seven days prior to being extracted and plated on a myelin substrate led to increased neurite outgrowth and demonstrated that the inhibitory properties of myelin could be overcome by elevating cAMP (Neumann et al., 2002; Qiu et al., 2002).

Intraganglionic injection of db-cAMP can also promote regeneration of dorsal column axons *in vivo*. L5 DRGs were injected with db-cAMP in the absence of a conditioning lesion one week prior to lesion of dorsal column axons. Transganglionic labeling with HRP one or two weeks following the dorsal column lesion revealed extensive regeneration of these axons (Qiu et al., 2002). A similar observation was made when L4/L5 DRGs were injected with db-cAMP 48 hours preceding a dorsal column lesion, with axons displaying robust regeneration into the lesion site whereas non-treated axons failed to regenerate into the lesion site, showing that increasing intracellular cAMP is sufficient to overcome inhibition (Neumann et al., 2002).

The role of cAMP in axonal regeneration is initially dependent on protein kinase A (PKA) activation, as PKA inhibitors applied 24 hours after a sciatic nerve lesion rendered DRG neurons incapable of overcoming inhibition by myelin (Qiu et al., 2002). One week following a lesion, however, the ability of cAMP to overcome myelin becomes independent of PKA (Qiu et al., 2002), relying instead on transcription via cAMP response element binding protein (CREB) (Lonze & Ginty, 2002). cAMP-induced transcription of the enzyme Arginase I (Arg I), which catalyzes the synthesis of polyamines, is capable of overcoming inhibition by myelin (Cai et al., 2002) and Arg I transcription in response to cAMP was found to be dependent on CREB (Gao et al., 2004). This was observed when CGNs and DRG neurons expressing a constitutively active form of CREB were no longer inhibited by myelin substrates and over 50% of Arg I expression was lost when

neurons expressing dominant-negative CREB were treated with db-cAMP (Gao et al., 2004). It was also found that injection of constitutively active CREB into DRGs four days prior to a dorsal column lesion allowed central processes to regenerate into and beyond the lesion centre (Gao et al., 2004). cAMP and its downstream targets thus play an important role in the conditioning lesion effect and understanding intrinsic factors that regulate CNS axon regeneration (Hannila & Filbin, 2008).

1.3.1.2 Astroglial scarring & chondroitin sulfate proteoglycans

Astrocytes are glial cells responsible for metabolic support of neurons and the regulation of synaptic plasticity, cerebral blood flow, and extracellular ion balance (Pellerin et al., 2007; Barres, 2008). Injury to the CNS induces reactive astrogliosis, a gradated response whereby astrocytes become hypertrophic, proliferate, and undergo transcriptional alterations culminating in the formation of a glial scar through which axons have difficulty regenerating (Rudge & Silver, 1990; Pekny & Nilsson, 2005). A few beneficial consequences of the glial scar include prevention of the spread of inflammation to surrounding healthy tissue and proper sealing of the blood-brain barrier (BBB) to prevent extravasation of harmful hematogenous leukocytes such as monocytes and lymphocytes (Bush et al., 1999; Faulkner et al., 2004).

CNS regeneration is limited not only by the glial scar but also by the elevated secretion of chondroitin sulfate proteoglycans (CSPGs) such as neurocan, brevican, and phosphacan from reactive astrocytes (McKeon et al., 1999; Jones et al., 2003). CSPGs are ECM proteoglycans possessing one or more covalently bound glycosaminoglycan (GAG) side chains (Avram et al., 2014) and are expressed by astrocytes in the CNS in response to cytokines such as transforming growth factor-β (TGFβ) and epidermal growth factor (EGF) (Smith & Strunz, 2005). Protein

tyrosine phosphatase-σ (PTPσ) and leukocyte common antigen-related phosphatase (LAR) are functional receptors for CSPGs (Shen et al., 2009; Fisher et al., 2011). CSPGs are vital throughout development for restricting neurite formation and elongation, synaptogenesis, gliogenesis, cell migration, and receptor binding (Bartus et al., 2012). Their expression is downregulated in the mature CNS and proves deleterious following CNS trauma as they render growth cones dystrophic and prevent regeneration via activation of the RhoA pathway (Monnier et al., 2003; Tom et al., 2004). Enzymatic degradation of the GAG chains of CSPGs using chondroitinase ABC promotes regeneration of axons in *in vitro* and *in vivo* models of CNS injury (Laabs et al., 2007; Bradbury & Carter, 2010). Careful laser-mediated CNS axotomy such that no glial scar forms, however, still fails to promote regeneration (Lorenzana et al., 2015). This observation could be explained by the effects of inhibitory proteins associated with myelin.

1.3.2 Myelin composition

Oligodendrocytes in the CNS and Schwann cells in the PNS produce the myelin that ensheathes axons. This phospholipid membrane has a dry mass composed of 70-75% lipids and 25-30% proteins (Cuzner & Norton, 1996; Simons & Nave, 2015). Proteolipid protein (PLP) and myelin basic protein (MBP) represent the most abundant proteins found within compacted CNS myelin, accounting for ~50% and ~30% of total myelin proteins, respectively (Cuzner & Norton, 1996). PLP is replaced by the type I integral membrane glycoprotein P0 as the most abundant (>70%) myelin protein in the PNS (Yin et al., 2006). The inhibitory nature of CNS myelin is attributed to specific myelin-associated inhibitors (MAIs) including myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Nogo-A (Chen et

al., 2000; GrandPré et al., 2000; Prinjha et al., 2000), and oligodendrocyte-myelin glycoprotein (OMgp) (Wang et al., 2002a).

1.3.3 Myelin-associated inhibitors

1.3.3.1 Myelin-associated glycoprotein

MAG is a 100 kDa type 1 transmembrane glycoprotein expressed exclusively in myelinating glia. It belongs to the sialic acid-binding family of immunoglobulin (Ig)-type lectins (Siglec), which are classified by their homologous amino acid sequences in the first four Ig-like domains (Kelm et al., 1998) and their selective binding affinity to sialoglycoconjugates (Crocker et al., 1998). The Siglec family includes five members to date: Schwann cell myelin protein (SMP), sialoadhesin, MAG, CD22, and CD33 (Crocker et al., 1996). Of these, MAG (Siglec-4a) is responsible for initiating myelination and maintaining both the integrity of the periaxonal space and appropriate myelin compaction in the healthy nervous system (Trapp et al., 1984; Schachner & Bartsch, 2000; Quarles, 2007). The role of MAG in mediating glia-axon interactions is supported by studies showing localization of MAG at the periaxonal membranes of myelin sheaths rather than within compacted myelin, which explains why MAG is considered "myelin-associated" rather than a component of myelin itself (Sternberger et al., 1979; Quarles, 2002). Studies of *Mag*$^{-/-}$ mice revealed aberrant myelination, decreased axon caliber, and axonal degeneration, indicating that MAG is an important factor in the survival and maintenance of healthy axons (Li et al., 1994; Montag et al., 1994; Fruttiger et al., 1995; Yin et al., 1998).

Structurally, MAG is comprised of five extracellular Ig-like domains, one transmembrane domain, and one cytosolic domain (Salzer et al., 1987, 1990). MAG exists in two developmentally regulated isoforms, S-MAG and L-MAG, as a result of alternative splicing. S-MAG contains a

short intracellular C-terminal sequence and L-MAG contains a long C-terminal sequence (Lai et al., 1987; Quarles et al., 1992; Georgiou et al., 2004). In mice, both splice variants are present in comparable amounts in the mature CNS, although L-MAG expression is elevated during early myelinogenesis whereas S-MAG expression predominates in the adult CNS. Conversely, the PNS is characterized by high expression of S-MAG throughout development (Tropak et al., 1988).

In 1994 the laboratory of Marie Filbin developed a neurite outgrowth assay using Chinese hamster ovary (CHO) cells transfected to express MAG in order to isolate its effects from those of myelin in general. CGNs of various ages grown on monolayers of these CHO cells displayed a ~70% reduction in neurite outgrowth compared to CGNs grown on control transfected CHO cells and an anti-MAG antibody was capable of reducing the inhibition by ~50% (Mukhopadhyay et al., 1994). Adult DRG neurons were also inhibited, albeit to a lesser extent, when plated on MAG-expressing CHO cells (Mukhopadhyay et al., 1994). The same year, another team corroborated the involvement of MAG in growth inhibition by demonstrating that recombinant NG108-15 cells failed to extend neurites when treated with MAG ectodomain and neurite growth was restored when MAG was immunodepleted from myelin (McKerracher et al., 1994). Together, these experiments provided the first evidence that MAG is responsible for inhibition of neurite outgrowth.

MAG operates bifunctionally, promoting neurite outgrowth during embryonic development yet inhibiting it in the adult CNS. The developmental timepoint at which this occurs, however, is dependent on cell type. Neurites from P1 DRG neurons cocultured for 12 hours with MAG-expressing CHO cells were nearly twice as long as DRG neurons plated on control cells, whereas outgrowth from adult DRG neurons was inhibited by MAG (Mukhopadhyay et al., 1994). While the developmental timepoint at which DRGs display a change in response to MAG is P3

(DeBellard et al., 1996), postnatal CGNs, hippocampal neurons, retinal neurons, spinal neurons, and superior cervical ganglion (SCG) neurons of any age are all inhibited by MAG (Johnson et al., 1989; Mukhopadhyay et al., 1994; DeBellard et al., 1996; Turnley & Bartlett, 1998). The switch from promotion to inhibition was found to be dependent on intracellular cAMP levels, which decline in concordance with the functional switch from promotion to inhibition (Cai et al., 2001). Artificial elevation of cAMP via db-cAMP completely reversed the inhibition of CGNs and DRG neurons on MAG-expressing CHO cells in a dose-dependent manner and allowed these neurons to extend neurites twice as long on a substrate of purified myelin as CGNs and DRG neurons that did not receive db-cAMP (Cai et al., 1999).

1.3.3.2 Nogo-A

In 1988, two CNS myelin fragments named NI-35 and NI-250 were identified as potent neurite outgrowth inhibitors (Caroni & Schwab, 1988b). Application of a monoclonal antibody raised against NI-35/250, IN-1, via implantation of IN-1-producing tumours or pumps allowed regenerative sprouting of CST axons *in vivo* (Schnell & Schwab, 1990; Bregman et al., 1995) and reduced the inhibitory effects of CNS myelin on SCG neurons and neuroblastoma cells *in vitro* (Caroni & Schwab, 1988a). This marked an important milestone in the field of nervous system plasticity as it demonstrated that the inhibitory effects of CNS myelin were a result of specific myelin-associated proteins. The antigen of IN-1 was identified a decade later as Nogo (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000), which consists of an extracellular hydrophilic 66-amino acid domain (Nogo-66) flanked by two hydrophobic segments and a variable-length amino-terminal segment (Oertle & Schwab, 2003; Yang & Strittmatter, 2007). Nogo-66 is responsible for the induction of growth cone collapse and abortive regeneration which can also be

induced, via a separate receptor, by the N-terminal amino-Nogo domain (Δ20) of Nogo-A (Fournier et al., 2001; Oertle et al., 2003).

Along with Nogo-A, Nogo-B and -C are the other transcript variants of the eukaryotic reticulon-4 (*RTN4*) gene, which is implicated in the regulation of blood vessel density and plasticity of the nervous system (GrandPré et al., 2000; Oertle et al., 2003). These isoforms share with their paralogues (*RTN1-3*) a reticulon (RTN) homology domain in their carboxyl-termini consisting of 188 amino acids (Oertle & Schwab, 2003; Schwab, 2010). The reticulon name originates from the localization of this protein to the endoplasmic reticulum when overexpressed in kidney cells (van de Velde et al., 1994). Nogo-A is the RTN-4 isoform enriched in oligodendrocytes (Huber et al., 2002; Wang et al., 2002) and it is expressed to a lesser extent in a variety of neurons including Purkinje cells, hippocampal neurons, retinal ganglion cells (RGCs), and motoneurons (Josephson et al., 2001; Liu et al., 2002; Hunt et al., 2003). Nogo-A-transfected cells added to chick embryonic day (E)12 DRG explants resulted in growth cone collapse and inhibition of neurite outgrowth (GrandPré et al., 2000). In *Nogo-A*$^{-/-}$ mice, adult DRG neurons and CGNs grow extended neurites on myelin substrates and injured CST axons display extensive sprouting and improved locomotor function, indicating that Nogo-A is a potent inhibitor of regeneration (Kim et al., 2003; Simonen et al., 2003).

1.3.3.3 Oligodendrocyte-myelin glycoprotein

The most recent MAI to be identified is OMgp (Wang et al., 2002a), a 433-amino acid glycosylphosphatidylinositol (GPI)-linked protein highly enriched at the oligodendroglial-axon junction. It is anchored to the outer leaflet of oligodendrocyte plasma membranes as well as adult mouse pyramidal neurons of the hippocampus, brainstem motoneurons, Purkinje cells, and anterior

horn motor neurons of the spinal cord (Mikol et al., 1990; Habib et al., 1998). It is a 110 kDa protein consisting of a series of tandem leucine-rich repeats (LRRs), a serine/threonine-rich domain, and a short cysteine-rich motif (Mikol et al., 1990). The potent inhibitory properties of OMgp *in vitro* were discovered when purified recombinant OMgp was found to inhibit neurite outgrowth from DRG neurons and CGNs in a dose-dependent manner (Wang et al., 2002a). Depleting OMgp from solubilized CNS myelin also significantly lowered the degree of inhibition (Wang et al., 2002a). Comparing myelin fractions enriched in either OMgp, MAG, or Nogo-A revealed that OMgp inhibits neurite outgrowth to a greater extent than MAG and to a similar extent as Nogo-A (Wang et al., 2002a).

1.4 Receptors & signaling pathways for myelin-associated inhibitors

MAG, Nogo-A, and OMgp exert their effects through several signaling mechanisms. Binding partners for MAG to date include microtubule-associated protein 1B (MAP1B) (Franzen et al., 2001), heparin (Fahrig et al., 1987), collagen (Probstmeier et al., 1992), fibronectin (Strenge et al., 2001), and tenascin-R (Yang et al., 1999). Known receptors for MAG include β1-integrin (Goh et al., 2008), Nogo-66 receptor 1 (NgR1) (Domeniconi et al., 2002; Liu et al., 2002), NgR2 (Venkatesh et al., 2005), low-density lipoprotein (LDL) receptor-related protein-1 (LRP1) (Stiles et al., 2013), and paired immunoglobulin-like receptor B (PirB) (Atwal et al., 2008). Binding partners for Nogo-A and OMgp include NgR1 (Fournier et al., 2001; Wang et al., 2002b) and PirB (Atwal et al., 2008).

1.4.1 Gangliosides

Given its structure, MAG was initially believed to bind to sialic acid-bearing glycosphingolipids called gangliosides on the cell surface, which was later confirmed by studies that demonstrated its high affinity binding to GD1a and GT1b (Kelm et al., 1994; Collins et al., 1997; Vinson et al., 2001; Vyas et al., 2002). Complex gangliosides such as these are ubiquitous in the outer leaflet of all cellular membranes and found abundantly in the plasma membranes of neurons (Al-Bashir et al., 2016). The sialic acid binding site of MAG is localized to arginine 118 (R118) in the first Ig-like domain and is independent of MAG's potent inhibitory effects, as mutations of R118 have no effect on inhibition (Tang et al., 1997). A truncated form of soluble MAG containing only the first three Ig-like domains (MAG1-3-Fc), however, binds to neurons in a sialic acid-dependent manner but fails to inhibit neurite outgrowth, indicating that inhibition is regulated by the fourth or fifth Ig-like domain (Tang et al., 1997).

MAG's ability to induce inhibition of neurite outgrowth was initially proposed to be a result of MAG-ganglioside interactions (Vinson et al., 2001). Supporting this idea were findings showing that removing sialic acids from the surfaces of CGNs via *Vibrio cholerae* neuraminidase treatment or depleting gangliosides via treatment with a glycosylceramide synthase inhibitor both reverse MAG-induced inhibition (Vyas et al., 2002). The ability of GT1b to contribute to MAG-induced inhibition, however, depends on its binding with NgR1 (Williams et al., 2008). The site mediating inhibition of neurite ougrowth was later localized to the fifth domain of MAG and it was found to operate independently of sialic acid-binding when MAG is membrane-bound but not when it is soluble (Tang et al., 1997; Cao et al., 2007; Al-Bashir et al., 2016).

1.4.2 Nogo-66 receptor 1 complex

The 473-amino acid NgR1 is a GPI-linked cell surface receptor composed of a signal sequence, eight LRRs, and a cysteine-rich C-terminal LRR flanking domain (Fournier et al., 2001). NgR1 is expressed in a variety of neurons, including olfactory epithelium neurons, CGNs, hippocampal neurons, Purkinje cells, and layer 5 pyramidal neurons of the sensorimotor cortex (Barrette et al., 2007; Funahashi et al., 2008; Richard et al., 2009). The GPI linkage anchors NgR1 to the plasma membrane and renders it dependent on a transduction partner to mediate neuronal growth inhibition. To date, two have been identified. The first to be discovered was the low affinity neurotrophin receptor p75 ($p75^{NTR}$) (Wang et al., 2002; Wong et al., 2002) followed by TAJ/TROY, another member of the tumor necrosis factor (TNF) receptor family (Park et al., 2005; Shao et al., 2005). All three myelin-associated inhibitors are functional ligands for NgR1 (Fournier et al., 2001; Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002a), although MAG binds with a higher affinity to its homologue NgR2 (Venkatesh et al., 2005; Liu et al., 2006; Giger et al., 2008). Ectopic expression of NgR2—normally found in P7 CGNs, adult CGNs, and Purkinje neurons (Zheng et al., 2005)—in neonatal DRG neurons leads to inhibition of neurite outgrowth in these neurons when exposed to MAG or myelin (Venkatesh et al., 2005), suggesting a possible role for NgR2 in MAG-mediated inhibition of neurite outgrowth.

The binding of all three MAIs to NgR1 is surprising due to the lack of structural or sequence homology between MAG, Nogo-A, and OMgp, presenting an important convergence point for the inhibitory effects of all MAIs. Blocking the function of this receptor using an anti-NgR1 antibody, soluble NgR1 ectodomain, or a dominant-negative form of NgR1 abolishes MAG's capacity to induce inhibition of neurite outgrowth in RGCs, hippocampal neurons, and DRG neurons (Domeniconi et al., 2002; Liu et al., 2002; Robak et al., 2009). An anti-NgR1

monoclonal antibody can also prevent binding of MAG, Nogo-A, and OMgp to NgR1 and allows P3 DRG neurons to extend neurites on a substrate of CNS myelin (Li et al., 2004). Overexpression of this receptor in embryonic RGCs, which are typically unresponsive to MAIs, confers responsiveness to these myelin proteins leading to neurite outgrowth inhibition (Fournier et al., 2001).

Genetic deletion of NgR1, however, does not completely remove the inhibitory effect of MAIs (Zheng et al., 2005; Chivatakarn et al., 2007). In fact, dissociated *NgR*$^{-/-}$ CGNs and DRG neurons cultured on a myelin substrate were inhibited to a similiar extent as neurons from NgR heterozygous mice. CGNs from *NgR*$^{-/-}$ mice grown on recombinant Fc-Nogo-66 fusion protein also failed to display significant increases in neurite outgrowth in comparison to control neurons. Genetic deletion of NgR1 does not significantly enhance regeneration *in vivo*, as dorsal hemisection resulted in a lack of CST axon regeneration in both *NgR*$^{-/-}$ and wild-type (WT) mice (Zheng et al., 2005). These results suggest that other receptors are involved in myelin-mediated inhibition. Because NgR1 is GPI-linked, it is necessary to consider the effects of its transduction partner p75^{NTR}.

p75^{NTR} is a 75 kDa low-affinity receptor for four neurotrophins: nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). These secreted growth factors regulate neuronal survival and differentiation throughout vertebrate development (Gentry et al., 2004). p75^{NTR} is a member of the TNF receptor superfamily and is known for its involvement in regulating neural cell death. It possesses four highly conserved extracellular cysteine-rich domains and an intracellular death domain responsible for inducing apoptosis in the absence of ligand binding (Bredesen & Rabizadeh, 1997; Rabizadeh & Bredesen, 2003). Conversely,

neurotrophin binding to $p75^{NTR}$ allows it to act as a co-receptor for tyrosine kinase (Trk) receptor family members TrkA, TrkB, or TrkC which prevent cell death.

Neurite outgrowth from $p75^{NTR-/-}$ DRG neurons and CGNs was no longer inhibited by MAG, providing the first evidence of $p75^{NTR}$'s role in mediating myelin-associated inhibition (Yamashita et al., 2002). $p75^{NTR}$ acts as a signal-transducing moiety within the NgR1 complex and activates the small GTPase RhoA (Yamashita & Tohyama, 2003). Immunoprecipitation assays determined that $p75^{NTR}$ could physically associate with NgR1 (Wang et al., 2002; Wong et al., 2002), and two years later LINGO-1 was identified as a third component of this receptor complex capable of direct binding to NgR1 (Mi et al., 2004). The ternary NgR1 complex induces greater RhoA-GTP activation in response to any of the three MAIs than any binary combination of NgR1, $p75^{NTR}$, or LINGO-1 (Mi et al., 2004). Upon binding of MAIs, this complex activates protein kinase C (PKC) within the cell (Sivasankaran et al., 2004) which enables Rho-guanine nucleotide exchange factors (GEFs) to catalyze the exchange of RhoA-bound GDP for GTP, which leads to the activation of Rho-associated protein kinase (ROCK). ROCK activation leads to phosphorylation of LIM kinase (LIMK) and cofilin resulting in cytoskeletal actin disassembly and eventual growth cone collapse (Mackay et al., 1995; Dergham et al., 2002).

The importance of $p75^{NTR}$ in limiting regeneration *in vivo* is unclear. One study found that $p75^{NTR-/-}$ mice do not display significant regeneration of injured CST axons (Song et al., 2004). P7 CGNs from these mice were, however, less inhibited by Nogo-66 *in vitro* than CGNs from control mice (Zheng et al., 2005). DRG neurons from $p75^{NTR-/-}$ mice are no longer inhibited by MAIs *in vitro* (Wang et al., 2002b); however the relevance of these findings *in vivo* is limited as $p75^{NTR}$ expression within the spinal cord is limited to a small subset of ascending sensory axons (Song et al., 2004; Shao et al., 2005). This led two groups to identify another TNF family member,

TAJ/TROY, which is capable of substituting for $p75^{NTR}$ in the ternary NgR1 complex and inhibiting neurite outgrowth via RhoA activation (Park et al., 2005; Shao et al., 2005). CGNs and DRG neurons in which TROY activity was inhibited were able to extend longer processes than untreated cells on substrates of Nogo-66, OMgp, or myelin (Park et al., 2005; Shao et al., 2005).

1.4.3 Paired immunoglobulin-like receptor B

In 2008, human leukocyte Ig-like receptor B2 (LILRB2), whose mouse orthologue is paired immunoglobulin-like receptor B (PirB), was identified as another functional receptor for MAIs by the Tessier-Lavigne group (Atwal et al., 2008). This type I transmembrane glycoprotein recognizes major histocompatibility complex class I (MHCI) molecules, known for their role in cellular-mediated immunity. PirB plays a role in limiting synaptic plasticity, evidenced by the increased plasticity observed in the visual cortex following monocular deprivation or enucleation in *PirB*$^{-/-}$ mice (Syken et al., 2006; Datwani et al., 2009). More recently, PirB's functions have been expanded to include inhibition of CNS axon regeneration, as disrupting its activity with anti-PirB antibodies or a truncated form of PirB partially reverses neurite outgrowth inhibition by MAG, Nogo-A, and OMgp (Atwal et al., 2008; Matsushita et al., 2011).

PirB shares 50% amino acid homology with LILRB2 and consists of six extracellular Ig-like domains, a hydrophobic transmembrane domain, and a cytosolic segment (Filbin, 2008; Gou et al., 2014). Within the CNS, subsets of neurons and astrocytes express PirB in the cerebral cortex, olfactory bulb, cerebellum, and hippocampus (Gou et al., 2014). It is not expressed in the adult cervical spinal cord (Omoto et al., 2010), although significant upregulation of PirB can be found surrounding the injury site within the damaged spinal cord (Bombeiro et al., 2016). Nevertheless, *PirB*$^{-/-}$ mice do not display enhanced regenerative sprouting of CST axons following a dorsal spinal

cord hemisection, suggesting that its contribution to restricting CNS axon regeneration is limited (Omoto et al., 2010; Nakamura et al., 2011). Likewise, disruption of PirB activity, either with anti-PirB antibodies or via targeted deletion of the *PirB* gene, only partially restores neurite growth in CGNs and DRG neurons exposed to Nogo-66 or MAG *in vitro* (Atwal et al., 2008). Concurrently blocking the actions of NgR1 and PirB by culturing CGNs from *NgR1*$^{-/-}$ mice in the presence of an anti-PirB antibody also proved to be ineffective at completely abolishing inhibition by Nogo-66, suggesting the presence of other receptors for this inhibitory Nogo-A fragment (Atwal et al., 2008). The effects of MAG or OMgp on *NgR1*$^{-/-}$ CGNs in the presence of a PirB antibody have not yet been examined, nor is it clear whether PirB belongs to a receptor complex or acts autonomously.

The possibility that PirB exerts its effects via RhoA signaling was established when it was observed that anti-PirB antibodies prevented the activation of ROCK that is normally induced following injury to cortical neurons via oxygen and glucose deprivation (Wang et al., 2012). Interestingly, PirB has been shown to associate with p75^{NTR}, as co-immunoprecipitation assays revealed that CGNs treated with MAG, Nogo, or OMgp enhance the interaction between p75^{NTR} and PirB (Fujita et al., 2011). Since p75^{NTR} is a known co-receptor of NgR1, these findings raise the possibility that PirB may directly or indirectly interact with the NgR1 complex in response to MAIs.

1.4.4 Low-density lipoprotein receptor-related protein 1

LRP1 is a 600 kDa type I transmembrane receptor. It is a cell signaling and endocytic receptor for ligands that include apolipoprotein E, amyloid Aβ, matrix metalloproteinase 9, activated α_2-macroglobulin (α_2M), and tissue-type plasminogen activator (tPA) (Kowal et al., 1989; Herz &

Strickland, 2001; Hu et al., 2006; Padmasekar et al., 2007; Mantuano et al., 2008; Holtzman et al., 2012). The interaction of LRP1 with these numerous ligands implicates it in diverse activities such as synaptic transmission, cellular metabolism, migration, survival, and BBB integrity (Hayashi et al., 2007; May et al., 2007; Polavarapu et al., 2007). α_2M or tPA binding to LRP1 was also shown to promote neurite outgrowth in CGNs and PC12 cells by downstream transactivation of the TrkA receptor via Src family kinase (SFK) activation (Shi et al., 2009). This was the first evidence of LRP1 involvement in regulation of neurite outgrowth and receptor transactivation (Shi et al., 2009). Targeting LRP1 may, therefore, prove useful in promoting neurite growth. Synthetic LRP1 agonists led to increased neurite outgrowth for DRG neurons in a permissive and non-permissive myelin environment *in vitro* and increased axonal regeneration following spinal cord injury *in vivo* via this Trk transactivation system (Yoon et al., 2013).

In 2013, co-immunoprecipitation experiments identified LRP1 as a high affinity receptor for MAG (Stiles et al., 2013). Inactivating LRP1 function by RNA interference (RNAi) or generating *LRP1*$^{-/-}$ mice reversed inhibition by MAG or myelin for CGNs, PC12, and N2a cells (Stiles et al., 2013). As with PirB and NgR1, LRP1 has also been found to associate with p75^{NTR} and activate RhoA in response to MAG, and MAG-mediated inhibition of neurite outgrowth via RhoA activation may therefore be dependent on both p75^{NTR} and LRP1 (Stiles et al., 2013). LRP1 is therefore involved in both the promotion or inhibition of neurite outgrowth and appears to be dependent on the signaling pathways initiated by the specific ligand. LRP1 was also found to bind Nogo-66 and OMgp, however it remains unclear whether these two MAIs restrict neurite outgrowth via LRP1 (Stiles et al., 2013). This receptor may prove a useful target for further study in CNS regeneration due to its expression in most neuronal populations, especially CGNs, cortical neurons, and hippocampal neurons (Wolf et al., 1992; Bu et al., 1994).

1.5 Transforming growth factor-β receptor & the Smad2/3 pathway

The number of receptors involved in neurite outgrowth inhibition via MAIs continues to increase, the transforming growth factor-β receptor (TGFβR) being one such example. Its eponymous ligand induces a variety of cellular responses in the developing embryo and mature nervous system that include cellular differentiation, proliferation, immune system regulation, and apoptosis (Shi & Massagué, 2003). This pleiotropic cytokine is a member of the transforming growth factor superfamily that includes TGFα and TGFβ isoforms TGFβ1-3. TGFβR is a cell surface serine/threonine kinase arranged as a heterotetramer composed of two type II and two type I monomers (Shi & Massagué, 2003). Structurally, each subunit possesses an extracellular N-terminal ligand-binding domain, a transmembrane domain, and a C-terminal serine/threonine kinase domain. Binding of TGFβ to the type II TGFβR induces the recruitment and phosphorylation of the type I receptor, culminating in the activation of ligand-specific signaling cascades (Massagué, 1998). This process is initiated by dimeric ligand binding to both TGFβRII monomers which allows for a conformational change of their intracellular kinase domains. This facilitates the phosphorylation of multiple serine and threonine residues in a specific cytoplasmic GS domain sequence (TTSGSGSG) of TGFβRI, thereby activating it and allowing it to phosphorylate intracellular signaling molecules known as receptor-regulated Smads (R-Smads) (Macías-Silva et al., 1996; Kretzschmar et al., 1997; Massagué, 1998).

The R-Smads (Smad1, 2, 3, 5, and 8) comprise one of three Smad protein classes along with the Co-Smad Smad4 and inhibitor Smads (I-Smads; Smad6 and 7). They lack any known structural motifs but possess highly conserved carboxyl-terminal MAD-homology-2 (MH2) domains. R-Smads and Co-Smads also possess a highly conserved amino-terminal MH1 domain (Massagué et al., 2005). Smad2 and Smad3 are signaling intermediates for TGFβR as well as the

activin and Nodal receptors, whereas Smads 1, 5, and 8 are intermediates for the bone morphogenetic protein (BMP) and anti-Muellerian receptors (Massagué et al., 2005).

The R-Smads Smad2 and Smad3 are immobilized near the inner plasma membrane leaflet by Smad anchor for receptor activation (SARA) (Tsukazaki et al., 1998). The activation of Smad2 by TGFβR1 is driven by phosphorylation of Ser^{465} and Ser^{467} in the SXS motif of the MH2 domain (Abdollah et al., 1997). Phosphorylation of Smad2/3 destabilizes its interaction with SARA and augments its affinity with Smad4, which is continuously shuttled into the nucleus along with Smad2/3 where they form a heterotrimeric complex that activates or inhibits gene transcription depending on the presence of cell type-specific proteins (Xu et al., 2000; Shi & Massagué, 2003). Upon dephosphorylation of R-Smads, they are recycled back into the cytoplasm to await receptor activation (Inman & Hill, 2002).

TGFβ1 and TGFβ2 are potent immunoregulators (Letterio & Roberts, 1998) whose expressions are upregulated following spinal cord injury in humans and animals (Buss et al., 2008). In the acute inflammatory phase following spinal cord injury, TGFβ1 mRNA is upregulated in hematogenous inflammatory cells and neurons, whereas increased TGFβ2 mRNA is observed in the subacute phase of astrogliosis, particularly in neovascular endothelial cells and reactive astrocytes, which supports the involvement of TGFβ2 in glial scar formation (Logan et al., 1994, 1999; Lagord et al., 2002; Buss et al., 2008). TGFβ1 contributes to regenerative failure by inducing CSPG expression in astrocytes and this can be countered with anti-TGFβ antibodies (Asher et al., 2000; Moon & Fawcett, 2001; Hamel et al., 2005). The TGFβR-Smad2 signaling pathway was therefore a plausible candidate for inducing CSPG expression (Susarla et al., 2011). Astrocytes treated with Smad2 or Smad4 siRNA nevertheless displayed increased neurocan, brevican, and phosphacan expression in response to TGFβ, suggesting the involvement of another TGFβR-

induced pathway (Jahan & Hannila, 2015). The phosphoinositide 3-kinase (PI3K)-Akt-mechanistic target of rapamycin (mTOR) pathway is a likely candidate, as astrocytes treated with TGFβ in the presence of a PI3K or mTOR inhibitor displayed significantly reduced expression of CSPGs (Jahan & Hannila, 2015).

TGFβ is a potent growth inhibitor in most cell types (Zhong et al., 2010) and inhibiting its function may be a method by which to promote growth. The Smad2 signal transducing pathway was first implicated in the restriction of neurite outgrowth in a study demonstrating that the E3 ubiquitin ligase Cdh1-anaphase-promoting complex (Cdh1-APC) could inhibit axon growth via degradation of its substrate, SnoN. Conversely, RNAi silencing of Smad2, which acts upstream of SnoN, enhances axonal growth by nearly 50% in cultured CGNs (Stegmüller et al., 2008). Expression of Smad6 or Smad7, which inhibit the Smad pathway, induced robust neurite outgrowth. Further evidence for the involvement of this pathway in neurite outgrowth inhibition came from an experiment in which knockdown of Smad2 with RNAi in CGNs reversed inhibition by myelin (Stegmüller et al., 2008). Lastly, Smad2 phosphorylation was significantly increased in CGNs following treatment with MAG or Nogo (Hannila et al., 2013). These findings allow the TGFβR and Smad2 signaling pathway to be added to the list of potential mediators of CNS neurite outgrowth inhibition by myelin-associated inhibitors.

Chapter 2

Hypothesis & Aims

Previous studies have demonstrated that knockdown of Smad2 enhances neurite outgrowth on an inhibitory myelin substrate (Stegmüller et al., 2008; Hannila et al., 2013) and that the myelin-associated inhibitors MAG and Nogo can induce Smad2 phosphorylation in CGNs (Hannila et al., 2013). Since Smad2 is phosphorylated as a result of TGFβR activation, these studies suggest that MAG and Nogo are either directly or indirectly activating TGFβR and, furthermore, that TGFβR may be an important receptor for myelin-mediated inhibition of CNS regeneration. Due to lack of structural resemblance to TGFβ, it is unlikely that MAG, Nogo, and OMgp bind directly to TGFβRII. A previous study demonstrated that MAG and Nogo could transactivate epidermal growth factor receptor (EGFR) in a NgR1-dependent manner to inhibit neurite outgrowth from CGNs and DRG neurons (Koprivica et al., 2005). Based on these results, we have hypothesized that MAG and Nogo may be inducing phosphorylation of Smad2 via a similar mechanism of TGFβRII transactivation, and since NgR1, PirB, and LRP1 are well-established receptors for MAG and Nogo, they are logical candidates to target as possible TGFβRII transactivators.

Aim #1: To characterize Smad2 phosphorylation in response to MAG and Nogo.

Aim #2: To determine if NgR1, PirB, and LRP1 are responsible for transactivating TGFβRII in response to MAG and Nogo.

Chapter 3

Experimental Design & Procedures

All animal procedures were approved by the Protocol Management and Review Committee of the University of Manitoba (protocol #14-020).

3.1 Cerebellar Granule Neuron cultures

P5-6 cerebella from female and male Long Evans rat pups were collected in 2 ml Neurobasal-A (NB; Life Technologies) media and incubated for 20 minutes with 5% papain (Sigma) at 37°C followed by another 20-minute incubation with 5% papain supplemented with 10% DNAse. Tissue digestion was stopped with a 1-minute treatment of NB supplemented with 10% Soybean Trypsin Inhibitor (SBTI; Sigma) followed by a 1-minute treatment with 40% SBTI. Tissues were washed three times in plain NB and triturated with NB containing 10% DNAse. Cells were passed through a 40 μm strainer, centrifuged for 5 minutes at 1000 rpm, and resuspended in NB supplemented with 2% B27 (Gibco), 1% L-glutamine (Gibco), and 1% Antibiotic-Antimycotic (AA; Life Technologies). CGNs were plated on poly-L-lysine (PLL; Sigma)- coated 60 mm plates (100 μg/ml) at a concentration of 2-3 million cells/ml (6-9 million cells/plate) and incubated at 37°C for 24 hours. The following day, the cells were serum-starved with 2 ml/plate plain Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) and incubated for another 24 hours prior to treatment application.

3.2 Treatments

3.2.1 Myelin-associated glycoprotein & Nogo timecourses

Serum-starved CGNs were treated with 20 μg/ml MAG fused to an immunoglublin Fc domain (MAG-Fc) or Nogo fused to alkaline phosphatase (AP-Nogo) for 1, 5, 10, 20, 30, 60, or 120 minutes.

3.2.2 Transforming growth factor-β receptor inhibitors

CGNs were treated with two selective TGFβRI inhibitors: 5 mM SB431542 or SB505124 (both Sigma) diluted in dimethyl sulfoxide (DMSO). Serum-starved CGNs were treated with 20 μM SB431542 or 5 μM SB505124 for 1 hour at 37°C followed by a 30-minute treatment with 20 μg/ml of either MAG-Fc or AP-Nogo. Additional plates received 10 μl/ml DMSO (control) alone or DMSO for 1 hour followed by 30-minute treatments with 20 μg/ml MAG-Fc or AP-Nogo.

3.2.3 Small interference RNA assay

In order to silence the expression of NgR and LRP1, 60 mm plates each containing 10-12 million CGNs were incubated at 37°C for 24 hours followed by treatment with either Accell SMARTpool rat NgR1 siRNA, Accell SMARTpool rat LRP1 siRNA, or Accell Non-targeting siRNA (Dharmacon). siRNAs were diluted to 1 μM in 1X siRNA Accell Delivery Media (Dharmacon). Cells were incubated with the siRNA for 72 hours at 37ºC and then exposed to 20 μg/ml MAG-Fc or AP-Nogo for 30 minutes.

3.3 *PirB*$^{-/-}$ mice

In order to explore the role of PirB in MAG- and Nogo-induced Smad2 phosphorylation, PirB WT and *PirB*$^{-/-}$ mice were obtained from Dr. Carla Shatz (Stanford University) and isolated as described above. 60 mm plates each containing 9-12 million CGNs from *PirB*$^{-/-}$ mice were treated with 20 μg/ml MAG-Fc, 20 μg/ml AP-Nogo, or 10 ng/ml TGFβ for 30 minutes. CGNs from WT mice were subjected to identical treatment conditions and used as controls.

3.4 Cell lysis & Western blotting

Plates were kept on ice and the media aspirated prior to rinsing with phosphate-buffered saline (PBS). Cells were immediately lysed with 1X RIPA Lysis Buffer (Millipore) containing 0.1% protease inhibitor (Calbiochem), 0.1% phosphatase inhibitor (Calbiochem), and 0.05% phenylmethylsulfonyl fluoride (PMSF; Sigma), collected, and stored at -80°C. Protein concentrations were measured using the Bio-Rad *DC* protein assay and samples containing 30-40 μg of protein were combined with Pierce™ Lane Marker Reducing 5X sample buffer containing dithiothreitol (DTT; Thermo), boiled for 5 minutes, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 8% gels for all experiments excepting the siRNA receptor knockdown experiment which used 6% gels. Proteins were transferred to nitrocellulose membranes at 75 V for 1 hour for low molecular weight proteins or 150 mA for 16 hours at 4°C for high molecular weight proteins, blocked for 1 hour at room temperature in 10% milk in PBS containing 0.05% Tween®-20 (PBS-T; Sigma), and incubated overnight at 4°C with primary antibodies diluted in 3% milk in PBS. Primary antibodies include rabbit anti-phospho Smad2 (Ser465/467; 1:1000; Cell Signaling), rabbit anti-Smad2/3 (1:1000; Cell Signaling), goat anti-NgR (1:2000; R&D Systems), rabbit anti-LRP1 (1:1000; Sigma), and rabbit anti-actin

(1:1000; Sigma). Membranes were incubated with secondary antibodies at room temperature for 1 hour and include horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2000; Cell Signaling) diluted in 3% milk in PBS, HRP-conjugated anti-mouse IgG (1:2000; Cell Signaling) diluted in 3% milk in PBS, and HRP-conjugated donkey anti-goat IgG (1:2000; Jackson ImmunoResearch) diluted in 3% milk in PBS. The membranes were treated with Pierce enhanced chemiluminescence (ECL) Western Blotting Substrate (Thermo) or SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo) and exposed to autoradiographic film (HyBlot CL, Denville Scientific Inc., New Jersey). Membranes were subsequently stripped with Restore™ Western Blot Stripping Buffer (Thermo) and reprobed with antibodies in succession.

3.5 Image acquisition

Autoradiographic films were scanned and densitometric analyses of proteins were performed using ImageJ software (NIH). Figures were compiled with Adobe Photoshop CS5.

3.6 Statistical analyses

All analyses were performed with GraphPad Prism (version 6). Comparisons of three or more data sets were achieved by one-ANOVA with Tukey's multiple comparison test. All data are represented as mean ± standard error of mean (SEM).

Chapter 4

Results

4.1 Myelin-associated glycoprotein and Nogo induce phosphorylation of Smad2

Previous studies have revealed that Smad2 siRNA knockdown resulted in significantly increased neurite outgrowth on a myelin substrate (Stegmüller et al., 2008; Hannila et al., 2013). To compare the degree of Smad2 phosphorylation between MAIs and TGFβ, we treated neurons with MAIs and other myelin proteins for 30 minutes and probed for phosphorylated Smad2 (pSmad2). P5-6 CGNs were treated with either 10 ng/ml TGFβ, 20 μg/ml MBP, 20 μg/ml MAG1-3-Fc, 20 μg/ml MAG-Fc, 20 μg/ml AP-Nogo, or 20 μg/ml myelin. MAG-Fc or AP-Nogo both induced high levels of Smad2 phosphorylation in these neurons (n= 3, Fig. 1) and pSmad2 is induced to a similar degree by either MAG-Fc or AP-Nogo as it is by TGFβ. There is an absence of pSmad2 in cells treated with MBP, the most abundant protein in CNS myelin, suggesting that pSmad2 is induced specifically by MAIs, however it does not preclude the possibility that other myelin proteins are also capable of pSmad2 induction. Curiously, Smad2 phosphorylation was induced by MAG1-3-Fc, a truncated form of MAG containing the first three Ig-like domains and believed to lack inhibitory properties (Tang et al., 1997). Our results indicate for the first time that MAG1-3 is capable of inducing Smad2 phosphorylation to a degree comparable to MAG-Fc and AP-Nogo.

4.2 Characterization of onset and duration of Smad2 phosphorylation by MAG and Nogo

To expand on the finding by Hannila et al. (2013) that Smad2 is phosphorylated by MAIs, we aimed to determine when Smad2 phosphorylation by MAIs is induced and the length to which it

is sustained. P5-6 CGNs were treated with 20 μg/ml MAG-Fc or AP-Nogo for 1, 5, 10, 20, 30, 60, or 120 minutes and subjected to Western blotting for detection of pSmad2. Cells treated with MAG-Fc (n=4, Fig. 2A) display increased levels of pSmad2 within 10 minutes of the treatment, peak at the 1-hour mark and return to baseline levels 2 hours post-treatment. Cells treated with AP-Nogo (n=3, Fig. 2B) also display increased pSmad2 levels beginning 10 minutes following treatment but peak levels are achieved within 30 minutes and remain elevated by the 2-hour mark.

4.3 TGFβR is required for MAG- and Nogo-mediated Smad2 phosphorylation

Our previous results indicate that MAG-Fc and AP-Nogo induce phosphorylation of Smad2 in CGNs (Fig. 1). To investigate whether Smad2 phosphorylation by MAIs is achieved via TGFβR, P5-6 CGNs were treated for 1 hour with either 10 μl/ml DMSO or one of two selective TGFβR type I inhibitors: 20 μM SB431542 or 5 μM SB505124. The doses for these TGFβR inhibitors were based on those used in previous studies in which CGNs treated with 20 μM SB431542 or 5 μM SB505124 significantly reduced levels of pSmad2 (DaCosta et al., 2004; Stegmüller et al., 2008). Neurons received these treatments followed by a 30-minute treatment with 20 μg/ml MAG-Fc and probed for pSmad2. CGNs treated with MAG-Fc and DMSO show a significant increase in pSmad2 (2.7-fold) levels which was significantly reduced in response to treatment with 20 μM SB431542 or 5 μM SB505124 (one-way ANOVA with Tukey's HSD, ****$p < 0.0001$, n=5) (Fig. 3A). CGNs treated with AP-Nogo show a similar increase in Smad2 phosphorylation (3.1-fold) which was significantly reduced by treatment with 20 μM SB431542 or 5 μM SB505124 (one-way ANOVA with Tukey's HSD, ****$p < 0.0001$, n=4) (Fig. 3B). These data indicate that both MAG and Nogo induce Smad2 activation via TGFβR, however it remains unknown whether MAG or Nogo bind directly to TGFβR to produce this effect.

4.4. NgR1 and LRP1 do not transactivate TGFβR in response to MAIs

The lack of structural similarity between MAIs and TGFβ indicate that they are unlikely to bind directly to TGFβR. Based on findings published by Koprivica et al. (2005) that showed MAIs are capable of transactivating EGFR, a possible scenario is the MAI-induced transactivation of TGFβR via a separate receptor. NgR1, LRP1, and PirB are confirmed receptors for MAIs and are thus plausible candidates as TGFβR transactivators. To investigate whether NgR1 or LRP1 act as transactivators of TGFβR, we employed siRNA to genetically silence these two receptors. P5-6 CGNs were treated for 72 hours with 1% non-targeting siRNA or siRNA for NgR1 or LRP1 followed by 30-minute treatments with 20 μg/ml MAG-Fc or AP-Nogo. SDS-PAGE revealed successful silencing of NgR1 and LRP1 (Fig. 4A). Cells treated with non-targeting siRNA followed by either MAG-Fc or AP-Nogo display elevated pSmad2 levels compared to control cells receiving only non-targeting siRNA. Likewise, cells treated with siRNA for NgR1 or LRP1 alone display no pSmad2 induction unlike cells that also received MAG-Fc or AP-Nogo treatments, which display a clear pSmad2 signal. Despite the clear visual pattern that emerges, quantification of the raw data did not result in statistically significant patterns due to large variability between exposure of the Western blot films (Fig. 4B). The presence of MAG- and Nogo-induced pSmad2 in neurons treated with NgR1 or LRP1 siRNA indicate that neither NgR1 nor LRP1 is responsible for transactivation of TGFβR by MAIs.

4.5 PirB does not transactivate TGFβR in response to MAIs

It is not possible to target PirB with commercial siRNA (C. Shatz, personal communication, 2016), therefore we obtained *PirB*$^{-/-}$ and WT mice from Dr. Carla Shatz. Serum-starved CGNs were treated for 30 minutes with 10 ng/ml TGFβ, 20 μg/ml MAG-Fc, or 20 μg/ml AP-Nogo and probed

for pSmad2. WT CGNs treated with MAG-Fc, AP-Nogo, or TGFβ all display high levels of pSmad2. Likewise, CGNs from *PirB*$^{-/-}$ mice treated with MAG-Fc, AP-Nogo, or TGFβ each exhibit pSmad2 levels similar to those displayed by the treated WT cells (Fig. 5A). Compared to untreated WT CGNs, there is a significant increase in pSmad2 expression in WT CGNs following treatment with MAG-Fc (3.2-fold; one-way ANOVA with Tukey's HSD, $*p < 0.05$, n=3) or AP-Nogo (3.8-fold; one-way ANOVA with Tukey's HSD, $**p < 0.01$, n=3) (Fig. 5). *PirB*$^{-/-}$ CGNs also display a similar increase in pSmad2 expression when treated with AP-Nogo (2.6-fold) (one way-ANOVA with Tukey's HSD, $*p < 0.05$, n=3) (Fig. 5). Importantly, there is no significant difference in the levels of pSmad2 induced by MAG-Fc or AP-Nogo between WT CGNs and *PirB*$^{-/-}$ CGNs (Fig. 5B). As with NgR1 and LRP1, PirB seems unlikely to act as a TGFβR transactivator and points to the presence of an unidentified receptor as the mediator of MAI-induced TGFβR transactivation.

Figure 1. Myelin-associated inhibitors induce phosphorylation of Smad2. Western blot of cell lysates from P5-6 CGNs treated with 10 ng/ml TGFβ, 20 μg/ml MBP, 20 μg/ml MAG1-3, 20 μg/ml MAG-Fc, 20 μg/ml AP-Nogo, or 20 μg/ml myelin for 30 minutes. TGFβ is used a positive control and induces significant pSmad2. MBP, the most abundant protein in myelin, fails to induce pSmad2 expression. MAG, MAG-1-3, and Nogo all induce expression of pSmad2 to a significantly higher degree than does myelin. The image is representative of 3 independent experiments.

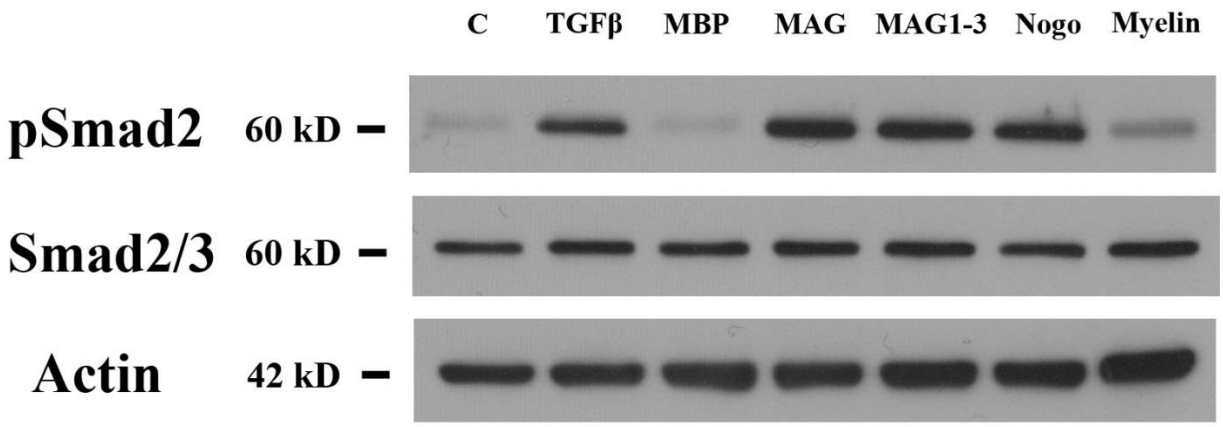

Figure 1. Myelin-associated inhibitors induce phosphorylation of Smad2.

Figure 2. Time courses of MAG- and Nogo-induced Smad2 phosphorylation. (A) Western blot of lysates from P5-6 CGNs treated with 20 μg/ml MAG-Fc for 1, 5, 10, 20, 30, 60, or 120 minutes. MAG-Fc induces an increase in the levels of pSmad2, beginning within 10 minutes and peaking within 1 hour. Image is representative of 4 independent experiments. (B) Western blot of cell lysates of P5-6 CGNs treated with 20 μg/ml AP-Nogo for 1, 5, 10, 20, 30, 60, or 120 minutes. AP-Nogo induces an increase in the levels of pSmad2, beginning within 10 minutes, peaking at 1 hour, and it is sustained for 2 hours. Image is representative of 3 independent experiments.

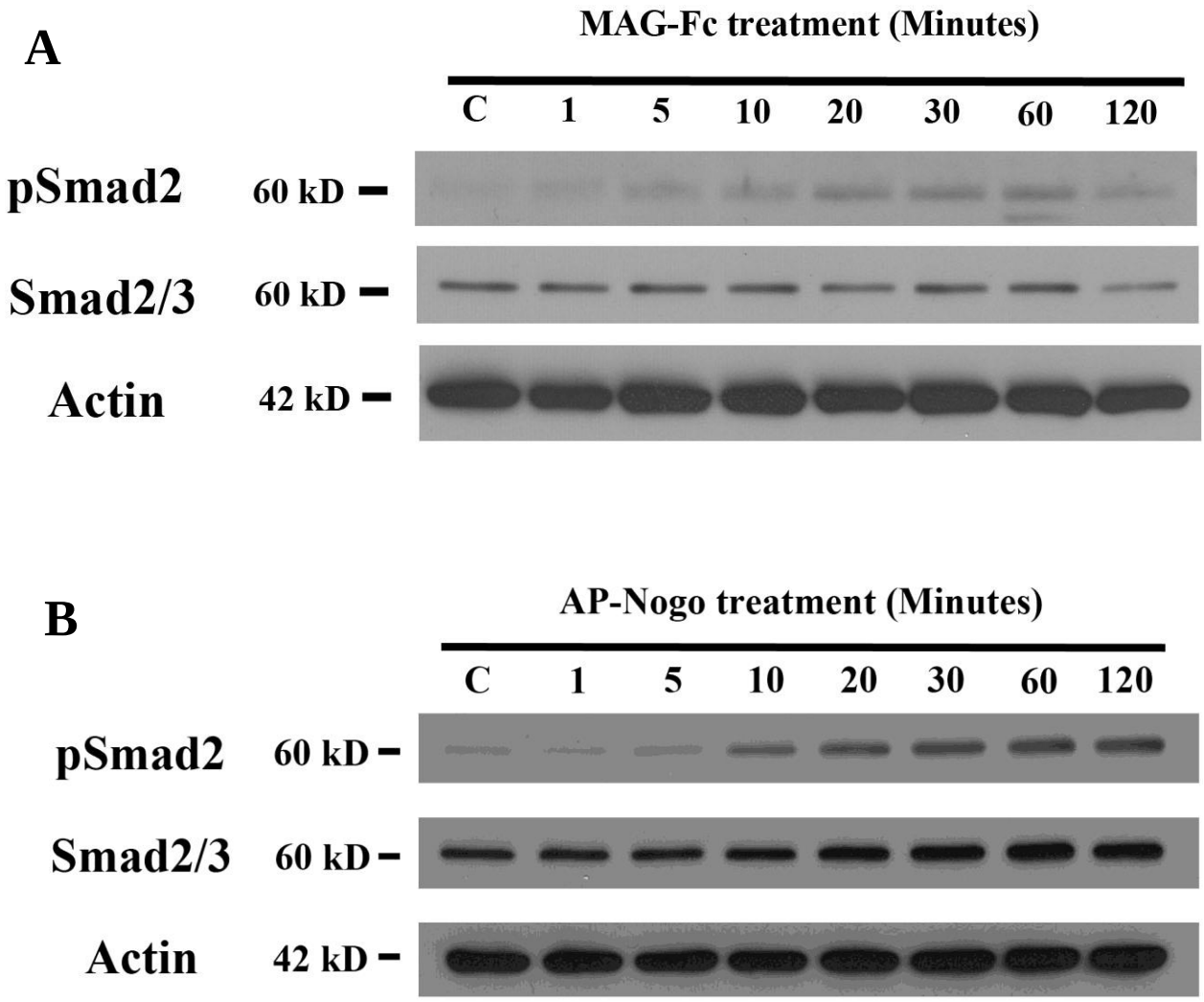

Figure 2. MAG or Nogo induce a gradual increase in phosphorylated Smad2.

Figure 3. TGFβ receptor inhibitors SB431542 or SB505124 abolish MAG- or Nogo-mediated Smad2 phosphorylation. (A) Western blot of lysates from P5-6 CGNs treated with 20 μM SB431542 or 5 μM SB505124 for 1 hour followed by 20 μg/ml MAG-Fc for 30 minutes. (B) MAG-Fc significantly increases pSmad2 expression and SB431542 or SB505124 both completely abolish this response. (C) Western blot of cell lysates of P5-6 CGNs treated with 20 μM SB431542 or 5 μM SB505124 for 1 hour followed by 20 μg/ml AP-Nogo for 30 minutes. (D) AP-Nogo significantly increases pSmad2 expression and SB431542 or SB505124 both completely abolish this response. Graphs represent average densitometric measurements ± SEM (****$p < 0.0001$). The data in graph B were obtained from 5 independent experiments and the data in graph D were obtained from 4 independent experiments.

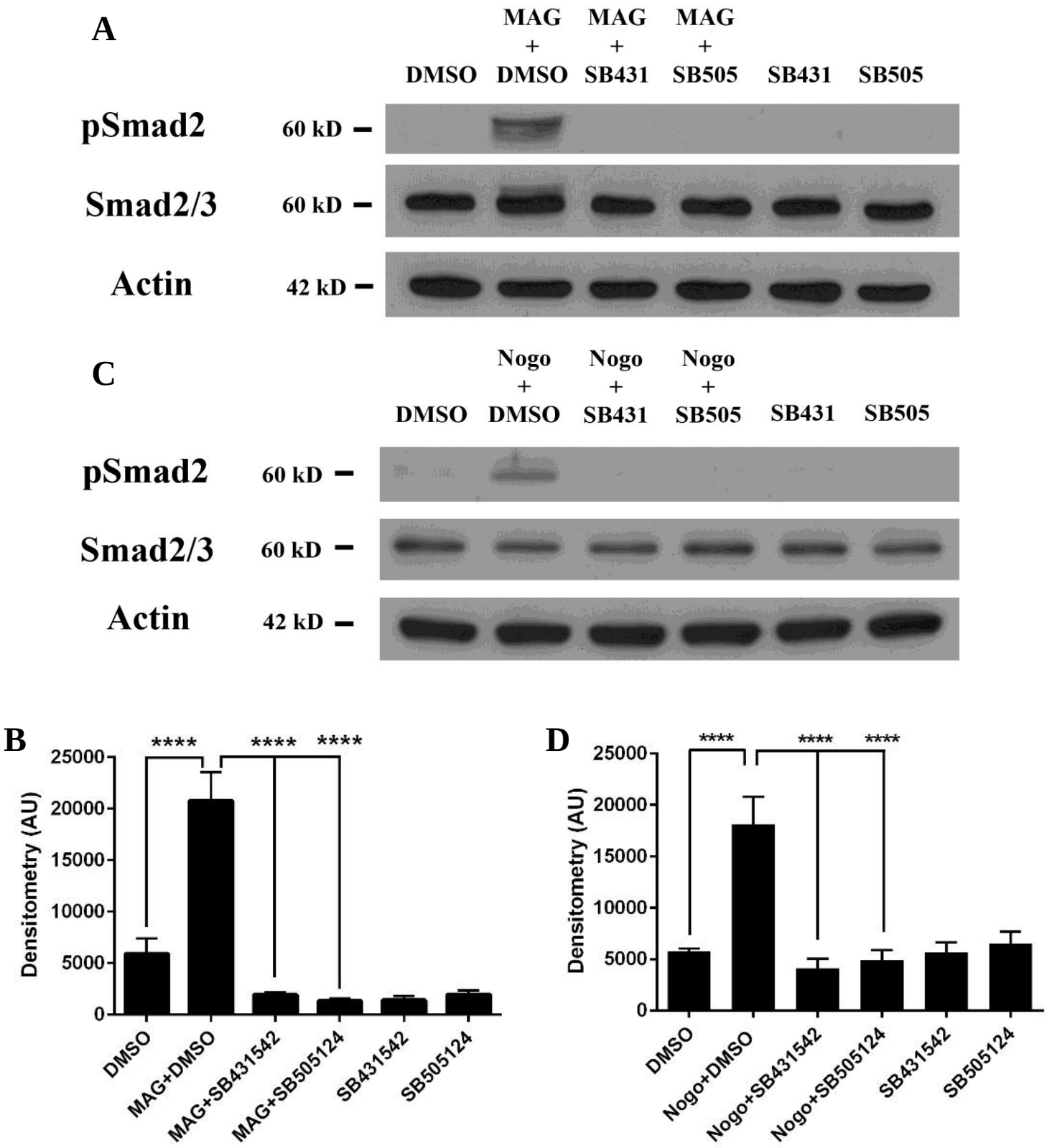

Figure 3. TGFβ receptor inhibitors SB431542 or SB505124 abolish MAG- or Nogo-mediated Smad2 phosphorylation.

Figure 4. siRNA knockdown of NgR1 or LRP1 does not affect MAG- or Nogo-induced Smad2 phosphorylation. (A) Western blot of lysates from P5-6 CGNs treated with 1% non-targeting (NT) siRNA or siRNA for NgR1 or LRP1 for 72 hours followed by 30-minute treatments with 20 μg/ml MAG-Fc or 20 μg/ml AP-Nogo. (B) pSmad2 induction in all conditions were non-significant in response to MAG-Fc or AP-Nogo. Graph represents average densitometric measurements ± SEM. The data in the graph were obtained from 3 independent experiments.

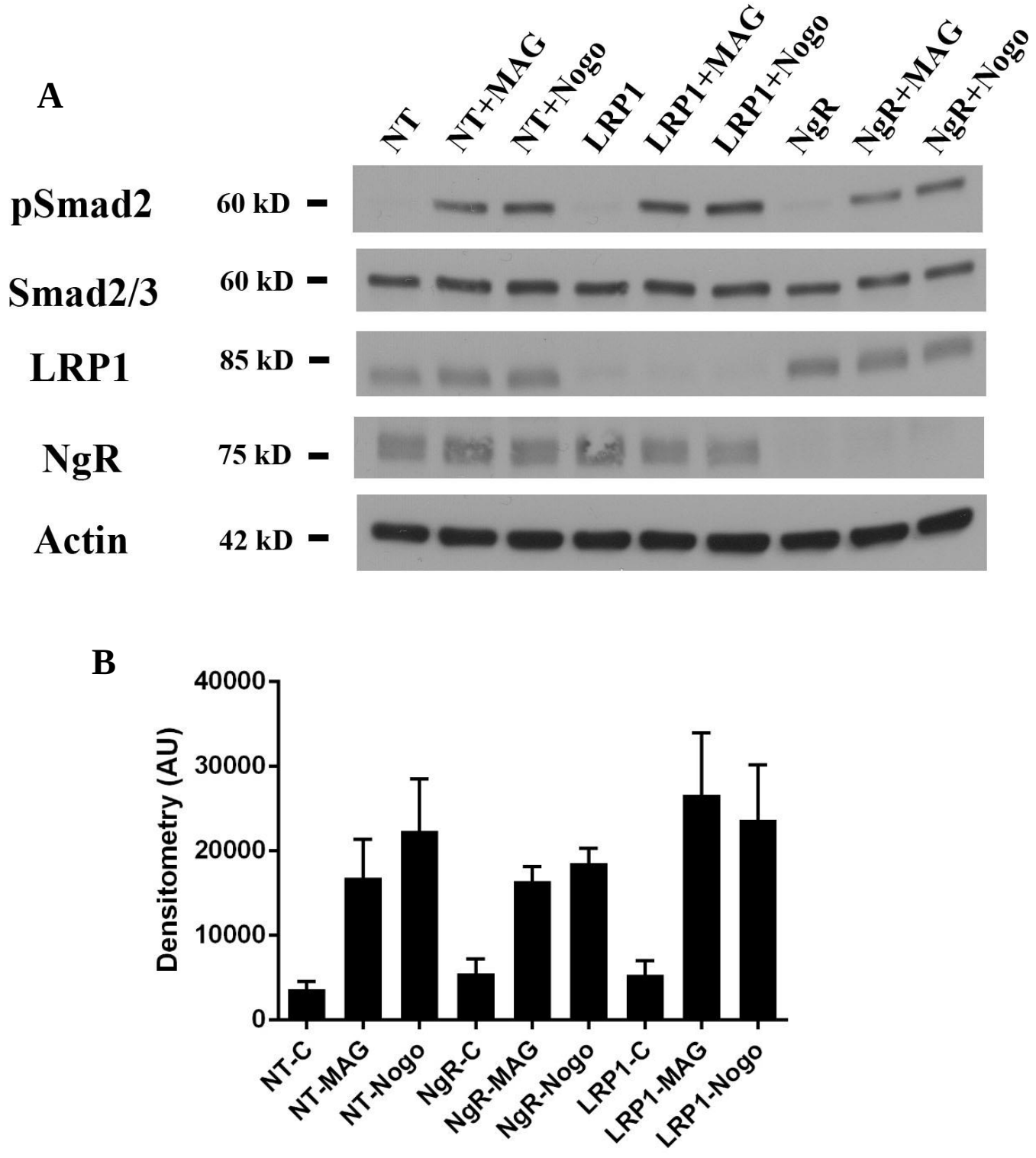

Figure 4. siRNA knockdown of NgR1 or LRP1 does not affect MAG- or Nogo-induced Smad2 phosphorylation.

Figure 5. Silencing of PirB does not affect MAG- or Nogo-induced Smad2 phosphorylation. (A) Western blot of lysates from WT and *PirB*$^{-/-}$ P5-6 CGNs treated with 10 ng/ml TGFβ, 20 μg/ml MAG-Fc, or 20 μg/ml AP-Nogo for 30 minutes. (B) Smad2 phosphorylation was significantly increased in WT CGNs in response to TGFβ, MAG-Fc, or AP-Nogo. Smad2 phosphorylation was also significantly increased in *PirB*$^{-/-}$ CGNs in response to AP-Nogo but was increased in a non-significant manner in response to MAG-Fc. There was no significant difference in pSmad2 levels in response to either MAG-Fc or AP-Nogo between WT and *PirB*$^{-/-}$ CGNs. Graph represents average densitometric measurements ± SEM (*$p < 0.05$, **$p < 0.01$). The data in the graph were obtained from 3 independent experiments.

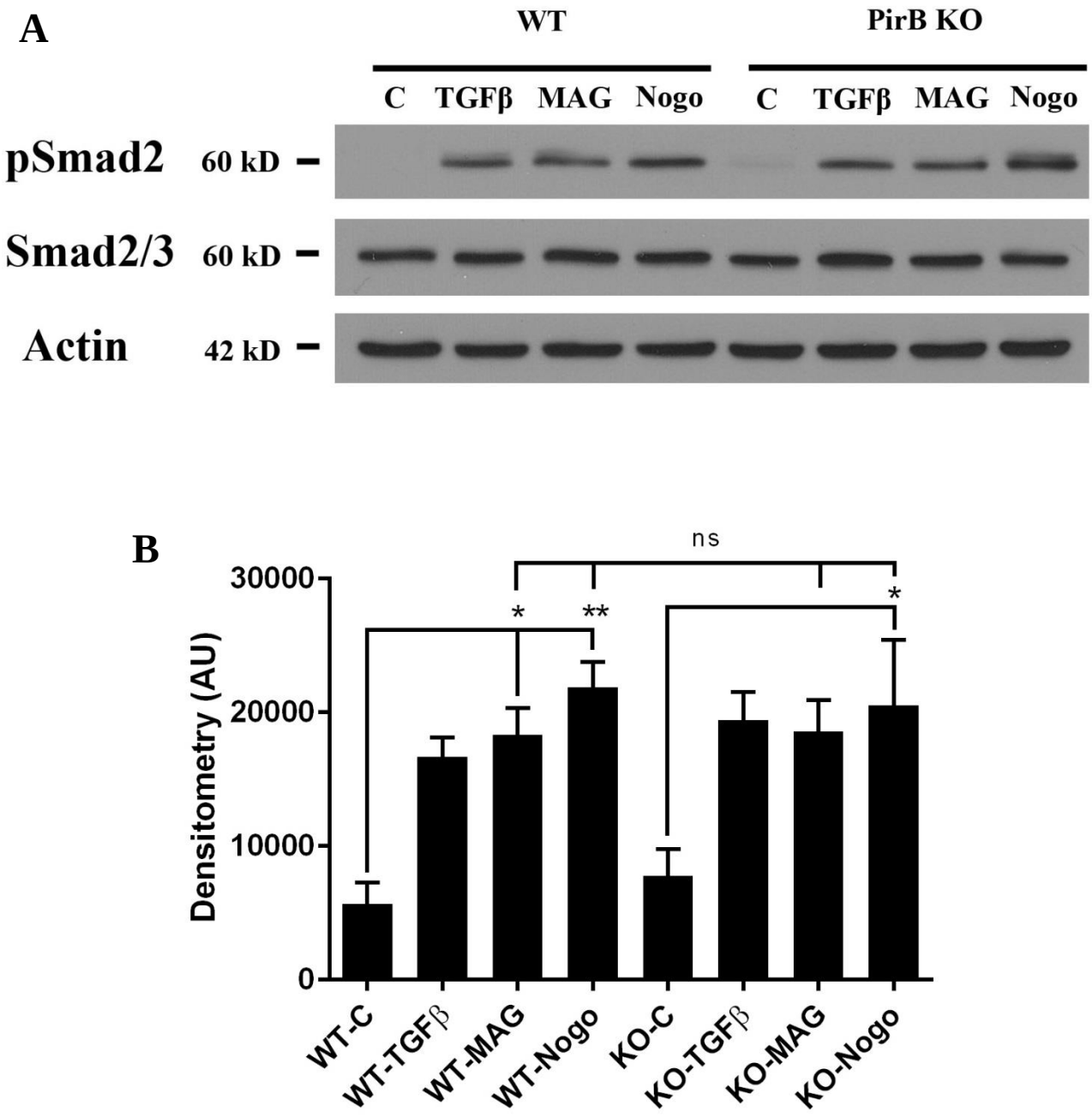

Figure 5. Silencing of PirB does not affect MAG- or Nogo-induced Smad2 phosphorylation.

Chapter 5

Discussion

Inhibition by MAIs presents an important obstacle to regeneration in the CNS and efforts have been made to elucidate the signal transduction mechanisms by which they act. MAG, Nogo-A, and OMgp induce inhibition by binding to NgR1 or PirB, while MAG is also capable of mediating inhibitory responses through LRP1 (Fournier et al., 2001; Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002b; Atwal et al., 2008; Stiles et al., 2013). NgR1 is a GPI-linked receptor and therefore depends on $p75^{NTR}$, TAJ/TROY, or LINGO-1 to transduce signals across the cell membrane (Wang et al., 2002; Wong et al., 2002; Mi et al., 2004; Park et al., 2005; Shao et al., 2005). This NgR1 complex is a major convergence point for MAIs; disrupting NgR1 function prevents inhibitory activity by MAG on several neuronal populations and allows DRG neurons to extend neurites on substrates of CNS myelin (Domeniconi et al., 2002; Liu et al., 2002; Li et al., 2004). Cells that are normally unresponsive to MAIs also become inhibited by these myelin proteins if NgR1 is overexpressed (Fournier et al., 2001). Likewise, interfering with PirB activity partially reverses the inhibitory effects of MAG, Nogo-A, and OMgp on neurite outgrowth (Atwal et al., 2008; Matsushita et al., 2011) and inactivating LRP1 function reversed inhibition by MAG or myelin in several cell types (Stiles et al., 2013).

The intracellular signaling protein Smad2 has recently been implicated in the modulation of axonal growth in CNS neurons. Silencing Smad2 activity stimulated neurite outgrowth in CGNs and rendered these neurons capable of overcoming inhibition by myelin (Stegmüller et al., 2008; Hannila et al., 2013). Smad2 phosphorylation was induced in CGNs in response to MAG or Nogo (Hannila et al., 2013), which implies activation of TGFβR and constitutes a novel signaling

pathway for MAIs. These findings have prompted us to investigate the precise mechanisms whereby MAIs such as MAG and Nogo act on the TGFβR-Smad2 pathway. Given that MAG and Nogo are structurally dissimilar from TGFβ and that several receptors are known to mediate MAG and Nogo signaling, we hypothesized that TGFβR is being transactivated by NgR1, LRP1, or PirB in response to these myelin proteins. Pharmacological disruption of TGFβR activity in neurons may therefore constitute an attractive target for promoting CNS regeneration.

Our experiments have confirmed that MAG and Nogo induce phosphorylation of Smad2 in CGNs. Because Smad2 is phosphorylated in response to TGFβR activation, we antagonized this receptor with SB431542 or SB505124 and found that pSmad2 expression returned to baseline levels in CGNs treated with either MAG or Nogo. These results conclusively demonstrate that MAG and Nogo are inducing Smad2 activation via TGFβR. In addition, we have characterized the onset and duration of Smad2 phosphorylation in response to MAG and Nogo and shown that phosphorylation is induced within 10 minutes of treatment administration and is sustained for approximately 1-2 hours. These results demonstrate that Smad2 signaling activity is initiated relatively soon following ligand binding to TGFβR.

Unexpectedly, we have also found that phosphorylated Smad2 was strongly expressed when CGNs were treated with MAG1-3. This truncated form of MAG contains only Ig-like domains 1-3 which were not previously implicated in Smad2 phosphorylation. Based on previous findings that disrupting Smad2 activity enhanced neurite outgrowth on an inhibitory myelin substrate in CGNs (Stegmüller et al., 2008; Hannila et al., 2013), our finding raises the possibility that Ig-like domains 1-3 of the MAG protein may play a role in mediating inhibition of neurite outgrowth in the CNS via TGFβR-Smad2 activation.

The primary aim of this study was to elucidate the mechanisms whereby MAG and Nogo activate the TGFβR and induce phosphorylation of Smad2. Given the structural heterogeneity between MAIs and TGFβ, binding of MAIs directly to TGFβR is highly unlikely. Transactivation is a more plausible mechanism as MAIs have already been shown to be capable of receptor transactivation (Koprivica et al., 2005). Based on these ideas, we wanted to confirm or reject the involvement of the three most well-characterized MAI receptors in the transactivation of TGFβR. We examined the effects of MAG or Nogo on neurons in which NgR1, LRP1, or PirB function was disrupted. NgR1 and LRP1 knockdown via siRNA did not result in any significant decrease in pSmad2 in CGNs. Likewise, CGNs from genetically modified mice lacking PirB expression did not display significant differences in pSmad2 when compared to control CGNs in response to treatments with MAG or Nogo.

These results are surprising given that NgR1, LRP1, and PirB are responsible for a significant proportion of the inhibition observed in CNS neurons in response to MAIs. NgR1 and PirB remain the only known receptors to date capable of mediating inhibition by all three MAIs and were therefore the most plausible candidates for TGFβR transactivation. This study strongly suggests that an unidentified MAI receptor is responsible for transactivating TGFβR. Future studies may consider employing technology such as LRC-TriCEPS to identify all neuronal receptors for MAG and Nogo and to perform subsequent immunoprecipitation experiments for TGFβR and the newly identified receptor(s). In summary, preliminary studies have implicated the Smad2 signaling cascade in overcoming the inhibitory effect of myelin on neurite outgrowth in neurons and we have confirmed TGFβR-Smad2 as a novel signal transduction pathway for MAG and Nogo. We anticipated that either NgR1, LRP1, or PirB was acting as a transactivator for TGFβR in MAG- and Nogo-mediated Smad2 phosphorylation but found, unexpectedly, that none

of these candidate receptors are involved in this process and suggests the involvement of another receptor. Future efforts to identify this receptor would allow for a more accurate understanding of the mechanisms by which inhibitory myelin proteins exert their effects in the CNS.

Chapter 6

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