MECHANISMS OF NEURITE OUTGROWTH INHIBITION BY MYELIN-ASSOCIATED GLYCOPROTEIN

By

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To my parents, São and Antonio, and my brother, Rodrigo,

for your constant love and support

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LIST OF ABBREVIATIONS

APP	Amyloid precursor protein
ASIA	American Spinal Injury Association
BBB	Blood brain barrier
C3	C3 exoenzyme
Cdk5	Cyclin-dependent kinase 5
CGN	Cerebellar granule neuron
CHO	Chinese hamster ovary
CK2	Casein kinase II
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycans
CST	Corticospinal tract
DRG	Dorsal root ganglion
GPI	Glycosyl phosphatidylinositol
GSK3	Glycogen synthase kinase 3
IGF-1	Insulin-like growth factor 1
LINGO1	LRR and Ig domain containing, Nogo receptor interacting protein
LRR	Leucine-rich repeat
MAG	Myelin-associated glycoprotein; S- (small) L- (large)
MAI	Myelin-associated inhibitor
MAP2	Microtubule-associated protein 2
MHCI	Major histocompatibility complex class I
MLC	Myosin light chain
MMP	Matrix metalloproteinases
mTORC	Mammalian target of rapamycin complex
N-CAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NgR	Nogo receptor
NSCISC	National Spinal Cord Injury Statistical Center
OMgp	Oligodendrocyte myelin glycoprotein
PAK	p21-activated kinase
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PI-PLC	Phosphatidylinositol-specific phospholipase C
PirB	Paired-immunoglobulin-like receptor B
PKA	Protein kinase A
PKC	Protein kinase C

PLCγ	Phospholipase C gamma
PNS	Peripheral nervous system
PrP	Prion protein fragment
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RGC	Retinal ganglion cell
Rho-GDI	Rho GDP dissociation inhibitor
Rho-GEF	Rho guanine nucleotide exchange factor
ROCK	Rho kinase, p160ROCK (ROCKI) and ROK-Rho-kinase (ROCKII)
SCG	Superior cervical ganglion
SCI	Spinal cord injury
Sema3A	Semaphorin 3A
Sema4D	Semaphorin 4D
SHIP	Src homology 2-domain-containing inositol polyphosphate 5'-phosphatase
SHP 1 or 2	Src homology 2–containing protein tyrosine phosphatase
TNFR	Tumor necrosis factor receptor
TRAF6	Tumor necrosis factor Receptor Associated Factor 6
TSC1	Tuberous sclerosis complex 1

CHAPTER I

INTRODUCTION

Overview

It has long been known that spinal cord injury (SCI) results in paralysis. The Edwin Smith Surgical Papyrus dating to 1550 BC and one of the oldest surviving surgical texts, describes spinal cord damage in Egyptian hieroglyphics as “One having a dislocation in a vertebra of his neck, while he is unconscious of his two legs and his two arms, and his urine dribbles. An ailment not to be treated.” (National Library of Medicine Archives). Although our understanding of the mechanisms preventing functional recovery following spinal cord injury has grown significantly since this early observation, spinal cord injury continues to be a devastating condition for which we have no cure.

In the central nervous system, axons fail to regenerate following injury due in part to the presence of growth inhibitory signals at the injury site. The focus of the research described here was to elucidate the mechanisms of axon outgrowth inhibition by Myelin-associated glycoprotein (MAG), an inhibitory protein expressed on myelin that contributes to lack of axonal regrowth. Chapter 1 will describe the clinical features and pathophysiology of spinal cord injury to underscore the importance of conducting studies such as those described here and to aid in the understanding of the disease process. It will summarize why regeneration is limited in the CNS with a

particular focus on the inhibitory nature of myelin. Chapter 1 will then move on to describe what is known about the biochemistry and function of MAG with regards to its normal functions as well as its role as an inhibitor of axon outgrowth. Chapter 2 will present studies carried out to identify mechanisms of cortical neuron outgrowth inhibition by MAG. Although these particular neurons are often damaged in spinal cord injuries, very little is known about how they are inhibited. Chapter 3 will describe the identification of a novel pathway involved in neurite outgrowth inhibition by MAG. Chapter 4 will present work done to elucidate how one particular form of MAG, soluble MAG, signals to inhibit axonal growth. Lastly, in Chapter 5 I will conclude by discussing important implications of this work for the regeneration field as well as other MAG functions and outline future experiments to identify novel axonal MAG receptors and enhance our understanding of how MAG signals.

Anatomy of the spinal cord

The spinal cord is encased along its length by the vertebral column and functions to connect the brain to the rest of the body. A very simplified view of the spinal cord encompasses axons carrying information from the cortex to the spinal column to regulate motor function and axons carrying information from the skin to the cortex for processing of sensory cues. The gray matter of the spinal cord is a butterfly shaped region containing neuronal cell bodies and the surrounding white matter contains axon tracts projecting to and from the brain. Cortical neurons of the motor cortex (upper motor neurons) project via the corticospinal tract (CST) to motor neurons in the ventral horns of the spinal column (lower motor neurons). Lower motor neurons

then project outside of the spinal cord to synapse on muscle tissue to initiate movement. Peripheral sensory nerves of dorsal root ganglion neurons carry sensory input from sensory receptors on skin to the spinal cord. Sensory neurons then transmit information to the somatosensory cortex via the dorsal column-medial lemniscus and anterolateral axon tracts (Figure 1.1) (Purves et al., 2002; Lin 2003).

In addition to neurons, the central nervous system (CNS) also contains glial cells, which include astrocytes, oligodendrocytes, and microglia. The main functions of glial cells are to provide an appropriate environment for neuronal function. Briefly, astrocytes play an important role in maintaining ion homeostasis and are an important component, along with endothelial cells, of the blood brain barrier. Oligodendrocytes are the myelin forming cells of the CNS. Oligodendrocytes extend a specialized, lipid-rich membrane called myelin that ensheaths axon fibers. Myelin will be discussed in more detail in later sections, but its main function is to insulate axons and allow for rapid and more efficient nerve conduction. Schwann cells play an equivalent role to oligodendrocytes in the peripheral nervous system (PNS). Microglia are similar to phagocytic macrophages and are important in protecting neurons as well as clearing debris during injury (Purves et al., 2001).

Spinal Cord injury

Epidemiology

It is estimated that 2.5 million people live with a SCI worldwide and according to the International Campaign for Cures of Spinal Cord Injury Paralysis (ICCP), a conservative estimate of the incidence of SCI is 22 people per million with

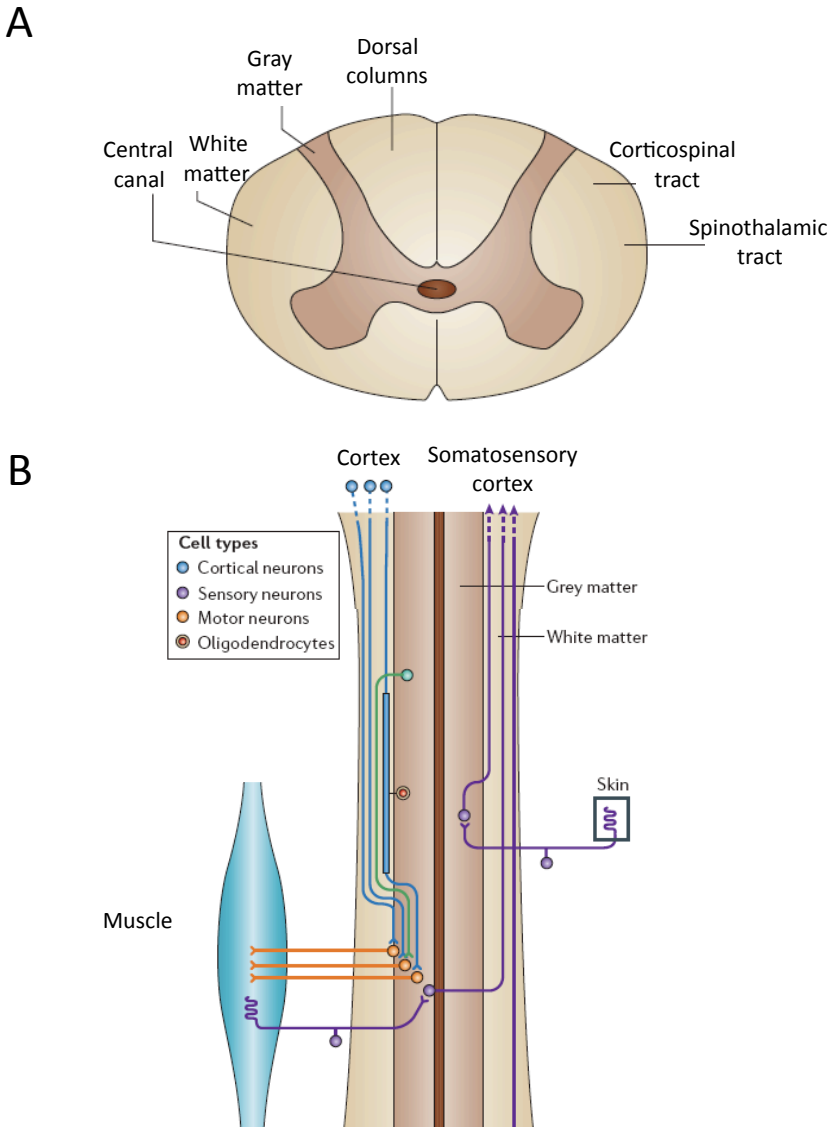


Figure 1.1 Illustration of spinal cord anatomy. ***A***, A transverse section of the spinal cord showing gray and white matter as well as the corticospinal tract. ***B,*** Cortical neurons project from the cortex via the corticospinal tract to motor neurons in the gray matter of the spinal cord. Motor neurons then signal to muscle to regulate movement. Sensory input from the skin and muscle projects through the PNS to the CNS where it ascends to the somatosensory cortex. Modified from Thuret et al. 2006.

approximately 130,000 people surviving a SCI annually (Thuret et al., 2006). In the United States, approximately 12,000 people survive a SCI per year, with an estimated 262,000 people living with spinal cord injury in 2009 (National Spinal Cord Injury Statistical Center, NSCISC, 2010). The average age of individuals afflicted with spinal cord injury is 40.2 years, with 80.8% of the cases occurring among males.

The major causes of traumatic spinal cord injury as of 2005 are motor vehicle crashes (41.3%), falls (27.3%), violence (mostly gun shot wounds, 15%), sports related activities (7.9%), and other/unknown causes (8.5%). There are also many non-traumatic causes of spinal cord injuries, including congenital, degenerative, metabolic, infectious, inflammatory, ischemic, and tumorigenic (Table 1.1) (McDonald and Sadowsky, 2002). The prevalence of non-traumatic SCIs is not known due to the heterogeneity of causes. However, it is thought that the overall prevalence of SCIs would be quadrupled if non-traumatic injuries were accounted for (McDonald and Sadowsky, 2002). My discussion of spinal cord injury will focus on traumatic injury for simplicity, but it is important to keep in mind that our understanding of the molecular mechanisms involved in preventing regeneration will aide in addressing lack of regeneration observed in traumatic and non-traumatic injury.

Clinical Features

The clinical manifestations of SCI depend on the location as well as extent of the injury. Severity of injury is classified according to the American Spinal Injury Association (ASIA) Scale (Table 1.2) (McDonald and Sadowsky, 2002). In most SCIs, both upper and lower motor neuron injuries are observed, due to damage to axon tracts (white matter) and spinal motor neurons (gray matter) respectively at the level of injury. Complete injury involves most of the spinal cord at a given level in the spinal column and results in paralysis and loss of sensation below that level. Approximately 56% of traumatic SCIs impact the cervical cord, the most flexible region of the spinal cord, and can result in quadriparesis or quadriplegia (Rowland et al., 2008). Various levels of incomplete injuries can occur depending on the neurons and axon tracts damaged. Injury to specific axon tracts results in different clinical signs (Figure 1.2) (Dietz and Curt, 2006). Sensation is usually retained to a greater extent than motor function due to the location of sensory tracts in a less susceptible, peripheral location in the cord.

The prognosis of an individual suffering from spinal cord injury depends on the person's age, level of injury, and severity of injury with overall life expectancy of individuals surviving a SCI being lower than the general population (NSCISC 2010). Compared to patients suffering a thoracic injury or lower, patients with a cervical cord injury have a significantly higher risk of death (6.6 fold for C1-C3 injury, 2.5 fold for C4-C5 injury, and 1.5 fold for C6-C8 injury) (Hansebout et al., 2009). The extent of improvement in motor function depends on the severity of injury. Only 10 to 15% of individuals with a complete injury improve, with only 3% improving to a Grade D

Table 1.1 Non-traumatic causes of spinal cord damage

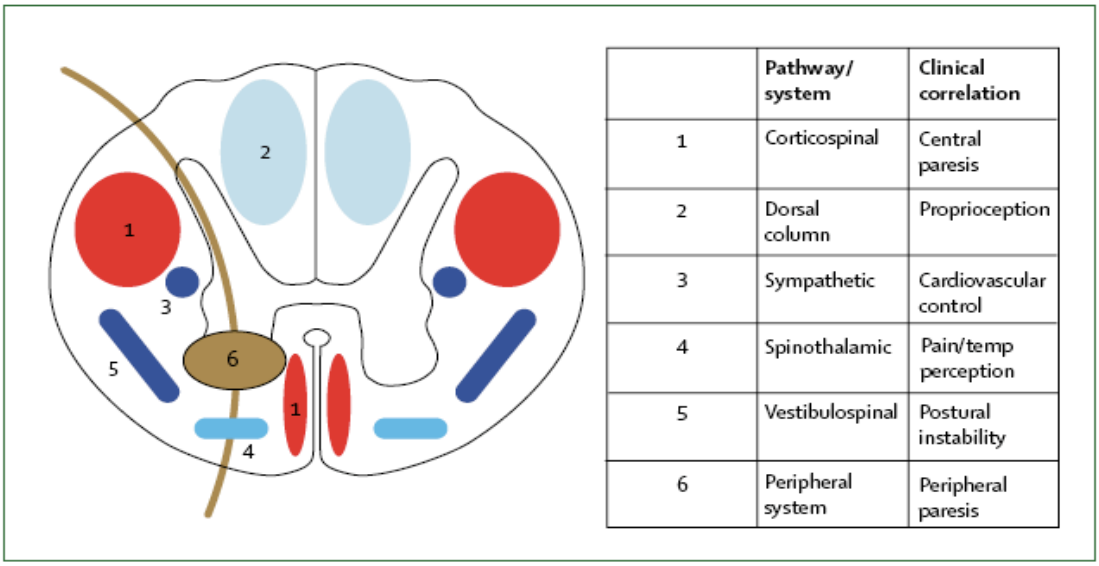
Cause	Disorder
Congenital and developmental	Cerebral palsy, dysraphism, diastematomyelia, spina bifida
Degenerative CNS disorders	Amyotrophic lateral sclerosis, Friedreich's ataxia, hereditary spastic paraparesis, infantile neuroaxonal dystrophy, Pelizaeus-Merzbacher disease, Canavan's disease, spinal muscular atrophy
Genetic and metabolic	Glutathione, β-methylcrotonylglycinuria, gangliosidosis, myelin protein zero, adrenomyeloneuropathy, abetalipoproteinaemia, vitamin B12 deficiency
Infectious	Most can cause transverse myelitis: viral (herpes simplex virus, varicella-zoster virus, cytomegalovirus, human T cell leukaemia virus-1, HIV, poliovirus); bacterial (Pott's disease, *Mycobacterium* spp); fungal (*Cryptococcus* spp); parasitic (*Toxoplasma gondii*, *Schistostoma mansoni*)
Inflammatory	Multiple sclerosis, transverse myelitis
Ischaemic	Arterial *vs* venous: aortic dissection, cardiac arrest, systemic hypotension, atherosclerosis, thrombosis, embolism, iatrogenic (aortic repair), arteriovenous malformations
Post-injury sequelae	Delayed functional loss, syringomyelia
Rheumatological and degenerative	Spondylolysis, stenosis, disc disease, Paget's disease, rheumatoid arthritis, posterior longitudinal ligament ossification
Toxic	Methotrexate, cytosine arabinoside, radiation
Tumours	Primary and metastatic (intramedullary and extramedullary)

Modified from McDonald et al. 2002

Table 1.2 ASIA scale: Severity of injury

American Spinal Injury Association Scale (ASIA)	
A	No motor or sensory function is preserved below the neurologic level through the sacral segments S4-5 (complete cord injury)
B	Sensory but not motor function is preserved below the neurologic level and extends through the sacral segments S4-5
C	Motor function is preserved below the neurologic level and the majority of key muscles below the neurologic level have a muscle grade less than 3 (ie, no antigravity movement)
D	Motor function is preserved below the neurologic level and the majority of key muscles below the neurologic level have a muscle grade of at least 3 (at least antigravity movement)
E	Motor and sensory functions are normal (no cord injury)

Modified from Hansebout et al. 2009



	Pathway/ system	Clinical correlation
1	Corticospinal	Central paresis
2	Dorsal column	Proprioception
3	Sympathetic	Cardiovascular control
4	Spinothalamic	Pain/temp perception
5	Vestibulospinal	Postural instability
6	Peripheral system	Peripheral paresis

Figure 1.2 Clinical features of spinal cord injury. Spinal cord injury can damage a number of pathways in the spinal cord. The extent of injury and the axonal tracts impacted by damage result in a variety of clinical manifestations. Shown here is a transverse section of the spinal cord highlighting key tracts impacted by spinal cord injury and the clinical correlations associated with those specific tracts. Damage to the corticospinal tract for example leads to paralysis. Modified from Dietz et al. 2006.

injury on the ASIA scale. 54% of Grade B injuries recover to Grade C or D and 86% of initial Grade C or D injuries will recover some walking ability. The major causes odeath in individuals with spinal cord injury are pneumonia, septicemia, and pulmonary emboli due to immobility (NSCISC 2010).

Pathophysiology

The pathophysiology of SCI is biphasic, consisting of an initial primary injury that is followed by a secondary injury (McDonald and Sadowsky, 2002; Rowland et al., 2008). The primary injury, which is often associated with damage to the vertebral column, results from the trauma itself. The mechanical insult can result in compression, contusion, laceration, or shearing of the spinal cord. Mechanical injury can directly damage axons, blood vessels, and cell membranes. Key features of primary injury are hemorrhaging, swelling, and ischemia.

The secondary damage begins approximately two hours after the primary event and consists of ischemia, hypoxia, inflammation, edema, excitotoxicity, loss of ion homeostasis, free radical production, and cell death (neurons, oligodendrocytes, and astrocytes) at the injury site (Thuret et al., 2006; Rowland et al., 2008). This second injury phase leads to a prolonged period of tissue damage that accounts for most of the pathology observed in SCI and consists of various stages: early acute, secondary subacute, intermediate, and chronic (Figure 1.3) (McDonald and Sadowsky, 2002; Norenberg et al., 2004; Rowland et al., 2008). The early acute phase, which lasts 2-48 hours, is characterized by ongoing hemorrhaging, rising edema, and inflammation. Furthermore, secondary injury processes that advance

axon damage and bring about cell death begin during this phase of SCI. Secondary injury progression is often accompanied with considerable neurological deterioration in the patient (Hansebout et al., 2009). Free radical production peaks at approximately 12 hours after injury and the resulting lipid peroxidation plays a role in axonal membrane damage and neuronal and glial cell death. The lack of ion homeostasis, especially calcium, is a key contributor to necrotic and apoptotic cell death at the injury site. Extracellular glutamate levels rise following injury due to cell damage and deregulation of ion and glutamate transporters, resulting in excitotoxicity. The high levels of glutamate receptor activation contribute to neuronal and glial cell death. The early acute phase is also characterized by infiltration of immune cells and inflammation. Various cell types (astrocytes, T cells, microglia, monocytes, and neutrophils) and inflammatory signals (TNFa, interleukins, and interferons) contribute to inflammation at the site of injury. Inflammation following injury is a very complex process as it is thought to contribute to secondary injury processes as well as aid in recovery by clearing debris and providing a neuroprotective function. Blood brain barrier (BBB) permeability is compromised during this phase of SCI due to the physical disruption of endothelial cells and astrocytes making up the barrier and to the effects of inflammation on vascular permeability. Death of oligodendrocytes results in demyelination of axons following injury, a process that in rodents peaks at 24 hours after injury.

The subacute phase of SCI lasts from 2 days to 2 weeks. During this phase, phagocytes actively clear debris at the injury site. Differences in the degree of clearance between the PNS and CNS may account for differences in capacity for

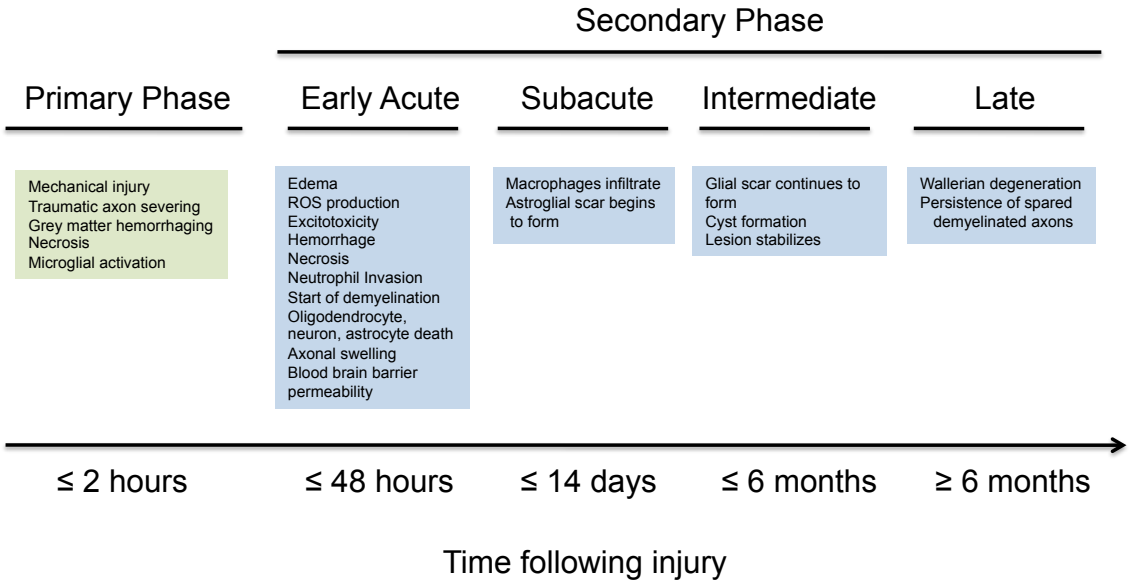


Figure 1.3 Pathophysiology of spinal cord injury. Pathophysiology of spinal cord injury consists of two phases, an immediate primary phase followed by a secondary phase that lasts throughout the lifetime of the individual. Shown here is a timeline of spinal cord injury highlighting key aspects of pathophysiology for each phase. Adapted from Rowland et al. 2008.

regeneration between the two systems. The glial scar also forms during the subacute phase. The glial scar is formed by reactive astrocytes at the outside edge of the lesion. Reactive astrocytes proliferate and become hypertrophic, extending many, large processes that entangle to form the glial scar, a significant physical barrier to regeneration in the CNS. The intermediate phase covers the time between 2 weeks to 6 months following injury. During this phase the glial scar matures and axonal sprouting can be observed. Although sprouting is minimal and does not occur over long distances, the sprouting observed during this phase indicates that there may be potential for regrowth in the CNS. The chronic phase begins approximately 6 months after injury and lasts throughout the patient's lifetime. During this phase, the injury stabilizes and the scar continues to form. The development of cavities or cysts that impede with ascending and descending axonal tracts occurs during the chronic phase and represent the final stages of injury. Dying back of axons and eventual neuronal death occurs over a period of years. Although CNS neurons can survive years following injury, one of the major barriers to regeneration and functional recovery in the CNS is the failure of axons to regrow past the injury site.

Understanding the pathophysiology of spinal cord injury and the mechanisms underlying the various processes involved is critical to developing effective therapies. Although numerous therapeutic interventions have been attempted for the various stages of spinal cord injury, they have had very limited success (Dietz and Curt, 2006; Thuret et al., 2006). Due to the complexity of the nervous system and SCI pathophysiology it is likely that effective therapy will encompass multiple approaches and targets. Enhancing regeneration of surviving neurons to reestablish

neuronal connectivity and function is an area of great interest and has thus far proven extremely challenging.

Regeneration in the CNS

Regeneration in the CNS and PNS

The lack of regeneration in the adult CNS of higher vertebrates was described in 1928 by Ramon y Cajal as “Once development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centers, the nerve paths are something fixed, ended, immutable. Everything may die. Nothing may be regenerated.” (Cajal 1928) While axons of peripheral nerves are capable of regeneration following injury, central nervous system axons are not. Ramon y Cajal went on to hypothesize that the capacity for regeneration of damaged axons in the CNS and PNS differs due to the environment in which the axons are located and not on the intrinsic ability of the neurons to regrow. Several studies have supported Cajal’s hypothesis by showing that CNS axons are capable of regrowth if presented with a favorable cellular environment.

When allowed to grow into transplanted peripheral nerves, injured CNS axons are able to elongate, demonstrating that these neurons have the intrinsic ability to regenerate. One study transected and removed a portion of the mid-thoracic spinal cord in rats and replaced it with a sciatic nerve graft (Richardson et al., 1980). Transected CNS axons were able to grow into and cross the graft (Figure 1.4). Similarly, CNS axons were able to regenerate over long distances across a sciatic nerve bridge connecting flanking ends of a spinal cord injury (David and Aguayo,

1981). Importantly, axons stopped growing when they encountered CNS tissue again, underscoring the inhibitory nature of the CNS milieu. Such studies provide evidence that the lack of regeneration observed in the CNS is not due to intrinsic properties of the CNS neurons, but to the environment their axons encounter. In reverse experiments, PNS neurons failed to extend processes into the CNS when optic nerves were implanted into sciatic nerves, further emphasizing that differences exist between the CNS and PNS tissues (Aguayo et al., 1978; Weinberg and Spencer, 1979). *In vitro*, when superior cervical ganglion (SCG) or dorsal root ganglion (DRG) neurons were plated in a chamber slide and allowed to grow into optic nerve explants or sciatic nerve explants, axons grew in the sciatic nerve explant but were completely excluded from the optic nerve explant (Schwab and Thoenen, 1985), suggesting a preference for sciatic nerve tissue. It was postulated that the CNS environment is not conducive to axon outgrowth, either due to the presence of negative regulators of outgrowth or the absence of growth promoting factors. To date, most evidence points to the presence of growth inhibitory molecules in the CNS as the primary restraint on regeneration.

Inhibitors of regeneration in the CNS

The glial environment in the CNS, which differs considerably from that in the PNS, is the main inhibitor of regeneration. Inhibitors of axon outgrowth in the CNS are present in the glial scar and on myelin at the injury site (Figure 1.5) (Yiu and He, 2006). The glial scar is a very important contributor to inhibition of regeneration in

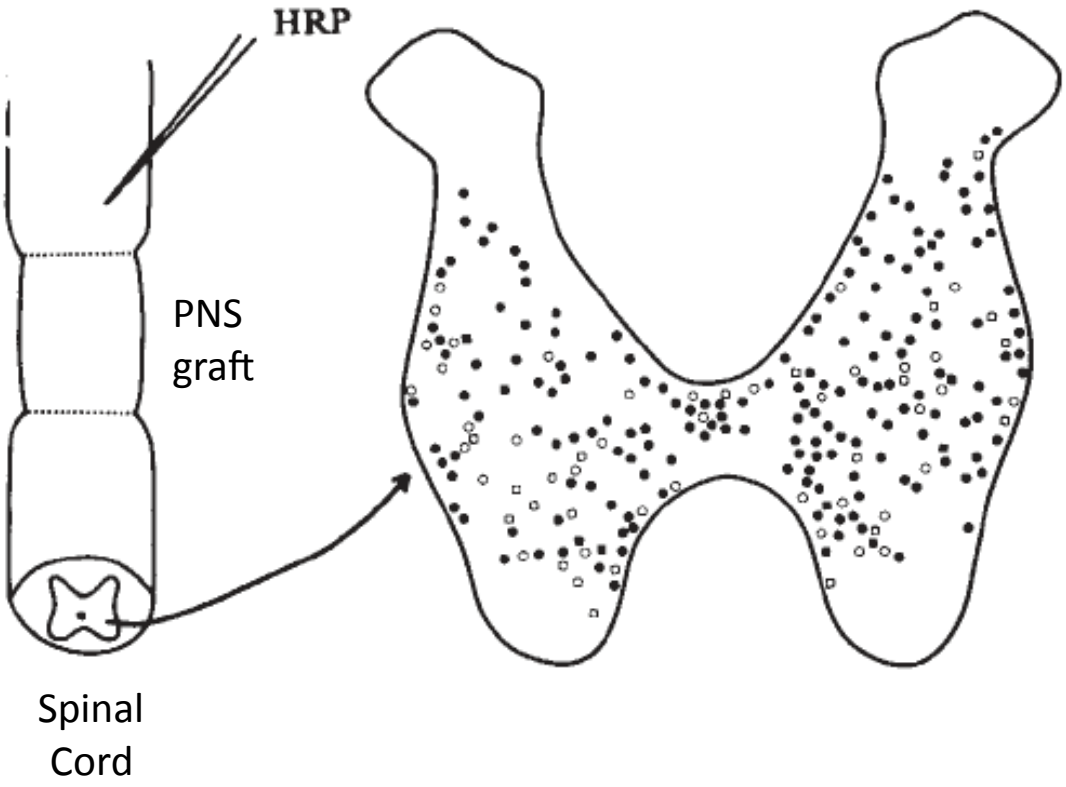
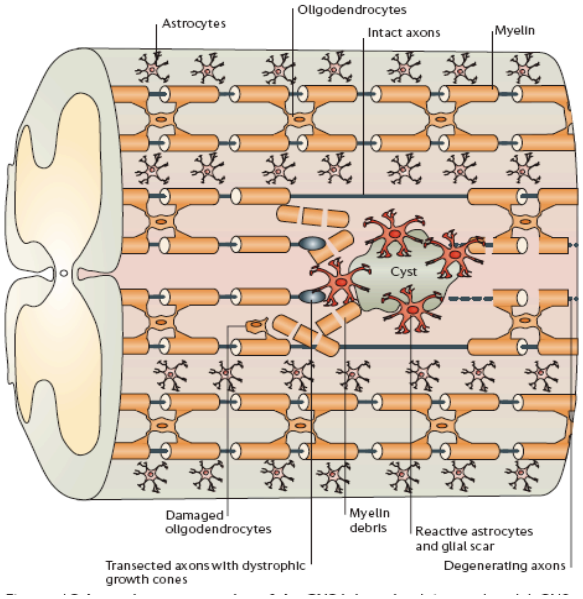


Figure 1.4 CNS axons can regenerate when presented with a favorable environment. A segment of rat spinal cord was removed and replaced with a sciatic nerve graft. 3-4 months post-injury, horseradish peroxidase (HRP) was injected rostral to the graft. 2 days later, spinal cords sections distal to the graft were analyzed for incorporation of HRP. The image on the right represents data from four animals (indicated by different symbols) showing labeled axons that were able to cross the injury site. Modified from Richardson et al. 1980.

A



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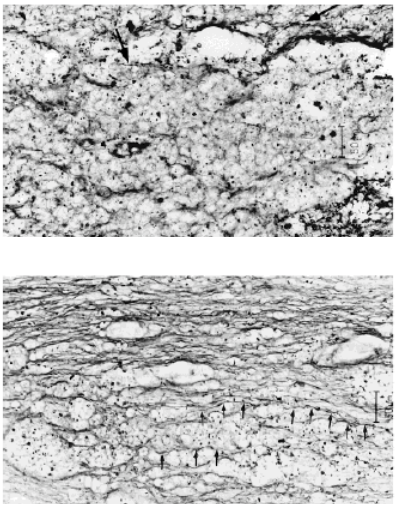

Figure 1.5 Inhibition of axon outgrowth following injury. ***A,*** Schematic of the injured spinal cord showing damaged axons with dystrophic growth cones as well as damaged surrounding tissue. Axon outgrowth past the lesion is restricted primarily by myelin debris, which expresses several inhibitory proteins, as well as by astrocytes that release inhibitors of outgrowth in addition to contributing to the formation of the glial scar. ***B,*** Longitudinal section of injured rat spinal cords 1 month after injury containing very few neurites as shown by immunostaining for neurofilament (arrow, upper panel). Myelin remnants at the injury site are shown by staining for MAG (arrows, bottom panel). Adapted from Yiu and He 2006 and Franzen et al. 1998.

the CNS by providing a physical barrier to growth. Furthermore, reactive astrocytes in and around the scar express and secrete inhibitory molecules that contribute to further to outgrowth inhibition (Rudge and Silver, 1990; Silver and Miller, 2004). One of the biggest contributors to restricting axon growth in the glial scar is the expression of chondroitin sulfate proteoglycans (CSPGs) by astrocytes (McKeon et al., 1991; Wang et al., 1997). CSPGs (family members include brevican, aggrecan, neurocan, NG2, and phosphacan) are proteins with attached sulphated glycosaminoglycans, moieties that are thought to be required for the inhibitory effects of CSPGs (Sandvig et al., 2004). A lot remains unknown regarding CSPG mechanisms of axon outgrowth inhibition with possible mechanisms including a masking of growth promoting signals in the extracellular matrix. However, there is evidence for activation of Rho GTPase by CSPG, a known negative regulator of regeneration, and for axonal receptors for NG2 that may mediate inhibitory signaling (Yiu and He, 2006). Interestingly, although the extent of glial scarring is considerable in rodents, it does not appear to be as prominent a feature in humans (Hagg and Oudega, 2006), raising a question as to the benefit of targeting glial scar components therapeutically in humans. The relative contributions of the glial scar and myelin, discussed in detail in the next section, to inhibition of regeneration remain controversial, but it is likely that both play some role in preventing regrowth of axons.

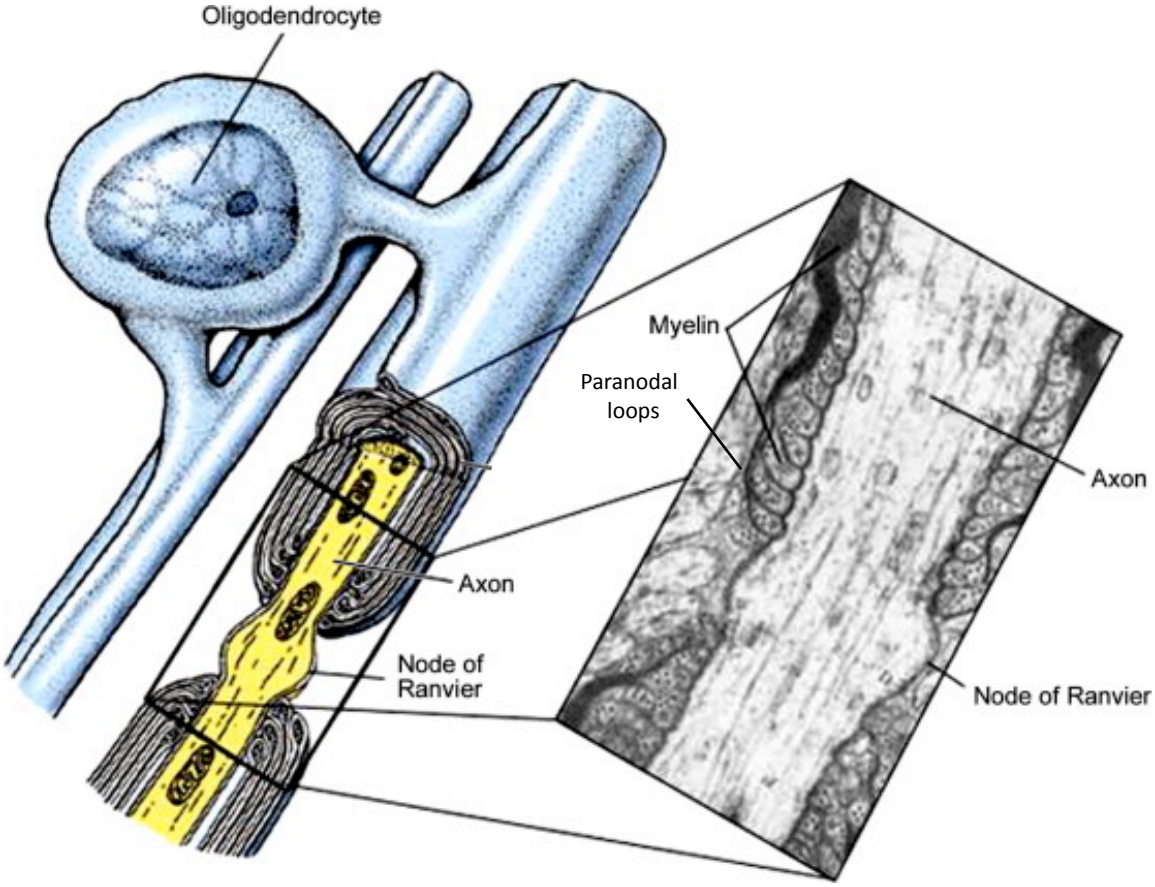

Figure 1.6 Schematic and ultrastructure of myelin in the CNS. Oligodendrocytes in the CNS extend processes that ensheath axons with a specialized phospholipid bilayer known as myelin. The main function of myelin is to facilitate saltatory conduction of action potentials from one Node of Ranvier to another and thus produce fast nerve conduction over long distances. Modified from Schnaar 2010.

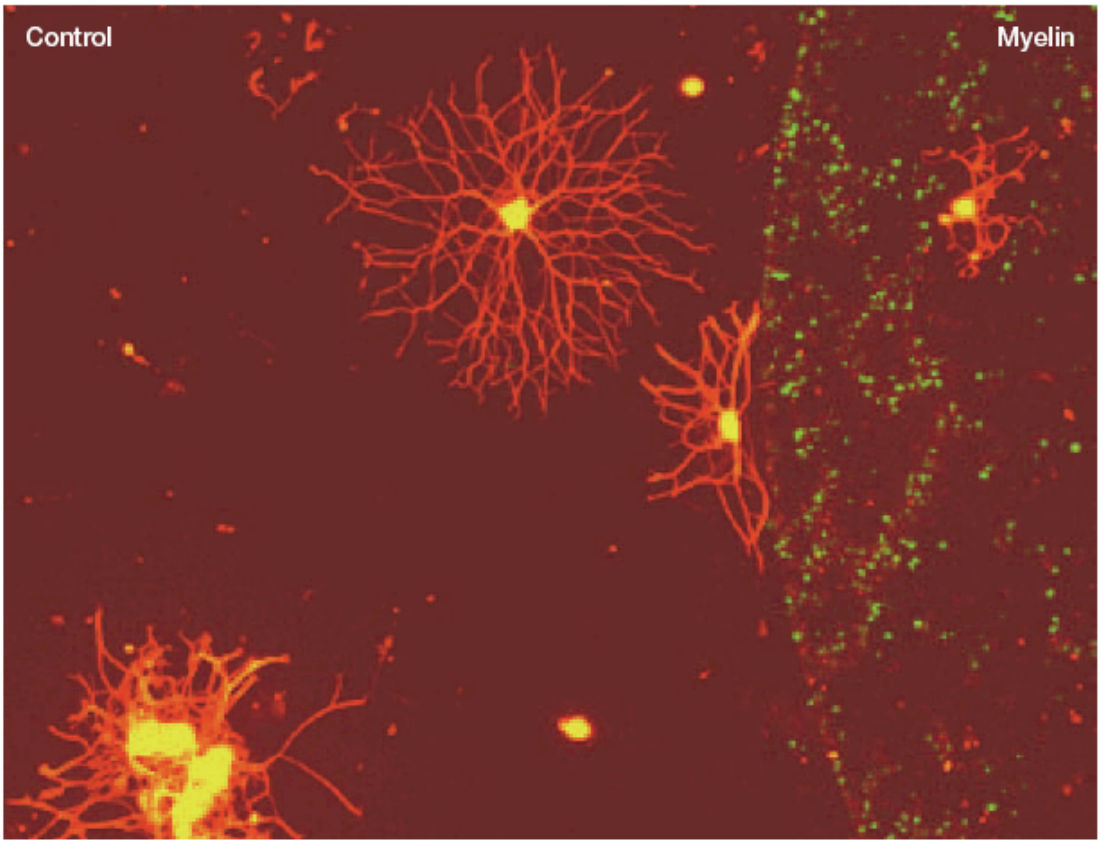

Figure 1.7 Neurite outgrowth is inhibited by myelin in culture. Myelin is a major contributor to neurite outgrowth inhibition in the CNS. *In vitro*, neurons grown on myelin substrate show reduced process outgrowth. DRGs were grown in a well containing control substrate on the left and myelin on the right. While neurite outgrowth was observed on control substrate, it was substantially reduced on myelin. (Red = neuronal specific marker, TuJ1; Green = myelin) (Lee et al 2004)

Inhibitory nature of myelin

The failure of axons to grow in the CNS is due to a significant extent to the inhibitory properties of myelin (Figure 1.6 and 1.7). Ramon y Cajal first postulated that white matter opposes CNS regeneration in 1928 (Cajal 1928). It wasn't until 60 years later that Caroni and Schwab (Schwab and Caroni, 1988) provided evidence that CNS myelin is not permissive to neurite outgrowth while PNS myelin is. Characterization of the effect of various CNS glial cells on neuronal process outgrowth revealed that astrocytes and immature oligodendrocytes are favorable substrates for neurite outgrowth. Superior cervical ganglion (SCG), dorsal root ganglion (DRG), or retinal ganglion neurons plated on astrocytes or immature oligodendrocytes in culture grew extensive neurites that formed dense neurite networks. However, neuronal adhesion and neurite outgrowth was dramatically reduced on mature oligodendrocytes. Similar results are observed with neurite outgrowth of neuroblastoma cells and with cell spreading of fibroblasts, showing that these inhibitory effects are not specific to primary neurons (Schwab and Caroni, 1988). Because mature oligodendrocytes are the myelin producing cells in the CNS, experiments were carried out to test the effect of plating neurons on myelin. Neurite outgrowth of SCG or DRG neurons was examined *in vitro* on myelin substrate. Both neuron types readily extended processes on PNS myelin but not CNS myelin, where neurons extended short processes that made minimal contact with myelin (Caroni and Schwab, 1988a).

The inhibitory nature of myelin has also been demonstrated *in vivo*. Mice immunized with myelin generate antibodies to myelin-associated inhibitors. Following a dorsal hemisection of the spinal cord of such mice, corticospinal tract fibers demonstrated

significant regeneration and the mice recovered significant hind limb motor function. Antisera from these mice also reversed the inhibitory effects of myelin on cerebellar neurons in culture (Huang et al., 1999). Interestingly, there is a correlation between the start of myelination and the loss of axon regeneration in the CNS. During development, myelination does not occur until axon outgrowth is for the most part complete (Lin 2003). The capacity for chick spinal cord regeneration following injury disappears at a developmental stage that correlates with the start of myelination (Keirstead et al., 1992). Furthermore, if the onset of myelination is delayed, the period of regrowth can be extended in the developing spinal cord, supporting the growth limiting nature of myelin (Savio and Schwab, 1990; Keirstead et al., 1992; Keirstead et al., 1995). When rats are x-irradiated over the spinal cord at birth, oligodendrocyte differentiation and myelin formation are prevented, while other cells in the spinal cord, such as astrocytes are not affected. *In vitro*, extracts from these myelin-free spinal cords were not inhibitory. Corticospinal tract lesions were performed on these myelin free rats and 2-3 weeks after injury, corticospinal tract fibers could be seen growing long distances past the injury site as compared to controls (Savio and Schwab, 1990).

The hypothesis that the CNS environment is nonpermissive for neurite outgrowth due to the lack of neurotrophic support is challenged by the demonstration that as in the PNS, neurotrophins are present in the developing and adult CNS and that increased neurotrophic activity is present at sites of injury (Needels et al., 1986; Whittemore et al., 1987). The differences in neurite outgrowth on CNS tissue (optic nerve explants) and PNS tissue (sciatic nerve explants) occurred in the presence of high concentrations of nerve growth factor (NGF) (Schwab and Thoenen 1985).

Furthermore, they observed similar results with previously frozen optic or sciatic nerves, which did not contain living cells capable of expressing and releasing neurotrophins.

In order to identify the myelin component(s) responsible for the inhibitory nature of CNS myelin, various fractions of myelin were tested for their ability to reduce neurite outgrowth. These studies led to the identification of 35kd (NI-35) and 250kd (NI-250) membrane proteins on CNS myelin with strong inhibitory properties and provided the first evidence for the existence of molecules on myelin capable of inhibiting neurite outgrowth (Caroni and Schwab, 1988a). Antibodies generated against NI-250 neutralized the inhibitory effects of myelin, oligodendrocytes, and optic nerve explants on SCG neurons *in vitro*, supporting that the inhibitory nature of myelin is due to particular inhibitory proteins (Caroni and Schwab, 1988b). NI-250 antibodies were also able to promote regeneration *in vivo*. Rats undergoing a complete transection of the corticospinal tract exposed to these antibodies showed axon elongation up to 7-11 mm distal to the lesion site as compared to control antibody treated rats that experienced elongation barely exceeding 1 mm (Schnell and Schwab, 1990). Importantly, only a small number of axons regenerated most likely due to the presence of other inhibitors.

Myelin-Associated Inhibitors

In the early 1990s several groups embarked on the search for myelin associated inhibitors (MAIs). In 1994, Myelin-associated glycoprotein (MAG) was the first MAI to be characterized. MAG is the focus of this thesis and will be discussed in detail in

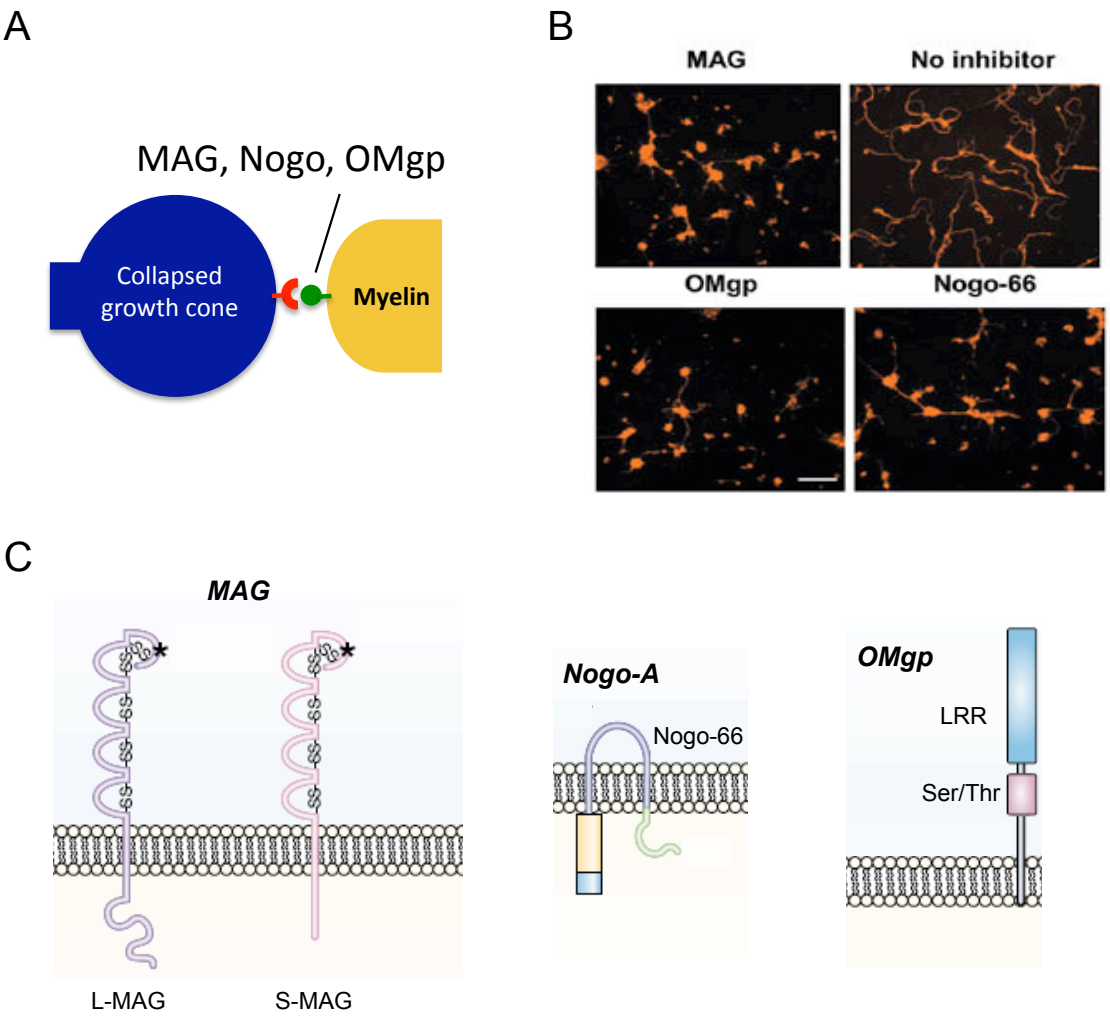

Figure 1.8 Function and structure of the major myelin-associated inhibitors. A, The classic MAIs expressed on myelin that interact with the axon to inhibit outgrowth are MAG, Nogo, and OMgp. ***B***, Cerebellar granule neuron outgrowth on MAG, Nogo or OMgp substrate is substantially inhibited compared to control substrate. ***C,*** Schematic of MAG, Nogo-A and OMgp structures. MAG exists as two isoforms, small MAG (S-MAG) and large MAG (L-MAG). The extracellular domain consists of 5 Ig-like domains. The sialic acid binding site (*) is present in the first Ig domain. The Nogo-A structure highlights its inhibitory 66 amino acid extracellular loop (Nogo-66). OMgp is a GPI-linked protein consisting of a leucine-rich repeat and a serine/threonine rich domain. Modified from Mi et al., 2004 and Filbin 2003.

the subsequent sections. The identity of NI-250 (see above) was established as Nogo-A (Nogo or RTN4) by three studies more than a decade after its characterization as a molecule capable of inhibiting axon outgrowth (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). Nogo is part of the Reticulun (RTN) family, which consists of three other members, RTN1, RTN2 and RTN3. Three Nogo protein isoforms, Nogo-A, Nogo-B and Nogo-C, are produced from the same gene by alternative splicing and differential promoter utilization. Nogo-A is enriched in oligodendrocytes and myelin and is the best characterized as an inhibitor of neurite outgrowth. Nogo-66 is a 66-amino acid extracellular loop of Nogo that potently inhibits neurite outgrowth and induces growth cone collapse (GrandPre et al., 2000). The third key MAI to be discovered was Oligodendrocyte myelin glycoprotein (OMgp), a protein that is expressed on myelin and neurons in the CNS. OMgp is a glycosyl phosphatidylinositol (GPI) linked protein consisting of a leucine-rich repeat (LRR) and serine/threonine rich region in its extracellular domain. Like Nogo and MAG, OMgp inhibits neurite outgrowth and induces growth cone collapse (Wang et al., 2002b). MAG, Nogo, and OMgp, although vastly different structurally, share receptors on the axon surface and are often referred to as the classic MAIs (Figure 1.8). Sema4D (Moreau-Fauvarque et al., 2003) and Ephrin-B3 (Benson et al., 2005) are also proteins expressed on myelin capable of inhibiting axon outgrowth.

Myelin-associated glycoprotein

Structure

MAG is a 100-kDa type I transmembrane glycoprotein belonging to the immunoglobulin-like (Ig) superfamily. MAG consists of five extracellular Ig-like domains, a transmembrane domain, and one of two alternatively spliced intracellular domains (Figure 1.8c). The two isoforms of MAG are large MAG (L-MAG) and small MAG (S-MAG), which are 626 residues (72kD) and 582 residues (67kD) respectively and differ only in their cytoplasmic tails (Salzer et al., 1987).

The extracellular domain of MAG contains eight N-linked glycosylation sites and is approximately 30% by weight carbohydrate. Within the ectodomain is an HNK-1 carbohydrate epitope, which is shared by many proteins involved in mediating cell-cell interactions, such as L1 and neural cell adhesion molecule (N-CAM) (McGarry et al., 1983; Kruse et al., 1984). MAG also contains an RGD sequence (Arg-Gly-Asp), a tripeptide sequence involved in interaction of various receptors with extracellular proteins (Arquint et al., 1987; Salzer et al., 1987). MAG is a member of a subgroup of the Ig superfamily known as the siglec proteins (Varki and Angata, 2006; Quarles, 2007). Siglec group members exhibit high homology in their two amino terminal Ig domains and bind to sialic acid moieties on oligosaccharides. MAG prefers α2,3-linked sialic acid containing glycoproteins and gangliosides. MAG is one of few known siglecs expressed outside of the immune system (Quarles, 2007).

Expression

The expression of MAG is restricted to Schwann cells and oligodendrocytes, the myelin forming cells of the PNS and CNS respectively. MAG was appropriately named for its localization in "myelin-associated" glial membranes as opposed to compact myelin. MAG is expressed on the cell body and processes of oligodendrocytes prior to and during the initial stages of axonal ensheathment (Figure 1.9a) (Bartsch et al., 1989; Trapp et al., 1989). After compact myelin is formed in the CNS, MAG is localized to the innermost periaxonal membrane, the myelin membrane adjacent to the axon surface, as visualized by electron microscopic immunocytochemistry (Figure 1.9b) (Sternberger et al., 1979; Bartsch et al., 1989). MAG is quantitatively a minor myelin protein, comprising 1% of CNS and 0.1% of PNS total myelin protein. Although MAG expression is higher in the CNS than the PNS, MAG appears to be more widely distributed in the PNS than the CNS, with expression occurring in paranodes, Schmitt-Lanterman incisures, lateral loops, and mesaxons of PNS myelin (Trapp et al., 1989). The presence of MAG at these locations in the CNS remains somewhat controversial. Although early studies did not detect MAG at locations other than periaxonal myelin sheaths in the CNS, MAG was detected in paranodal regions of the optic nerve (Bartsch et al., 1989). More recently, expression of a GFP tagged MAG showed localization of MAG in CNS paranodes, although to a lesser extent than PNS paranodes (Erb et al., 2006).

MAG expression begins with the onset of myelination and continues throughout adulthood, suggesting a role for MAG in the initial process of myelin formation as well as the maintenance of myelin. L-MAG and S-MAG expression is regulated developmentally and differs between the CNS and PNS. In rodents, L-MAG

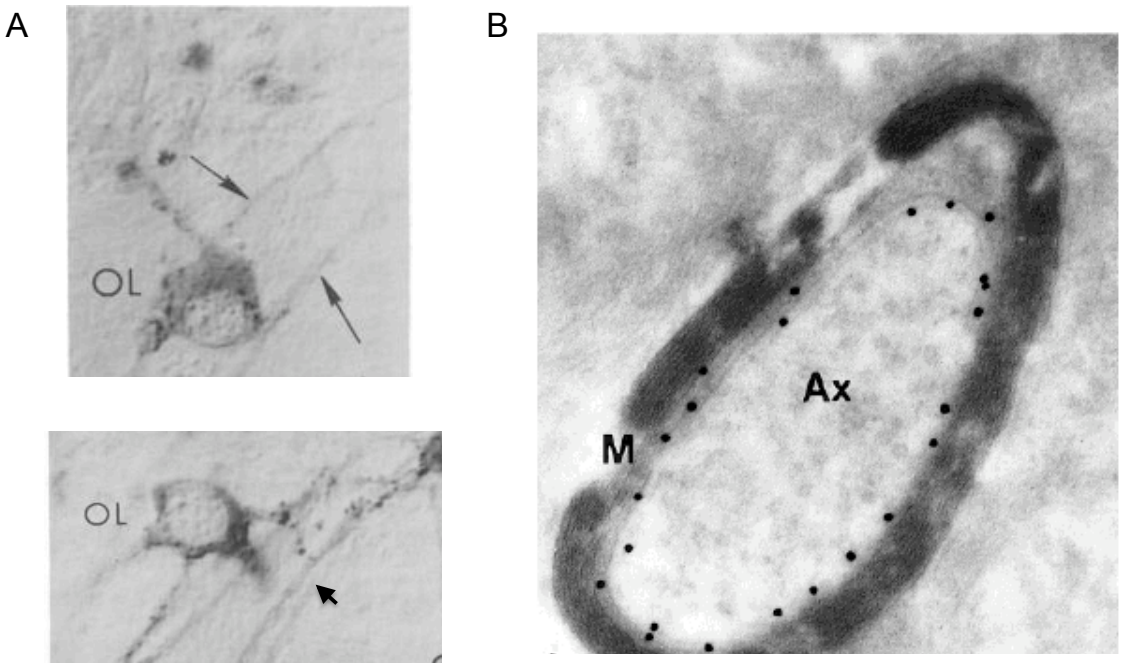

Figure 1.9 MAG is expressed on oligodendrocytes and myelin membranes. A, Immunostaining for MAG in rat brain sections showing expression of MAG in the cytoplasm and processes (arrows) of oligodendrocytes (OL) in the newborn (top panel) and also along myelin sheaths (arrow) at postnatal day 5 (bottom panel). ***B,*** Immunoelectron microscopy of the mouse optic nerve shows compact myelin (M) surrounding an axon fiber (Ax). MAG immunoreactivity can be seen at the interface between the inner myelin sheath and the axon. From Sternberger et al. 1979 and Bartsch et al. 1989.

expression is predominant in the CNS during development and decreases in adulthood. Equal amounts of S-MAG and L-MAG were detected in the adult rodent CNS (Salzer et al., 1987). However, S-MAG is the prevalent form of MAG in the PNS during development and in the adult. In humans, L-MAG is the major form found in the adult brain and S-MAG is the predominant form in the PNS (Miescher et al., 1997). Interestingly, the two isoforms of MAG are also differentially expressed by various subtypes of oligodendrocytes (Butt et al., 1998). The relevance of developmental changes in MAG isoform expression as well as cell-type specific expression is not understood.

Function

Although this thesis will focus on the negative role of MAG in axonal regeneration in the CNS, MAG also has positive functions in the developing and mature nervous system in maintaining axon-myelin stability, structuring Nodes of Ranvier, and regulating the axonal cytoskeleton.

The localization of MAG at periaxonal glial membranes and its extracellular features (Ig domains, RGD sequence, and HNK-1 epitope) suggested a role for MAG in axon-glial interactions. One of the first studies pointing to a role for MAG in maintaining the axon-glial interface arose from studies of the Quaking mouse mutant. The Quaking mouse exhibits a demyelinating phenotype and expresses only 15% of the normal levels of MAG. Interestingly, these mice have an abnormally wide space between the axon and the innermost myelin membrane in regions that do not show positive MAG staining by immunohistochemistry (Trapp et al., 1984). Cell culture

studies were critical in ascertaining a role for MAG in regulation of axonal-glial interactions. MAG antibodies blocked neuron-oligodendrocyte contact while having no impact on astrocyte-oligodendrocyte interactions (Poltorak et al., 1987). Moreover, MAG is a binding ligand in neuronal-oligodendrocyte interactions, as recombinant L-MAG or S-MAG, incorporated into fluorescent liposomes, bound to neurons when applied to cultures of spinal cord or DRG neurons (Poltorak et al., 1987; Johnson et al., 1989). The binding was specific, as liposomes without MAG or with a control protein did not bind (Johnson et al., 1989). Furthermore, MAG containing liposomes did not bind to other nervous system cells including astrocytes, fibroblasts, or macrophages (Poltorak et al., 1987). MAG containing liposomes bound preferentially to neurites of larger neurons (larger axons are preferentially myelinated) and binding to unmyelinated neurons, in particular cerebellar neurons, was either not observed or occurred to a lesser extent than neurons that are myelinated *in vivo* (Poltorak et al., 1987; Johnson et al., 1989; Sadoul et al., 1990). Binding of MAG incorporated liposomes to neurites in culture was not affected by antibodies to the HNK1 epitope or RGD containing peptides (Sadoul et al., 1990), suggesting that these extracellular features did not play an essential role in MAG binding to axons. MAG binding also did not depend on the presence of Ca^{2+}, indicating that it is a Ca^{2+} independent adhesion molecule (Sadoul et al., 1990).

The role of MAG in myelin-axon stability was further supported by studies of MAG knockout mice. The effect of MAG knockout on myelination is subtle and is more extensive in the CNS than the PNS. However, studies of MAG knockout mice revealed a role for MAG in the initiation of myelination, formation of structurally intact myelin, and the ongoing maintenance of myelin (Li et al., 1994; Montag et al., 1994;

Schachner and Bartsch, 2000; Chen et al., 2006; Quarles, 2007). As mentioned previously, MAG expression commences as the glial cell begins to wrap the axon. If MAG were important in this initial contact we would expect that knockdown of MAG expression would impact the initiation of myelin formation. Although compact myelin formation is normal, there is a delay in the formation of myelin during development in the CNS, but not the PNS in *Mag-/-* mice. The optic nerves of 10-day-old *Mag-/-* mice demonstrated a 50% decrease in the density of retinal ganglion axons encircled by compact myelin compared to control animals (Montag et al., 1994). Adult *Mag-/-* mice also showed hypomyelination of the optic nerve, with 34.7% of axons remaining hypomyelinated at 2 months in the knockout mice compared to 11.7% of axons in age-matched wild-type controls (Bartsch et al., 1997). This difference decreased with age with 9-month-old *Mag-/-* mice having 18.2% unmyelinated retinal ganglion axons compared to 8.5% of control mice.

Overall, myelin tracts formed normally in *Mag-/-* mice as observed by immunocytochemistry for MBP and compact myelin lamellar organization was normal as demonstrated by X-ray diffraction (Li et al., 1994). Furthermore, myelin thickness and caliber of myelinated fibers were similar between *Mag+/+* and *Mag-/-* mice (Li et al., 1994). However, more subtle defects in myelination were detected by electron microscopy. While one report observed a normal periaxonal space in *mag-/-* mice (Montag et al., 1994), a different study found that the periaxonal space of *mag-/-* mice was dilated in the CNS and PNS compared to wild-type mice, indicating axonal separation from their myelin sheaths (Li et al., 1994). A consistent defect observed in the CNS of *Mag-/-* mice was missing or disorganized periaxonal cytoplasmic collars (Li et al., 1994; Montag et al., 1994). Only 5% of *Mag-/-* mice

contained a fully developed periaxonal cytoplasmic collar, compared to 75% in wild-type mice (Montag et al., 1994). The absence of MAG resulted in other abnormalities, including redundant compact myelin, the presence of oligodendrocyte cytoplasm within compact myelin, and the occurrence of supernumerary myelin sheaths (Li et al., 1994; Montag et al., 1994). Not unexpectedly, the nerve and motor function defects of *Mag-/-* mice were also subtle. Electrophysiological evaluation of *Mag-/-* sciatic nerves revealed a slight, but significant reduction in conduction velocity compared to wild-type sciatic nerves (Fruttiger et al., 1995; Weiss et al., 2000). Overall motor function and posture appeared normal in open field experiments. However under conditions that require fine motor coordination or balance, such as crossing a bar-cross apparatus, exploratory sniffing, or grooming, decreased motor activity was observed (Li et al., 1994).

Mag-/- mouse pathology became more prominent with maturity and effects on PNS myelination became more evident. Studies of aging *Mag-/-* mice revealed findings similar to those found in demyelinating conditions that are consistent with a breakdown of the myelin-axon interaction, resulting in glial and axonal pathology. Biochemical analysis of myelin and myelinated axons demonstrated that several molecular changes occur in CNS myelin that are consistent with oligodendrogliopathy (Weiss et al., 2000). Comparison of 14-month-old *Mag-/-* and *Mag+/+* mice revealed decreases in 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), neural cell adhesion molecule (N-CAM), tubulin, Na^+K^+ATPase, and Fyn tyrosine kinase in *Mag-/-* mice, all indicative of changes in oligodendrocyte health. These findings are supported by the morphological finding of degenerating periaxonal oligodendroglial processes in 8-month-old *Mag-/-* mice (Lassmann et al.,

1997). Degenerative changes observed in oligodendrocyte processes of *Mag-/-* mice include the presence of multivesicular bodies, an abundance of mitochondria, lipofuscin granules and a variety of inclusions. Further support for the role of MAG in maintaining properly myelinated axons is the presence of degenerating axons in older *Mag-/-* mice. Axonal pathology occurred in the PNS and CNS of older *Mag-/-* mice and was characterized by axonal swelling, myelin figures, ovoids, decreased axonal diameter, decreased expression and phosphorylation of neurofilaments, and ultimately axonal degeneration (Fruttiger et al., 1995; Yin et al., 1998; Weiss et al., 2000; Loers et al., 2004; Pan et al., 2005). Older mice also exhibited abnormal motor behavior. *Mag-/-* mice performed worse than wild-type mice on rotarod tests, which gauge balance and motor control (Pan et al., 2005).

MAG also contributes to the timely structuring and maturation of the Nodes of Ranvier (Figure 1.6). Formation of intact Nodes of Ranvier involves the development of three discrete regions within the node: the node itself, where sodium channels are highly expressed, the contiguous paranodes, which are characterized by Caspr expression, and the juxtaparanodes which are rich in potassium channels (Susuki and Rasband, 2008). In *Mag-/-* spinal cords, immunostaining for these various components of the node revealed a more diffuse distribution of expression, with overlap occurring amongst the various regions (Marcus et al., 2002). Nodal structural abnormalities in *Mag-/-* mice include a reduction in reversed lateral glial loops and transverse bands, which are likely due to defects in axonal-glial interactions. Importantly, these defects were not detected in mature mice, suggesting that the proper organization of the nodes is delayed but not prevented.

As mentioned above, lack of MAG expression in aging mice resulted in axonal degeneration. One of the changes observed in aged *Mag-/-* axons was modification of neurofilament expression and phosphorylation. Myelination influences neurofilament organization in normal axons (Hsieh et al., 1994). Myelinated segments of DRG axons have a larger caliber, increased neurofilament expression, and increased space between neurofilaments compared to unmyelinated regions (Hsieh et al., 1994). There is evidence that MAG is a component of myelin that contributes to the regulation of the axonal cytoskeleton. Decreased axonal caliber, diminished neurofilament spacing, and lower neurofilament phosphorylation were detected in the sciatic nerves of *Mag-/-* mice compared to wild-type mice (Yin et al., 1998). To verify that the effects of MAG knockout on the axonal cytoskeleton were directly due to MAG signaling and not a secondary effect of changes in the axonal-glial interaction, studies were conducted *in vitro*. Embryonic DRG neurons were plated on MAG expressing CHO cells and subsequently stained for various neuronal components (neurofilament, microtubule-associated protein B1, MAP2, and tau) all of which were upregulated on MAG. Neurons on MAG expressing cells also had increased expression of phosphorylated neurofilament. These results were confirmed by the detection of increased phosphorylated neurofilaments in PC12 cells treated with soluble MAG (Dashiell et al., 2002).

MAG reverse signaling

When glial cells and neurons interact, MAG is capable of both reverse and forward signaling, influencing glial and axonal biology respectively. Reverse signaling, which

for the most part remains obscure, will be briefly discussed here. Forward signaling by MAG, which has received considerably more attention, will be discussed in detail later with regards to the effect of MAG on neurite outgrowth.

The intracellular domains of L-MAG and S-MAG contain several putative phosphorylation sites (Arquint et al., 1987; Salzer et al., 1987), the phosphorylation of which impacts their ability to interact with glial cytoplasmic components. Both S-MAG and L-MAG can be phosphorylated on various serine and threonine residues on their cytoplasmic domains (Edwards et al., 1988; Kirchhoff et al., 1993). Protein kinase A (PKA) and protein kinase C (PKC) have both been identified as candidate kinases for MAG (Kirchhoff et al., 1993; Kursula et al., 2000). L-MAG is tyrosine phosphorylated, whereas S-MAG is not. Tryptic digest analysis of ^{32}P-labeled MAG revealed that one of the major tyrosine phosphorylation sites on MAG in oligodendrocytes is Tyr-620 (Jaramillo et al., 1994). Phosphorylated MAG interacts with phospholipase C gamma (PLCγ) *in vitro* and *in vivo* and phosphorylation at Tyr-620 is required for this interaction.

MAG also interacts with Fyn, a tyrosine kinase. Fyn can be co-immunoprecipitated with L-MAG and S-MAG from total brain lysates (Jaramillo et al., 1994; Umemori et al., 1994), an interaction that is mediated by the SH2 and SH3 domains of Fyn. Crosslinking the extracellular domain of MAG with a MAG antibody resulted in increased Fyn phosphorylation and activity in COS cells expressing MAG, but not other tyrosine kinases such as Src (Umemori et al., 1994). Fyn and MAG also colocalized by immunostaining in mouse axon tracts and oligodendrocytes during myelination (Umemori et al., 1994). The interaction between Fyn and MAG is thought to contribute to axonal-glial interactions during myelination. Additional

intracellular binding partners such as S100β (Kursula et al., 1999) and tubulin (Kursula et al., 2001) have been identified for L-MAG and S-MAG respectively. To date, very little is known regarding the functional significance of the aforementioned interactions and the biological relevance of MAG phosphorylation.

Inhibition of neurite outgrowth by MAG

Regulation of neurite outgrowth by MAG

The search for myelin-associated inhibitors occurred at a time when very few proteins with neurite outgrowth inhibitory properties had been characterized. MAG was the first myelin-associated inhibitor to be identified (McKerracher et al., 1994; Mukhopadhyay et al., 1994). MAIs were purified by subjecting myelin extracts to diethylaminoethyl anion exchange column chromatography followed by salt gradient elution (McKerracher et al., 1994). Eluted fractions were utilized as a substrate for NG108-15 cells (neuroblastoma cell line) and tested for their ability to inhibit neurite outgrowth of these cells. One inhibitory fraction that dramatically reduced the percent of cells with neurites was enriched with a 100kDa protein. The 100kDa protein was confirmed by Western blot analysis to be MAG. Furthermore, immunodepletion of MAG from the column eliminated the inhibitory activity of this particular inhibitory fraction. The inhibitory effect of MAG on neurite outgrowth was confirmed more directly by experiments showing that the extracellular domain of MAG also inhibited neurite outgrowth of NG108-15 cells when used as a substrate. Interestingly, poorly glycosylated MAG, lacking the L2/HNK-1 epitope, still dramatically inhibited neurite outgrowth, suggesting that carbohydrates on MAG are

likely not essential for outgrowth inhibition. To investigate the contribution of MAG to the inhibitory effect of myelin, MAG was immunodepleted from myelin extracts. Neurite outgrowth on MAG depleted myelin was recovered to 63% of control levels, suggesting that MAG contributes significantly to the inhibitory activity of myelin. These findings also revealed that additional inhibitors exist on myelin to inhibit neurite outgrowth. This is supported by the presence of other inhibitory fractions in column chromatography experiments of myelin extracts (McKerracher et al., 1994).

CHO cells expressing MAG on the cell surface were used to test the effect of MAG on neurite outgrowth of primary neurons (Mukhopadhyay et al., 1994). Postnatal cerebellar granule neuron (CGNs) and adult dorsal root ganglion (DRG) neuron outgrowth was inhibited (decreased average neurite length) on MAG expressing CHO cells when compared to control CHO cells or CHO cells expressing a control myelin protein. MAG antibodies reversed the inhibitory effect of MAG on neurite outgrowth, confirming that inhibition by MAG expressing CHO cells was due to MAG. MAG also inhibited neurite outgrowth of a variety of other primary neuron populations, including retinal ganglion, superior cervical ganglion, hippocampal, and spinal neurons (Figure 1.10a and 1.10b) (DeBellard et al., 1996).

Interestingly, MAG is a bifunctional molecule in regards to axon outgrowth, acting to both promote and inhibit it (Figure 1.10c). These contrasting effects depend on the type of neuron as well as its maturity. The outgrowth promoting effects of MAG were first reported on perinatal DRG neurons (Johnson et al., 1989). Postnatal day 1 DRG neurons were plated on MAG expressing fibroblasts and found to have longer processes than neurons plated on control fibroblasts (Johnson et al., 1989). While

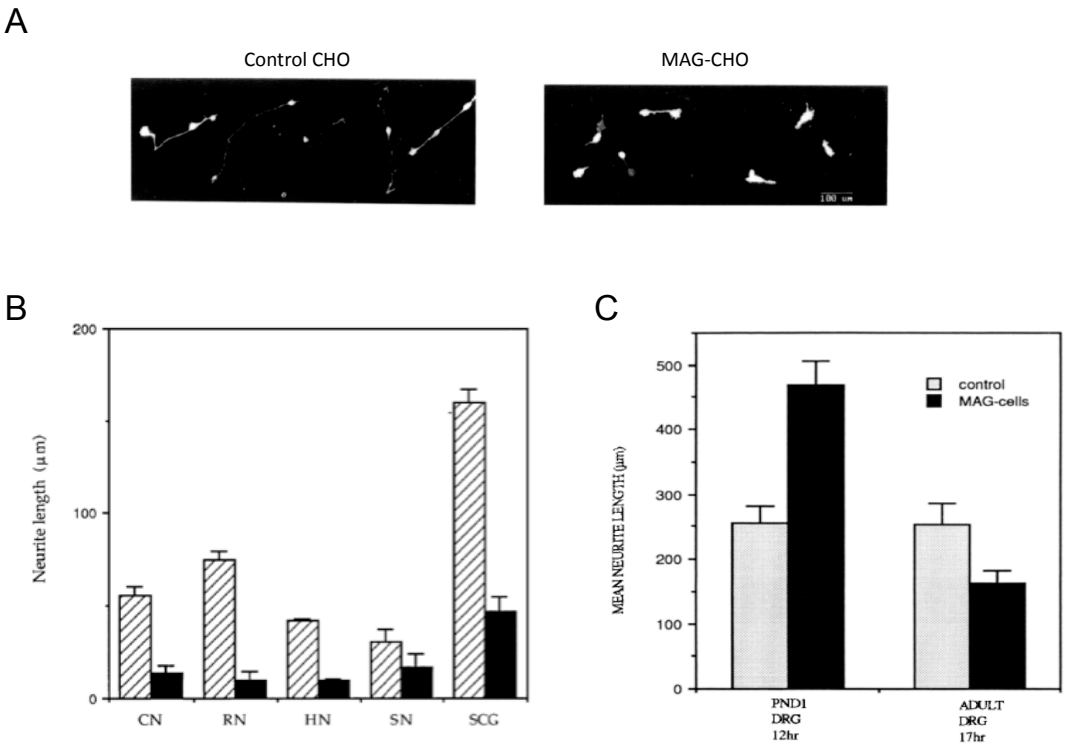

Figure 1.10 MAG is a bifunctional regulator of neurite outgrowth. A, B, MAG inhibits neurite outgrowth of various postnatal neuronal populations. Neurons were grown on MAG expressing (black bars) or control CHO (hatched bars) cells and immunostained for GAP-43, a neuronal marker. ***A,*** Images of cerebellar neurons on control CHO or MAG-CHO cells. ***B,*** Neurite outgrowth was assessed by measuring neurite length. CN = cerebellar RN = retinal ganglion HN = hippocampal SN = spinal SCG = superior cervical ganglion neurons ***C***, MAG has been shown to positively regulate neurite outgrowth of young neurons and negatively regulate neurite outgrowth of more mature neurons. While postnatal day 1 DRG neurite outgrowth was increased on MAG-CHO cells compared to control CHO cells, adult DRG neurite outgrowth was reduced. Modified from DeBellard et al., 1996 and Mukhopadhyay et al., 1994.

newborn DRG neurite outgrowth is promoted by MAG, adult DRG neuron outgrowth is inhibited (Mukhopadhyay et al., 1994; Cai et al., 2001). DRGs demonstrate a switch from outgrowth promoting to outgrowth inhibiting effects by MAG at postnatal day 3 (DeBellard et al., 1996). MAG also promotes the outgrowth of several types of embryonic neurons, including retinal ganglion cells (RGCs) (Cai et al., 2001), spinal neurons (Turnley and Bartlett, 1998), and raphe nuclei neurons (Cai et al., 2001), while inhibiting these same neurons postnatally. The mechanisms of enhancement of neurite outgrowth by MAG are not known and require future investigation. However, due to the relevance of MAG inhibition of neurite outgrowth to CNS injury, much effort has gone into understanding how MAG inhibits neurite outgrowth in the CNS.

MAG Receptors

Several neuronal receptors have been identified for MAG (Figure 1.11) (Xie and Zheng, 2008; Schnaar and Lopez, 2009; Cao et al., 2010). I will introduce these various receptors and their signaling partners and also discuss the controversies surrounding the role of individual receptors in neurite outgrowth inhibition by MAG.

The best characterized receptor on the axon surface that binds Nogo, MAG, and OMgp is Nogo receptor 1 (NgR1 or NgR), an 85kDa GPI anchored protein that participates in a tripartite-signaling complex to transduce growth inhibitory signals intracellularly (Figure 1.12). NgR is a brain-specific member of the leucine-rich repeat (LRR) superfamily made up of 8 LRRs and a cysteine-rich LRR flanking

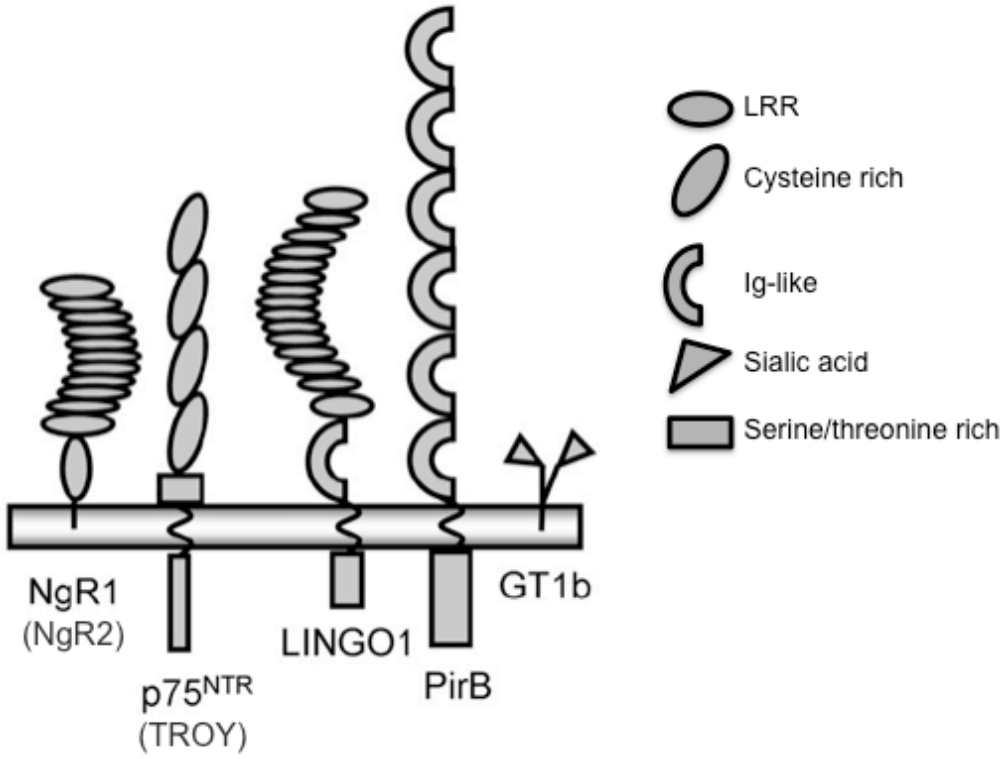

Figure 1.11 Schematic of axonal receptors involved in neurite outgrowth inhibition by MAG. Several receptors have been implicated in MAG signaling. NgR1, NgR2, PirB and gangliosides have been characterized as binding partners for MAG on the axon surface. p75 and TROY are co-receptors for NgR1 involved in transducing MAG's inhibitory signal intracellularly. Adapted from Cao et al 2010.

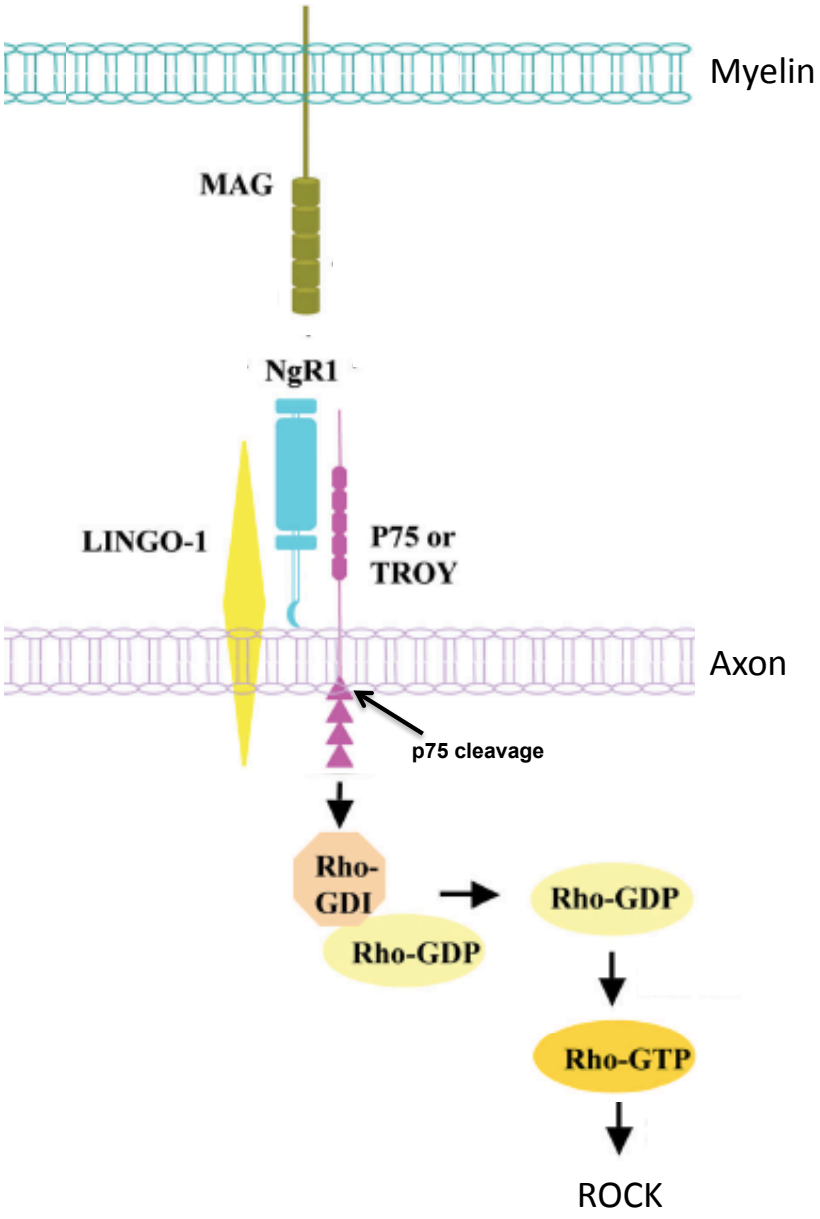

Figure 1.12 A tripartite signaling complex mediates Rho activation and neurite outgrowth inhibition by MAG. MAG binds to NgR1 on the axon surface, which forms a complex with p75 and LINGO1 to signal intracellularly. The binding of Rho-GDI to p75 and proteolysis of p75 have both been implicated in Rho activation and subsequent ROCK activation by MAG. Modified from Xie and Zheng 2008.

region in its extracellular domain and a GPI link (Fournier et al., 2001; McGee and Strittmatter, 2003). NgR is most highly expressed in neurons and axons of the CNS but is also expressed in the PNS (Josephson et al., 2002; Wang et al., 2002c). NgR was first identified as an axonal receptor for Nogo-66 (Fournier et al., 2001) and subsequently established as a receptor for MAG (Domeniconi et al., 2002; Liu et al., 2002). Application of phosphatidylinositol-specific phospholipase C (PI-PLC) to CGNs or DRG neurons rendered them less resistant to inhibition by MAG, supporting a role for a GPI linked receptor in inhibition by MAG (Domeniconi et al., 2002; Liu et al., 2002). MAG binds to NgR1 and interfering with NgR1 function by way of a function blocking antibody or a soluble ectodomain of NgR reversed inhibition of DRG or CGN neurite outgrowth inhibition by soluble MAG (MAG-Fc) or MAG expressing CHO cells (Domeniconi et al., 2002). Interestingly, expression of NgR in embryonic neurons that are not normally inhibited by MAG, rendered them responsive to MAG inhibition (Liu et al., 2002). Two mammalian homologs have been described for NgR1, NgR2 and NgR3 (Lauren et al., 2003). All three NgRs are highly expressed in the CNS and PNS and exhibit both unique and overlapping patterns of expression. NgR2, but not NgR3, can also function as a receptor for MAG (Venkatesh et al., 2005). MAG interacts with NgR2 with high affinity and exogenous application of recombinant NgR2 reversed neurite outgrowth of DRG neurons and CGNs on detergent extracts from MAG-CHO cells. Unlike NgR1 and others, NgR2 appears to serve as a receptor selectively for MAG, as it does not bind Nogo or OMgp. Of note, cleavage of GPI linked proteins by PI-PLC did not completely eliminate the susceptibility of many neurons to inhibition by MAG,

suggesting the presence of other, non-GPI-linked, receptors on the axon surface for MAG (Niederost et al., 2002).

Since NgR is GPI anchored it requires a co-receptor to signal intracellularly. Two such co-receptors have been identified for NgR, p75 neurotrophin receptor (p75) and TROY (also known as Taj), both members of the tumor necrosis factor receptor (TNFR) superfamily. P75 is a type I transmembrane receptor with the characteristic four cysteine-rich extracellular repeats of TNFR family members, a highly conserved transmembrane domain, and an intracellular "death domain". Best known as a neurotrophin receptor, p75 also binds additional ligands including pro-neurotrophins, prion protein fragment (PrP), and Aβ-peptide of the amyloid precursor protein (APP) (Hasegawa et al., 2004a). P75 is a multifunctional receptor, interacting with numerous transmembrane and cytoplasmic proteins and playing a role in a range of biological functions (Dechant and Barde, 2002; Hasegawa et al., 2004a). The foremost function of p75 is in developmental and disease related neuronal cell death, however other functions for p75 are emerging in processes such as promotion of axonal elongation, myelin formation, synaptic transmission, and neurite outgrowth inhibition.

Several lines of evidence support a role for p75 in transducing the growth inhibitory signals initiated by several MAIs. Crossing a *p75* knockout mouse with an NGF overexpressing transgenic mouse demonstrated robust outgrowth of *p75* null sympathetic neurons into myelinated portions of the cerebellum in comparison to wild-type neurons, suggesting that in the presence of p75, an inhibitory cue exists to prevent neurite outgrowth in response to myelin proteins (Walsh et al., 1999). There is evidence that p75 is up-regulated following axotomy or lesions (Ernfors et al.,

1989; Roux et al., 1999), indicating that although not normally expressed in high levels in many adult neurons, p75 expression can be regulated following injury to mediate inhibition of outgrowth. Furthermore, a role for p75 was shown in neurite outgrowth stimulation in response to NGF. Expression of the receptor activated Rho GTPase in the absence of ligand, but neurotrophin binding to p75 stimulated axon outgrowth and inactivated Rho (Yamashita et al., 1999). The first evidence backing a role for p75 in MAI signaling was that *p75* knockout DRG or CGN neurite outgrowth was not inhibited by MAG-Fc whereas p75 wild-type neurons were significantly inhibited (Yamashita et al., 2002). Subsequent work demonstrated that cultured neurons from *p75* knockout mice were less responsive to growth inhibitory signals induced by all known NgR ligands (Wang et al., 2002a; Yamashita et al., 2002). Interfering with p75 function also abolished *Xenopus* neuronal growth cone turning by MAG (Wong et al., 2002). p75 participated in a physical complex with NgR, forming an interaction that is strengthened by the presence of MAG (Wang et al., 2002a; Wong et al., 2002; Yamashita et al., 2002). Furthermore, disrupting the interaction between p75 and NgR via application of p75-Fc (the extracellular domain of p75 fused to Fc) to cultures or neuronal overexpression of NgR that is incapable of binding p75, blocked neurite outgrowth inhibition and Rho activation by MAG-Fc (Wang et al., 2002a). p75 is also necessary for regulation of Rho GTPase signaling downstream of several MAIs. CGNs treated with MAG showed increased activation of Rho, whereas p75 mutant CGNs did not exhibit increased Rho activity in response to MAG (Yamashita et al., 2002). The lack of Rho activation in *p75* null neurons was also shown with Nogo, OMgp, and myelin extracts (Wang et al., 2002a).

We have since gained significant insights into the mechanism by which p75 activates Rho. Overexpression of a p75 mutant lacking the receptor's cytoplasmic tail in CGNs desensitized the neurons to inhibition by MAG-Fc, suggesting that the intracellular domain of p75 is required for inhibitory signaling by MAG (Wang et al., 2002a). P75 interacted with Rho GDP dissociation inhibitor (Rho-GDI), an interaction that was strengthened by MAG-Fc (Yamashita and Tohyama, 2003). The binding of Rho-GDI by p75 releases Rho from this inhibitor, thereby facilitating GTP binding and activation. Overexpression of Rho-GDI or Pep5, a peptide that disrupts the Rho-GDI / p75 interaction abolished the effect of MAG-Fc on neurite outgrowth of DRG neurons and CGNs as well as preventing activation of Rho by MAG-Fc (Yamashita and Tohyama, 2003).

MAG-Fc can induce regulated intramembrane proteolysis of p75 by the γ-secretase complex in CGNs and, inhibitors of the protease reversed neurite outgrowth inhibition by MAG-CHO cells as well as MAG-Fc (Domeniconi et al., 2005). Secretase inhibitors also blocked activation of Rho by MAG-Fc in CGNs and expression of a noncleavable mutant of p75 in DRGs prevented the inhibitory effects of MAG-CHO cells. These results led the authors to suggest that cleavage of p75 is an important step in neurite outgrowth inhibition. The mechanisms by which p75 proteolysis regulates Rho have yet to be determined.

The third member of the NgR/p75 complex is LRR and Ig domain containing, Nogo receptor interacting protein (LINGO1). Like NgR, LINGO1 is a member of the LRR superfamily with an extracellular domain consisting of 12 LRRs and an Ig domain, but unlike NgR, LINGO1 also has a transmembrane domain and an intracellular domain. It is postulated, (but unknown) that the intracellular domain of LINGO may

play a role in signaling events downstream of MAIs. Not much is understood regarding the function of LINGO1, except that its presence in the NgR-p75-LINGO1 complex is required for inhibition of neurite outgrowth and the regulation of Rho GTPases by several MAIs (Mi et al., 2004).

p75 can function as a signaling partner for NgR in the inhibition of neurite outgrowth, but many populations of mature neurons do not express p75 (Park et al., 2005). Furthermore, although p75 knockout neurons are less responsive to MAIs, there is still some inhibition of neurite outgrowth (Wang et al., 2002a) suggesting the presence of another receptor capable of transducing MAI signaling. A search for TNFR family members, other than p75, that are expressed in the CNS and could interact with NgR lead to the identification of TROY as a member of the NgR complex (Mandemakers and Barres, 2005; Park et al., 2005; Shao et al., 2005). TROY was identified as a co-receptor for NgR that can functionally substitute for p75 in neurite outgrowth inhibition signaling (Park et al., 2005; Shao et al., 2005). Co-immunoprecipitation experiments show that TROY forms a complex with NgR and LINGO1. When expressed in CHO or COS-7 cells, with NgR and LINGO1, TROY mediates activation of Rho by MAIs. Neuronal over expression of a mutant TROY lacking its intracellular domain or application of soluble TROY in neurite outgrowth assays reversed outgrowth inhibition by Nogo-66, OMgp, or myelin (Park et al., 2005; Shao et al., 2005). TROY is more widely expressed in mature neurons than p75 (Park et al., 2005) and may be important for mediating MAI signaling in neurons lacking p75. Notably, even when p75 and TROY function is abolished, there is still some residual neurite outgrowth inhibition, hinting at additional receptors involved in this process. It is important to note that a role for TROY in MAG signaling has not

been shown, as the experiments mentioned above were conducted with MAIs other than MAG.

Gangliosides have also been proposed as receptors for MAG, although the exact contribution and role of gangliosides in MAG function remain largely controversial (McKerracher, 2002; Schnaar and Lopez, 2009; Cao et al., 2010). Gangliosides are sialic acid containing glycosphingolipids that are highly and broadly expressed in the brain (Schnaar, 2010). MAG contains a sialic acid binding site in its extracellular domain and binds various gangliosides (Schnaar et al., 1998). A mutant soluble MAG lacking its sialic acid binding capacity inhibited hippocampal neuron outgrowth to a lesser extent than wild-type MAG (Vinson et al., 2001). Two groups identified gangliosides GD1a and GT1b as receptors for MAG in the inhibition of neurite outgrowth of CGNs and hippocampal neurons (Vinson et al., 2001; Vyas et al., 2002). Treatment of neurons with neurominidase, inhibitors of ganglioside biosynthesis, or class specific ganglioside antibodies to GD1a or GT1b significantly reversed neurite outgrowth inhibition by MAG-CHO membranes or MAG-Fc. Additionally CGNs obtained from mice lacking complex gangliosides were less susceptible to inhibition by MAG (Vyas et al., 2002).

It has been postulated that MAG inhibits neurite outgrowth by clustering gangliosides on the cell surface, as antibody provoked clustering of GD1a or GT1b recapitulated neurite outgrowth inhibition by MAG (Vinson et al., 2001; Vyas et al., 2002). The mechanism by which gangliosides mediate neurite outgrowth inhibition is unknown. A role for the Rho GTPAse effector Rho kinase (ROCK) was suggested by demonstrating that the inhibition obtained by antibody induced ganglioside clustering can be reversed by Y27632 (a ROCK inhibitor) (Vinson et al., 2001). Conversely,

several studies have questioned the importance of gangliosides in MAG signaling (Tang et al., 1997a; Cao et al., 2007) and future studies will be needed to shed light on these differences.

The most recently identified receptor for MAG is Paired-immunoglobulin-like receptor B (PirB). PirB is a major histocompatibility complex class I (MHCI) receptor that along with MHCI functions in the remodeling or stabilization of neural circuits (Syken et al., 2006). Specifically, mice lacking *PirB* show increased cortical ocular-dominance plasticity, indicating that PirB normally functions to restrict plasticity in the CNS. PirB is expressed in various subsets of neurons including cortical, hippocampal, and cerebellar neurons. PirB was identified as a receptor for Nogo-66, MAG, and OMgp following an expression cloning screen for Nogo-66 binding partners (Atwal et al., 2008). A soluble extracellular domain of PirB or a function blocking PirB antibody partially reversed CGN or DRG neurite outgrowth inhibition by soluble Nogo-66 or MAG as well as myelin. Moreover, neurons from PirB mutant mice, bearing a loss-of-function *PirB* allele, showed decreased inhibition by MAG, Nogo-66, and OMgp.

To address whether PirB and NgR jointly mediate inhibition by MAIs, a function-blocking antibody was added to cultures of *NgR-/-* neurons in the presence of Nogo-66 or myelin. Blocking the function of both receptors fully reversed inhibition by myelin but not Nogo-66 (Atwal et al., 2008). Similar studies were not conducted with MAG. The mechanism by which PirB inhibits neurite outgrowth is not known. However, PirB complexes with src homology 2–containing protein tyrosine phosphatases (SHP-1 and SHP-2) in the brain (Syken et al., 2006). SHP-2 acts as a "stop" signal for neurons approaching their targets (Chen et al., 2002). Recruitment

of SHP-1 or SHP-2 to PirB in response to myelin inhibitors may play a role in mediating neurite outgrowth inhibition.

Recent controversies

It was initially presumed that the classic NgR complex, NgR-p75/TROY-LINGO1, was responsible for mediating inhibition by Nogo, MAG, and OMgp in most neuronal populations. However, over the last few years, MAG signaling in particular, has become increasingly complex, with many studies casting doubt on the extent to which these receptors function *in vitro* and *in vivo*. With the development of *NgR1* knockout mice, the latest studies suggest that the role of NgR in MAI signaling is more complicated than initially anticipated. Genetic deletion of *NgR1* reduced the extent of growth cone collapse elicited by soluble Nogo, MAG and OMgp in culture in one study (Kim et al., 2004). On the other hand, wild-type and *NgR1-/-* CGNs and DRGs were similarly susceptible to neurite outgrowth inhibition by Nogo-66 or myelin substrate (Zheng et al., 2005). These discrepancies may be explained by a recent study investigating neurite outgrowth inhibition by MAG and OMgp in *NgR-/-* neurons and neurons where *NgR* was knocked down by shRNA (Chivatakarn et al., 2007). Growth cone collapse, an acute effect where the growth cone loses its fan shaped structure and the axon retracts, in response to soluble ligands and inhibition of outgrowth, where the axon is exposed to the ligand long term and fails to advance, in response to membrane bound ligands were evaluated. While NgR was required for acute growth cone collapse by soluble MAG and OMgp, it was not required for the more chronic effect on neurite outgrowth when neurons were plated on cells

expressing MAG or OMgp. Together, these studies are supportive of a role for NgR in the acute effects of myelin-associated inhibitors, but indicate that other receptors are likely contributing to the inhibition of process outgrowth.

There is emerging evidence of neuronal type-specific mechanisms of neurite outgrowth inhibition by MAG. Wild-type and *p75 null* RGCs and CGNs were equally inhibited by MAG-CHO cells, bringing into question the role of p75 in MAG mediated neurite outgrowth inhibition in these two neuron populations (Venkatesh et al., 2007). However, *p75-/-* DRG neurons were significantly less inhibited by MAG-CHO cells as compared to wild-type neurons, supporting a role for the receptor in one of the neuron types investigated. The contribution of NgR, gangliosides and p75 to the inhibition of various neuron types by MAG substrate was analyzed using diverse enzymes and pharmacological inhibitors (Mehta et al., 2007). They found that while NgR and p75 largely mediated inhibition of DRG neuron outgrowth, CGN inhibition was mostly mediated by gangliosides and did not appear to require p75. Our current understanding of mechanisms of inhibition by MAG, especially with regards to its receptors, has become somewhat confounded. Inhibition by MAG depends on the age and type of neuron and whether growth cone collapse or inhibition of outgrowth is assessed. As new receptors are identified, such as PirB, our comprehension of MAG signaling will undoubtedly become clearer. Importantly, these recent discoveries indicate that reversing inhibition of regeneration of particular axon tracts will require further investigation of specific neuron populations.

The effects of MAIs on cortical neurons, one of the principal neuronal types impacted by SCI, remains poorly characterized. Many efforts to regrow the corticospinal tract (CST) following injury have proven unsuccessful. Regeneration of the CST was not

observed in *NgR-/-* mice following a dorsal hemisection (Zheng et al., 2005). Likewise CST regeneration was not observed following injury of *p75-/-* mice (Song et al., 2004). It is likely that if neuronal-specific mechanisms of outgrowth inhibition occur, we have yet to identify all receptors and signaling pathways required for inhibition of the corticospinal tract. The mechanisms identified thus far have been characterized in neurons other than cortical neurons. The work that will be presented in the subsequent chapters, set out to characterize the mechanisms of cortical neuron outgrowth inhibition by MAG in the hope of identifying molecules and pathways that can be targeted to reverse injury to this specific neuron type.

MAG signaling in neurite outgrowth inhibition

Understanding axonal signaling mechanisms downstream of MAG and other inhibitors of axon outgrowth is an area of great interest due to implications for therapeutic interventions. In order to cause growth cone collapse and axon outgrowth inhibition, many inhibitors ultimately act on the cytoskeleton to regulate actin dynamics. To date, Rho GTPase family members, which include Rho, Rac, and Cdc42, are the best-characterized axonal signaling intermediates involved in MAI regulation of the cytoskeleton (Sandvig et al., 2004; Govek et al., 2005). Rho GTPase is a common target of various inhibitors of axon outgrowth including those found on myelin or in the glial scar. RhoA stimulates actinomycin contractility and stress fiber formation in neuronal cells, inducing growth cone collapse (Hall, 1998; da Silva and Dotti, 2002). Rac and Cdc42 positively regulate axon growth, Rac contributing mostly to formation of lamellipodia and Cdc42 to the formation of

filopodia at the leading edge of the axon. Rac and Cdc42 contribute to the polymerization of actin, formation of cell protrusions, and provide the force necessary for movement of the growth cone (da Silva and Dotti, 2002; Govek et al., 2005).

Neuronal stimulation by MAG and other MAIs results in increased Rho activity (Niederost et al., 2002; Yamashita et al., 2002; Borisoff et al., 2003; Dubreuil et al., 2003; Fournier et al., 2003). Rho GTPase pulldown assays showed that soluble MAG and Nogo-66 can activate Rho in PC12 cells, CGNs, and DRGs (Niederost et al., 2002; Fournier et al., 2003). The balance of Rho and Rac GTPases seems to be important for determining whether axon outgrowth can occur, with Rho negatively regulating neurite outgrowth and Rac enhancing neurite outgrowth. While MAIs counter neurite outgrowth by activating Rho, there is also evidence that they simultaneously inhibit Rac (Niederost et al., 2002).

A critical role for Rho is supported by studies in which MAI mediated outgrowth inhibition is reversed with C3 exoenzyme (C3), a specific pharmacological inhibitor of Rho (Lehmann et al., 1999; Dergham et al., 2002). Primary RGCs treated with C3 extended more and longer neurites on MAG substrate (Lehmann et al., 1999). Complete reversal of CGN neurite outgrowth inhibition by MAG-CHO cells has been previously demonstrated following C3 application (Niederost et al., 2002). Rho signals through various effector proteins, molecules that bind to the active GTP bound form of Rho and regulate multiple downstream pathways (O'Donnell et al., 2009). Two Rho effectors that have been implicated in neurite outgrowth inhibition are p160ROCK (ROCK-I) and ROK-Rho-kinase (ROCK-II). Treatment of cortical neurons grown on myelin with Y27632, an inhibitor of ROCK-I and ROCK-II,

reversed the effect of myelin on the average length of the longest neurite (Dergham et al., 2002). Reversal of CGN neurite outgrowth inhibition by MAG-CHO cells and DRG inhibition by soluble MAG was observed in the presence of Y27632 (Niederost et al., 2002). LIM kinase and its target Cofilin, an actin modifying protein, are downstream of ROCK in the inhibition of axon outgrowth by MAIs (Alabed et al., 2006). Myosin light chain (MLC) has also been implicated in ROCK mediated inhibition of neurite outgrowth by MAIs (Alabed et al., 2006).

In addition to a clear role in inhibition by MAIs *in vitro*, Rho plays a role *in vivo*. Rho is activated following spinal cord injury (Dubreuil et al., 2003; Madura et al., 2004). GTP-Rho pull-down assays of spinal cord homogenates from rats with various injuries (contusions, hemisections and transections) revealed increased Rho activity in injured spinal cords when compared to control spinal cords (Dubreuil et al., 2003). Activation of Rho in spinal cord injuries was also confirmed by in situ visualization of increased GTP bound Rho in injured spinal cords compared to sham-operated cords (Dubreuil et al., 2003; Madura et al., 2004). Inhibition of Rho or ROCK *in vivo* has established these signals as negative regulators of axon regeneration. C3 treatment of crushed optic nerves resulted in significant axon growth past the lesion site into white matter (Lehmann et al., 1999). Enhanced sprouting of CST fibers and significant locomotor recovery were observed with Y27632 treatment (Fournier et al., 2003). However, in this study axonal outgrowth of corticospinal tracts following injury was not observed with C3 treatment, possibly due to ineffective delivery of the inhibitor. Application of C3 or Y27632 at the site of a spinal cord dorsal hemisection resulted in significant regeneration of CST fibers as well as functional recovery in locomotion and coordination (Dergham et al., 2002). More recently, genetic

manipulation of ROCKII revealed that this kinase plays a role in the restriction of axon outgrowth following spinal cord injury (Duffy et al., 2009). While many axons were found to cross the lesion site with inhibition of Rho or ROCK, it remains unknown what other mechanisms of inhibition exist and whether pursuing additional targets could improve the regeneration thus far observed with C3 or Y27632 treatment.

Although considerably less well understood, additional neuronal intracellular signals have been implicated downstream of MAG. MAG induced an increase in intracellular Ca^{2+} when applied to HEK 293 cells expressing NgR and p75 (Wong et al., 2002). When applied to *Xenopus* spinal neurons or CGNs, MAG produced an increase in intracellular calcium levels, which was required for growth cone repulsion in spinal neurons (Song et al., 1998; Wong et al., 2002). Conventional isoforms of PKC were also reported to contribute to neurite outgrowth inhibition by MAIs, including MAG, and CSPGs associated with the glial scar (Hasegawa et al., 2004b; Sivasankaran et al., 2004). Pharmacological inhibition of PKC reversed CGN neurite outgrowth inhibition by soluble MAG. Furthermore, application of soluble MAG to CGNs resulted in increased PKC activation, as substantiated by elevated levels of phosphorylated PKC (Hasegawa et al., 2004b; Sivasankaran et al., 2004). Interestingly, while one study found that PKC is required for Rho activation by soluble MAG in CGNs (Sivasankaran et al., 2004), another found that Rho activation by MAG is independent of PKC in the same neuronal population (Hasegawa et al., 2004b).

Evidence for soluble MAG

I have mentioned the use of soluble MAG (MAG extracellular domain) experimentally at several points thus far. Although MAG is assumed to interact with the axon as a membrane bound protein, several studies support the occurrence of soluble MAG *in vitro*. The release of MAG from the surface of oligodendrocytes is supported by the presence of a smaller molecular weight MAG in oligodendrocyte culture medium (Yim and Quarles, 1992). Following mechanical disruption of rat myelin and incubation, the extracellular domain of MAG was detected in the supernatant, supporting the notion that MAG can be released from myelin (Tang et al., 1997b). Proteolytic cleavage and release of MAG from MAG expressing CHO cells can be induced by recombinant matrix metalloproteinases, specifically MMP-2, MMP-7, or MMP-9 (Milward et al., 2008). Several studies have gone on to show that MAG released from myelin or the cell surface inhibits neurite outgrowth in neuronal cultures (Tang et al., 1997b; Tang et al., 2001; Milward et al., 2008). Many of the early studies investigating mechanisms of MAG signaling in outgrowth inhibition utilized soluble MAG. More recent observations, some of which seem contradictory to initial findings (see above section) may be due to differences in how MAG is presented to the axon, that is, as a soluble versus membrane bound form. The extent of MAG cleavage that occurs *in vivo*, under normal conditions and following injury, and the mechanism of cleavage remains unclear.

Aims of the dissertation

I hypothesize that given the very limited success in stimulating corticospinal tract regeneration *in vivo*, cortical neurons are particularly susceptible to outgrowth inhibitors, such as MAG. Given the emerging evidence for neuronal cell-type specific mechanisms of inhibition by MAG, I propose that cortical neurons may be inhibited by unique or multiple mechanisms. I set out to address this hypothesis with the following Specific Aims:

Specific Aim 1: Characterize cortical neurite outgrowth inhibition by MAG

a. Investigate the response of cortical neurons to MAG inhibition

b. Examine the role of known MAG receptors in inhibition of cortical neurite outgrowth

c. Explore whether the Rho / ROCK pathway mediates neurite outgrowth inhibition by MAG in cortical neurons

Specific Aim 2: Investigate a role for PTEN in cortical neurite outgrowth inhibition by MAG

a. Study the effect of PTEN knockdown or knockout on responsiveness of cortical neurons to MAG

b. Examine whether increasing phosphorylated Akt levels in cortical neurons impacts inhibition by MAG

c. Analyze whether MAG induces changes in Akt activation in cortical neurons

Specific Aim 3: Explore mechanisms of neurite outgrowth inhibition by soluble MAG

a. Investigate whether TRAF6 plays a role in outgrowth inhibition by soluble MAG

b. Delineate the mechanism by which TRAF6 mediates neurite outgrowth inhibition by soluble MAG

CHAPTER II

CHARACTERIZATION OF NEURITE OUTGROWTH INHIBITION BY MAG IN CORTICAL NEURONS

Introduction

The limited regrowth of axons following injury in the mammalian central nervous system (CNS) can result in debilitating and often permanent neurological deficits. Myelin, produced by oliogodendrocytes to facilitate saltatory conduction, expresses a number of proteins that inhibit axonal growth and are thought to contribute to preventing regeneration. These myelin-associated inhibitors include Nogo A, Myelin-associated glycoprotein (MAG), Oligodendrocyte myelin glycoprotein (OMgp), Ephrin-B3, and Sema4D (Filbin, 2003; Yiu and He, 2006).

MAG, one of the most extensively studied myelin-associated inhibitors, is a transmembrane glycoprotein expressed on periaxonal myelin membranes in the peripheral and central nervous systems. It plays a physiological role in maintaining myelinated axons as well as contributing to the pathology of demyelinating diseases and the inhibition of CNS regeneration (Filbin, 2003; Quarles, 2007). Several neuronal receptors have been identified for MAG, including the Nogo receptors (NgR1 and NgR2) (Domeniconi et al., 2002; Liu et al., 2002; Venkatesh et al., 2005), gangliosides GD1a and GT1b (Yang et al., 1996; Vinson et al., 2001; Vyas et al.,

2002), and the more recently identified paired immunoglobulin-like receptor B (PirB) (Atwal et al., 2008).

Understanding how the various receptors contribute to the inhibition of axon outgrowth by MAG has proven remarkably complex. NgR1 is a glycosylphosphatidylinositol-linked protein that binds MAG and signals through interaction with the p75 neurotrophin receptor (p75NTR) (Wang et al., 2002a; Wong et al., 2002) or a homologous protein, Taj/TROY (Park et al., 2005; Shao et al., 2005), and the novel transmembrane protein LINGO1 (Mi et al., 2004). The NgR complex was recently reported to function in an additive manner with the PirB receptor, such that inhibiting both receptors was necessary to fully reverse the effects of myelin (Atwal et al., 2008). The effects of MAG depend on an interaction with gangliosides, at least in some neurons (Vyas et al., 2002; Mehta et al., 2007); however, this remains controversial (Cao et al., 2007; Schnaar and Lopez, 2009). It has been suggested that MAG's inhibitory effects involve different receptors depending on the type of neuron and the nature of the inhibition, such as acute growth cone collapse versus more long-term axon extension (Chivatakarn et al., 2007; Mehta et al., 2007; Venkatesh et al., 2007).

How these various MAG receptors transduce their signals to inhibit axon growth is also not well understood. The NgR1 complex can activate the GTP binding protein RhoA, which regulates the actin-cytoskeleton, leading to growth cone collapse and the prevention of neurite outgrowth (Hall, 1998; Niederost et al., 2002; Wang et al., 2002a; Yamashita et al., 2002). However, a role for intracellular calcium (Song et al., 1998; Wong et al., 2002) and protein kinase C (PKC) (Hasegawa et al., 2004b; Sivasankaran et al., 2004) has also been suggested downstream of NgR-p75NTR-

LINGO1. The mechanisms by which gangliosides and PirB signal have yet to be determined, although an association between PirB and the phosphatases Shp-1 and Shp-2 has been reported (Syken et al., 2006).

We set out to investigate the receptors and intracellular signaling pathways involved in MAG-mediated inhibition of neurite outgrowth, specifically in cortical neurons. One of the pathways often damaged in spinal cord injury is the corticospinal tract, resulting in debilitating clinical manifestations including paralysis (Joosten, 1997; McDonald and Sadowsky, 2002). However, very few studies have investigated how cortical neurons respond to myelin-associated inhibitors and the mechanisms underlying their inhibition.

Our results demonstrate that cortical neuron process growth is robustly inhibited by MAG, but the inhibition could not be reversed by deletion of *p75NTR* or by blocking gangliosides or PirB. Surprisingly, inhibition of Rho signaling only partially reversed the effect of MAG, indicating the presence of additional intracellular signals.

Experimental Procedures

Primary neuron cultures

Cerebellar granule neuron (CGN) cultures were prepared from postnatal day 4-7 mice. Cerebella were isolated, dissociated in 0.125% trypsin (Worthington) at 37^{o}C for 15 min, washed in PBS, triturated and subsequently plated in Neurobasal media (Gibco) containing B27 supplement (Gibco), 25mM KCl, 33mM dextrose, 2mM glutamine, and 100U/ml penicillin / 100μg/ml streptomycin (Gibco). Cortical neurons

were isolated at E15-E17 or postnatal day 1-3, dissociated in 0.06% trypsin (Worthington) for 30 min at room temperature, triturated and plated in Neurobasal media with B27 supplement on dishes coated with 2μg/ml laminin (Invitrogen) or on CHO cells for neurite outgrowth assays.

Immunostaining

Cells were fixed in 3.7% formalin, blocked with 10% normal goat serum in PBS containing 0.2% triton X-100, incubated with primary antibody overnight and visualized with fluorescently labeled secondary antibodies and mounted with medium containing DAPI (Vectashield, Vector Labs). For MAG immunostaining, the permeabilization step was omitted. Primary antibodies used: anti-TuJ1 (1:500, Covance), anti-MAG (10 μg/ml, Chemicon) and anti-PirB (A-20, 1:50, Santa Cruz). Secondary antibodies used: goat anti-mouse Alexa 488 (1:500, Invitrogen) and donkey anti-goat Alexa 488 (1:500, Invitrogen).

Neurite outgrowth assay

Neurite outgrowth assays on MAG transfected CHO cells have been previously described (Mukhopadhyay et al., 1994). Cortical neurons or CGNs (40,000/well) were plated on confluent monolayers of MAG-CHO or control CHO cells in 8 well tissue culture slides (Lab-Tek, Nalge Nunc International). After incubation for ~20 hours, co-cultures were fixed and immunostained with anti-TuJ1, as described above. Where indicated, compounds were added at the time of neuronal plating: cell

permeable C3 exoenzyme (1μg/ml, Cytoskeleton, CT04), Y27632 (100μM, Sigma), neurominidase (40mU/ml, MP Biomedicals), function blocking PirB antibody (50μg/ml, kindly provided by M. Tessier-Lavigne, Genentech, CA). The percent of neurons with neurites was quantified in a blinded manner using a Zeiss fluorescence microscope at 40x magnification. A neurite was defined as a process at least twice the length of the cell body. For each experiment, 2-4 wells were used for each condition and at least 250 neurons were counted per condition. Neurite lengths were determined using ImageJ software analysis of images taken with a Zeiss fluorescence microscope at 40x magnification. The longest neurite ≥1 cell body was measured for each neuron, with at least 145 neurites evaluated for each experimental condition.

Mice

CD1 mice (Charles River) and C57BL/6 mice with the p75 gene deleted (Lee et al., 1992) were used, as indicated. All experiments with animals were approved by the Animal Care and Use Committee at Vanderbilt University.

HEK293 cell transfections

HEK293 cells, maintained in DMEM with 10% fetal bovine serum, were transfected with constructs expressing p75 (in pcDNA3) or TROY (in pFLAG-CMV-1) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. The TROY

plasmid was generously provided by Zhigang He (Harvard Medical School, Boston, MA) (Park et al., 2005).

Western blot analysis

Cells were lysed in NP40 lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol) containing a protease inhibitor cocktail (Complete Mini, Roche). Cell lysates were subjected to SDS-PAGE and Western blotting using anti-p75NTR (1:1000, generated from a GST fusion protein with the p75NTR intracellular domain), anti-TROY (1:500, E19, Santa Cruz) or anti-α-tubulin (1:1000, Calbiochem). Secondary antibodies conjugated to horseradish peroxidase included anti-rabbit (1:3000, Thermo Scientific) and anti-mouse (1:3000, Promega). For CHO cell membrane isolation, MAG-CHO cells or control CHO cells were harvested, incubated on ice for 30 min in homogenization buffer (20 mM Hepes, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitor cocktail), homogenized with a dounce homogenizer, and spun down at 3,000 rpm for 10 min at 4^{o}C. The resulting supernatant was then centrifuged at 16,000rpm for 1 hr at 4^{o}C. The pellet was analyzed by non-reducing Western analysis for MAG expression (10 μg/ml anti-MAG, Covance).

Apoptosis analysis

Cortical neurons were plated on control CHO or MAG-CHO cells. ~20 hrs following initial plating, neurons were fixed and stained with TuJ1 antibody and mounted with

DAPI containing medium. Apoptotic cells were analyzed by counting condensed, pyknotic nuclei and the ratio of pyknotic nuclei to total neurons counted per field are represented as % apoptotic nuclei.

Results

Neurite outgrowth in cortical neurons is potently inhibited by MAG

To evaluate the effects of MAG on neurite outgrowth from cortical neurons, embryonic neurons (E15-17) isolated from mouse cortex were plated on CHO cells stably expressing MAG on the cell surface (MAG-CHO cells) or control CHO cells (Figure 2.1). After approximately 20 hours, neurons were assessed for neurite outgrowth. Neurites were detected on 43.0% of the neurons on the control CHO cells; however, only 6.7% of the neurons on MAG-CHO cells displayed neurite outgrowth (Figure 2.2a). Neurite lengths were also considerably diminished on MAG-CHO cells, with a 53.6% reduction in neurite length compared to control CHO cells (Figure 2.2a). Since the corticospinal tract does not form until after birth in rodents (Bastmeyer and O'Leary, 1996), we also decided to analyze the effects of MAG on early postnatal cortical neurons. Using cortical neurons from P1-P3 pups, we found a comparable reduction in neurite outgrowth by MAG (Figure 2.2a). The dramatic reduction in neurite outgrowth was not due to cell death as there was no difference in the percent of apoptotic neurons on MAG-CHO cells (13.6%) as compared to control CHO cells (14.3%) (Figure 2.2b).

We performed similar neurite outgrowth experiments with CGNs, a neuron type often used to study inhibition of axonal growth by myelin proteins such as MAG. As

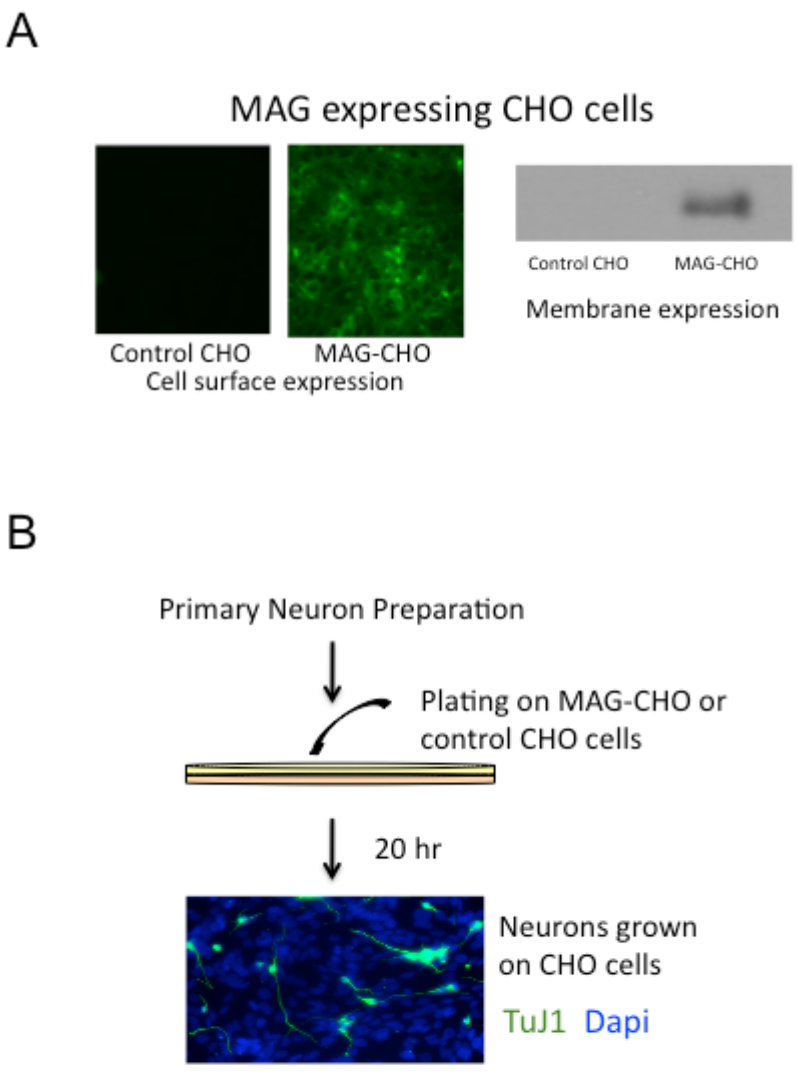

Figure 2.1. Neurite outgrowth assay on MAG expressing CHO cells. ***A,*** Non-permeabilized CHO cells and MAG-CHO cells were immunostained with anti-MAG to verify cell surface expression of MAG. CHO cell and MAG-CHO cell membranes were isolated and analyzed by Western blot analysis for MAG expression. ***B,*** Overview of neurite outgrowth assay. Primary neurons are plated on MAG expressing CHO cells or control CHO cells, grown for 20 hours, fixed and immunostained for Tuj1, a neuronal specific marker. A representative image is shown of neurons on CHO cells (shown by Dapi).

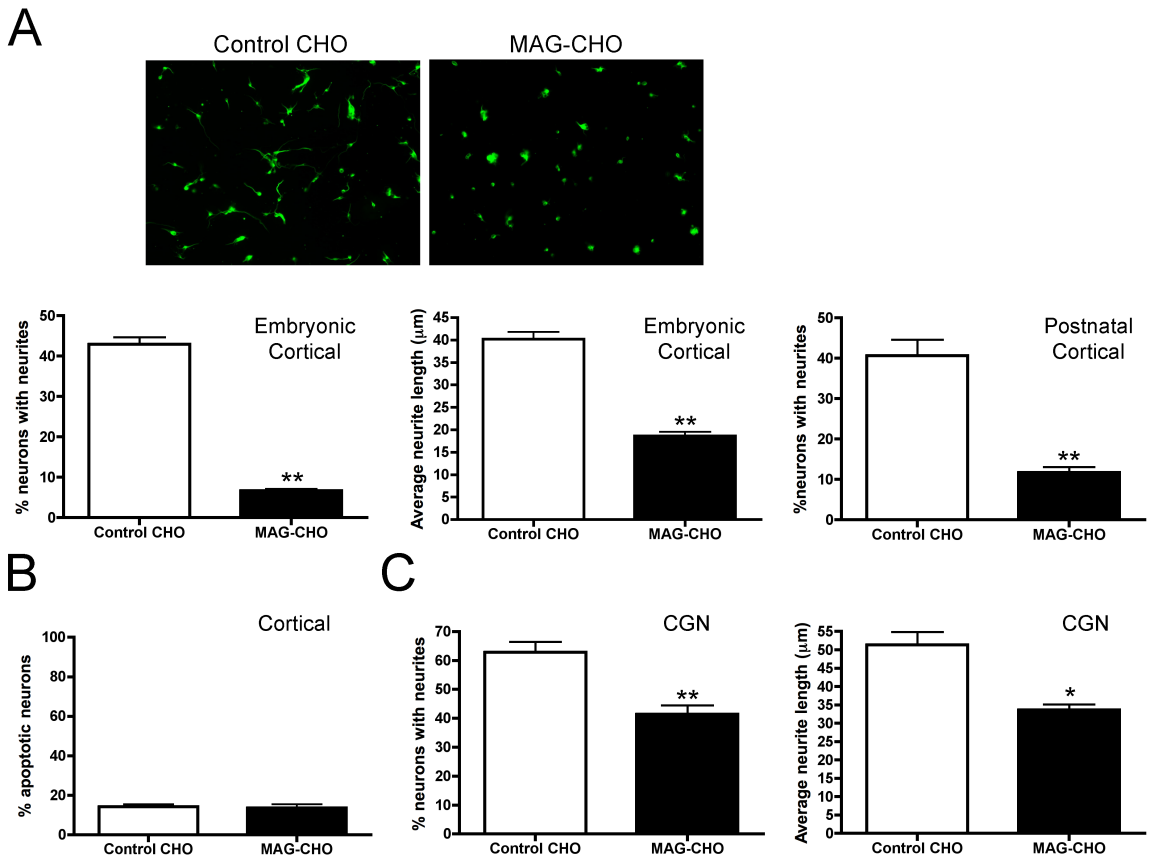

Figure 2.2. Cortical neuron outgrowth is dramatically inhibited by cell surface expressed MAG. ***A,*** Embryonic (E15-17) or postnatal (P1-3) cortical neurons were plated on control CHO or MAG-CHO cells for ~20 hrs then fixed and stained for TuJ1. Neurite outgrowth was measured as the percent of neurons with neurites or length of the longest neurite per neuron. Representative images are shown of embryonic cortical neuron cultures and the quantified data depicted below. Data represents mean ± SEM. For % neurons with neurites, n=8 for embryonic neurons and n=2 for postnatal neurons with 2-3 replicates per experiment. For length analysis, n=3 with 2 replicates per experiment. ***B,*** The percent of apoptotic neurons on control CHO and MAG-CHO cells was determined for embryonic cortical neurons. There was no significant difference between conditions based on a Student's t-test. Data represent mean ± SEM (n=4). ***C,*** Neurite outgrowth of CGNs on control CHO and MAG-CHO cells was quantified. Data represents mean ± SEM (n=6 for % neurons with neurites, n=3 for neurite length). (*, $p<0.01$, **, $p<0.0003$ based on a Student's t-test.)

expected, neurite outgrowth was significantly reduced (Figure 2.2c); however, the response of cortical neurons to MAG was substantially greater than that of CGNs. We observed an 86.0% reduction in the percent of embryonic cortical neurons with neurites, a 71.1% reduction in outgrowth of postnatal cortical neurons, but only a 34.1% reduction in outgrowth of CGNs. Similarly, we detected a 34.5% reduction in neurite length in CGNs on MAG-CHO cells relative to control CHO cells, compared to 53.6% for cortical neurons. These results suggest that cortical neurons are particularly sensitive to inhibition by MAG, raising the question as to the underlying mechanisms.

The effect of MAG on cortical neuron outgrowth is independent of its known receptors

MAG signals through a complex of the NgR1, p75NTR and LINGO1 in several types of neurons (Schnaar and Lopez 2009). In order to characterize the receptors playing a role in cortical neurite outgrowth inhibition by MAG, we first analyzed the expression of p75NTR and TROY in embryonic mouse cortex and cortical cultures, since these are the signal transducers in the tripartite complex. P75NTR was detected in cortical tissue as well as cortical neuron cultures, but there was no detectable signal for TROY (Figure 2.3a). Therefore, we investigated whether p75NTR was necessary for mediating the effects of MAG in cortical neurons. When plated on MAG expressing CHO cells, neurite outgrowth from both *p75ntr+/+* and *p75ntr-/-* neurons was similarly inhibited (Figure 2.4a), indicating that this receptor is

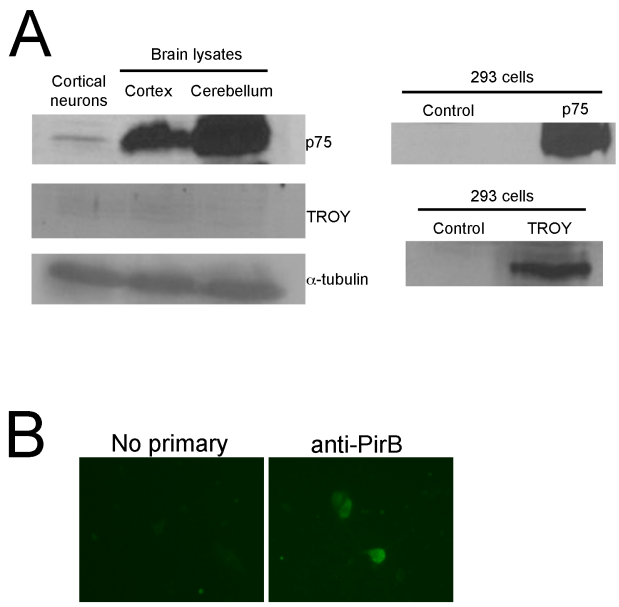

Figure 2.3. Expression of MAG receptors in cortical neurons. A, Lysates from E15 cortex, P6 cerebellum or cortical neuron cultures were analyzed by Western blot with antibodies to p75NTR or TROY. HEK 293 cells overexpressing p75NTR or TROY served as positive controls. ***B,*** Embryonic cortical neuron cultures were immunostained with an antibody to PirB. Staining in which primary antibody was omitted is included as a control.

not required for the response to surface bound MAG. As a comparison, we analyzed outgrowth *p75ntr+/+* and *p75ntr-/-* CGNs on MAG-CHO cells and found a similar response in both genotypes (Figure 2.4a). These results suggest that membrane bound MAG does not signal through the p75NTR-NgR-LINGO1 complex in cortical neurons or CGNs.

Gangliosides may also contribute to the inhibitory effect of MAG on neurite outgrowth, particularly in CGNs and hippocampal neurons (Mehta et al., 2007). Since GT1b and GD1a are expressed in cortical neurons (Lee et al., 2007), we investigated whether gangliosides mediate the MAG effect in cortical neurons. Neurons plated on MAG-CHO or control CHO cells, were treated with neurominidase to cleave off sialic acid moieties, which are required for MAG binding to GT1b and GD1a (Vyas et al., 2002). In agreement with previous studies (DeBellard et al., 1996; Venkatesh et al., 2007), we observed significant reversal of CGN neurite outgrowth on MAG-CHO cells in the presence of neurominidase. However, even with concentrations as high as 40 mU/ml there was no significant increase in outgrowth of cortical neurons on MAG-CHO cells (Figure 2.4b), indicating that membrane bound MAG signals independent of gangliosides in these neurons.

Recently, Atwal et al (2008) identified PirB as a functional receptor for the myelin-associated inhibitors, including MAG. In agreement with previous findings (Syken et al., 2006), we detected PirB in cortical neurons (Figure 2.3b); therefore we investigated its role in mediating the effects of MAG on neurite outgrowth. Cortical neurons were plated on control CHO or MAG-CHO cells in the presence of a PirB

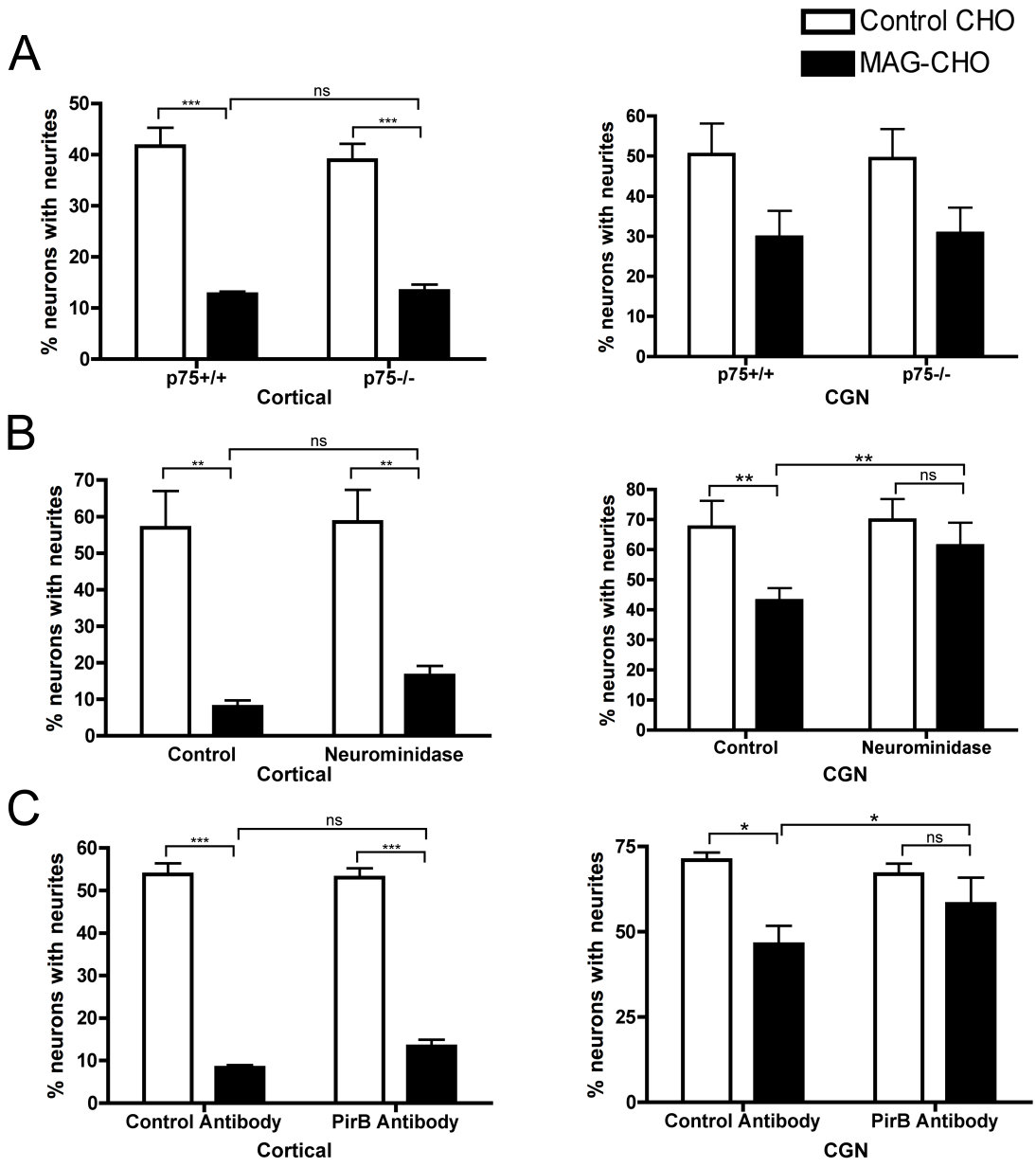

Figure 2.4. Inhibition of neurite outgrowth in cortical neurons by MAG is not mediated by p75NTR, gangliosides or PirB. A, *p75ntr+/+* or *p75ntr-/-* postnatal cortical neurons or CGNs were plated on control CHO or MAG-CHO cells for ~20 hrs, at which point the cells were fixed and stained for TuJ1. Neurite outgrowth was measured as the percent of neurons with neurites. Data represent mean ± SEM (n=2, 2-4 replicates per experiment). ***B,*** Cortical neurons or CGNs were plated on control CHO or MAG-CHO cells in the presence of neurominidase (40 mU/ml). Significant difference in the inhibition of neurite outgrowth was detected for CGNs, but not for cortical neurons. Data represents mean ± SEM (n=2, 2 replicate wells per experiment). ***C,*** Cortical neurons or CGNs were plated on control CHO or MAG-CHO cells in the presence of 50μg/ml of control antibody (human IgG) or PirB antibody. In CGNs, anti-PirB significantly reduced the inhibitory effects of MAG (n=3). However, there was no significant difference in the inhibition of neurite outgrowth by MAG in cortical neurons treated with anti-PirB (n=4). Data represents mean ± SEM. (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ by two way ANOVA).

function-blocking antibody that reversed the inhibitory effects of myelin proteins in dorsal root ganglia (DRG) neurons and CGNs (Atwal et al., 2008).

However, blocking PirB did not significantly attenuate the inhibition of neurite outgrowth by membrane bound MAG (Figure 2.4c). Increasing the concentration of the antibody 4-fold did not provide any further increase in neurite growth (data not shown). In contrast, there was a significant, although only partial, rescue of CGN neurite outgrowth on MAG-CHO cells in the presence of the PirB antibody (Figure 2.4c) in agreement with Atwal et al (2008). Taken together, these results demonstrate that membrane bound MAG prevents axonal growth in cortical neurons independent of the known receptors, suggesting there is another receptor system yet to be identified.

Inhibiting Rho or ROCK partially rescues cortical neuron outgrowth on a MAG substrate

Previous studies demonstrated that the GTPase RhoA and its effector Rho kinase (ROCK) are key mediators of the inhibitory effects of MAG in CGNs and DRG neurons (Lehmann et al., 1999; Niederost et al., 2002; Fournier et al., 2003; Alabed et al., 2006) and the NgR1-p75-LINGO1 complex can activate this pathway (Wang et al., 2002a; Yamashita et al., 2002; Yamashita and Tohyama, 2003). Therefore, to determine whether this cascade also contributes to the inhibition of neurite outgrowth by MAG in cortical neurons we treated the cells with C3 exoenzyme (C3), a Rho inhibitor, or Y27632, a ROCK inhibitor. As a positive control, we confirmed that both compounds could fully reverse the inhibition of neurite outgrowth by MAG in CGNs

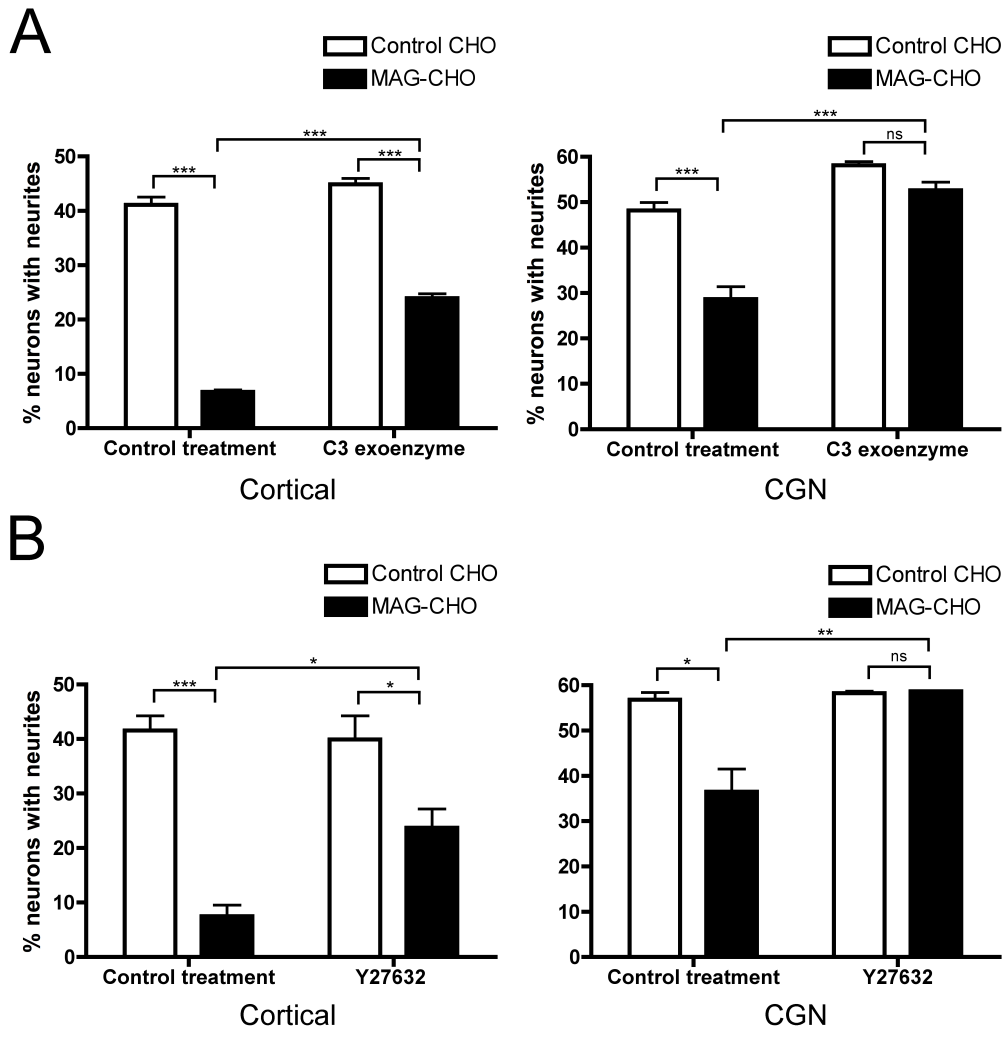

Figure 2.5. Inhibition of neurite outgrowth in cortical neurons by MAG is partially reversed by blocking Rho signaling. A, Cortical neurons (left panel) were harvested and plated on control CHO or MAG-CHO cells in the presence of vehicle or cell permeable C3 exoenzyme (1µg/ml). 20 hrs after plating, neurons were fixed and immunostained for TuJ1. Neurite outgrowth was measured as the percent of neurons with neurites. Data represents mean ± SEM (n=4). Similar experiments were conducted with CGNs (right panel) (n=2). ***B,*** Cortical neurons (left panel) or CGNs (right panel) were plated on control CHO or MAG-CHO cells in the presence of vehicle or the ROCK inhibitor Y27632 (100µM). 20 hrs after plating the neurons were fixed and immunostained for TuJ1. Neurite outgrowth was measured as the percent of neurons with neurites. Data represent mean ± SEM (n=2). (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ by two way ANOVA).

(Figure 2.5a and 2.5b, respectively). Surprisingly, C3 or Y27632 only partially reversed the inhibition of neurite outgrowth in cortical neurons (Figure 2.5a and 2.5b). Both compounds rescued process extension to an equivalent extent, consistent with Rho and ROCK acting in the same pathway. Thus, although Rho and ROCK play a significant role in mediating the effects of MAG in cortical neurons, there must be other signaling pathways involved.

Discussion

Cortical neurons are frequently damaged in spinal cord injury and fail to regenerate, in part, due to the presence of growth inhibitory proteins at the site of injury. Furthermore, recent findings suggest that inhibitors expressed by myelin may normally function in the refinement of cortical circuitry during development (McGee et al., 2005). However, very little is known about how cortical neurons respond to specific, endogenous inhibitors of axon growth. Here, we investigated the response of cortical neurons to MAG, a myelin protein known to prevent axonal outgrowth in other neurons. Our results indicate that both embryonic and postnatal cortical neurons are particularly sensitive to inhibition of neurite outgrowth by MAG. Interestingly, the effects of MAG were not mediated by any of its known receptors and inhibition of the Rho/ROCK pathway only partially reversed neurite outgrowth inhibition by MAG.

MAG is a multifunctional, transmembrane protein expressed on the periaxonal surface of myelinating cells in both the peripheral and central nervous system. It is

important for long-term axon-myelin stability and for proper structuring of Nodes of Ranvier (Quarles, 2007; Schnaar and Lopez, 2009). In addition, MAG regulates axonal growth, being able to promote it or inhibit it depending on the type of neuron as well as the developmental stage. MAG enhances neurite outgrowth from a number of embryonic neurons, including retinal ganglion cells (Cai et al., 2001), spinal neurons (Turnley and Bartlett, 1998; Cai et al., 2001), and dorsal root ganglia (DRG) neurons (Johnson et al., 1989; Mukhopadhyay et al., 1994; DeBellard et al., 1996; Cai et al., 2001), whereas postnatal axon outgrowth of these neurons is inhibited. In cortical neurons, we observed dramatic inhibition of neurite outgrowth by MAG at both embryonic (E15 to E17) and postnatal (P1-3) ages.

The mechanisms by which MAG mediates these various signals are poorly understood. Due to the relevance for spinal cord injury, much attention has focused on elucidating the signals responsible for inhibiting axonal growth. The best characterized receptor complex mediating the effects of MAG is the one formed by the gpi-linked Nogo receptor, p75NTR or TROY and LINGO1. However, we found that p75NTR was not involved in preventing neurite outgrowth in cortical neurons and no expression of TROY was detectable (Fig. 2). These results are in agreement with those of Chivatakarn et al (2007) who found that cortical neurons from mice with NgR1 deleted were as responsive to MAG-CHO cells as wild-type neurons. Since MAG can also bind NgR2 (Venkatesh et al., 2005), it is possible these results could be explained by NgR2 substituting for NgR1. However, taken together with our findings, the more likely conclusion is that the NgR-p75NTR-LINGO1 complex is not required for the inhibition of neurite out growth by membrane-bound MAG in cortical neurons.

The role of the NgR-p75NTR-LINGO1 complex in mediating the effects of MAG appears to depend on the type of neuron and the form of MAG used. For example, wild-type and *p75ntr* null retinal ganglion cells (RGCs) and CGNs were equally inhibited by MAG-CHO cells; however, *p75ntr-/-* DRG neurons were significantly less inhibited than wild-type neurons, suggesting that membrane-bound MAG signals through p75NTR in DRGs but not RGCs or CGNs (Venkatesh et al., 2007). In contrast, numerous reports have demonstrated a requirement for p75NTR in mediating the response of CGNs to a soluble fragment of MAG containing the extracellular domain (Wang et al., 2002a; Yamashita et al., 2002; Yamashita and Tohyama, 2003). A soluble form of MAG has been detected following incubation of spinal cord extracts *in vitro* (Tang et al., 2001), but whether this cleaved product of MAG is generated after neuronal injury is not known. Ultimately, a major objective of studies on MAG's ability to inhibit neurite outgrowth is to understand how regeneration could be facilitated following axonal damage *in vivo*. However, *p75ntr-/-* mice do not exhibit any significant improvement in regeneration of the CST following spinal cord injury, indicating that this receptor does not have a major role in preventing axonal growth from cortical neurons *in vivo* (Song et al., 2004; Zheng et al., 2005).

Two other receptors have been reported for MAG, the gangliosides GT1b and GD1a, and PirB, a major histocompatibility complex class 1 receptor. However, we found that neither of these molecules significantly contributed to the inhibitory effects of MAG in cortical neurons. Of course we cannot rule out the possibility that the concentration of PirB antibody used was not high enough to disrupt the interaction of MAG with cortical neurons. Although not statistically significant, there was a trend

toward a partial reversal of the inhibition by MAG with anti-PirB. However, taken together, these results suggest that there exists an additional, yet to be identified receptor for membrane-bound MAG that transduces a signal preventing neurite outgrowth in cortical neurons.

Down stream of many signals inhibiting process outgrowth and extension in cells are the small GTPase Rho and its effector kinase ROCK. Increased Rho activity has been detected in neurons following spinal cord injury (Dubreuil et al., 2003; Madura et al., 2004) and application of the Rho inhibitor C3 or the ROCK inhibitor Y27632 at the site of injury resulted in significant, although limited, regeneration of corticospinal tract fibers as well as functional improvement in locomotion and coordination (Dergham et al., 2002; Fournier et al., 2003). Similarly, genetic deletion of ROCKII revealed that this kinase plays a role in restricting axon outgrowth following spinal cord injury (Duffy et al., 2009). MAG increased Rho activity in neurons (Lehmann et al., 1999; Niederost et al., 2002; Yamashita et al., 2002; Fournier et al., 2003) and, not unexpectedly, we found that inhibiting Rho or ROCK significantly reversed the effect of membrane-bound MAG on cortical neurons. However, we were surprised to find that blocking Rho or ROCK was only partially effective in the cortical neurons; significant inhibition of neurite outgrowth remained in the neurons plated on MAG-CHO cells when C3 or Y27632 was added. This result suggested that membrane-bound MAG was activating additional growth inhibitory signals.

The results shown here indicate that membrane bound MAG inhibits outgrowth of cortical neurons through novel mechanisms. In particular, unidentified MAG receptors are present on the axonal membrane to mediate inhibition by MAG. Also,

in addition to Rho, other intracellular signals exist to mediate cortical neurite outgrowth inhibition by MAG.

CHAPTER III

MAG INHIBITS NEURITE OUTGROWTH IN CORTICAL NEURONS THROUGH A PTEN DEPENDENT MECHANISM

Introduction

The balance of positive and negative growth signals is critical to proper neuronal wiring during development and effective regeneration following injury. Of particular interest to the regeneration field is the characterization of inhibitory cues and signaling pathways that prevent axonal outgrowth in the CNS following injury. Several axonal signals have been identified downstream of growth repulsive cues including Rho GTPase, Cdk5, PKC, calcium and PTEN (Hou et al., 2008). Our results from Chapter II indicate that in addition to Rho and its effector ROCK, MAG utilizes additional signals in cortical neurons to prevent neurite outgrowth. We considered a role for PTEN, previously shown to negatively regulate axonal outgrowth *in vitro* and *in vivo*, in MAG mediated inhibition.

Biochemistry and function of PTEN

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid phosphatase and tumor suppressor that regulates numerous crucial biological processes including cell survival, proliferation, growth, chemotaxis, polarity, angiogenesis and migration (Endersby and Baker, 2008; Keniry and Parsons, 2008).

Mutations in *PTEN* are associated with many types of human cancers, including prostate, breast, and brain. Furthermore, PTEN plays an important role in nervous system development. The PTEN hamartoma tumor syndromes, which include Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome, are associated with germline *PTEN* mutations. In addition to multiple hamartomas, clinical manifestations of these syndromes include increased intracranial pressure, seizures, ataxia, learning deficits, and autistic behavior. Many of these symptoms have been attributed to neuronal abnormalities such as increased neuronal size, defects in neuronal migration, and irregular synapses (Endersby and Baker, 2008). PTEN mutations have also been identified in individuals with autism spectrum disorders (Goffin et al., 2001; Butler et al., 2005).

PTEN is a 47kDA protein that can generally be subdivided into a catalytic N-terminal domain and a regulatory C-terminal domain (Knobbe et al., 2002) (Figure 3.1a). The N-terminal domain is homologous to tensin and auxilin and contains the phosphatase domain, with activity towards both phosphoinositides and phosphoproteins. The C-terminal domain is important for the regulation of PTEN (Georgescu et al., 1999; Lee et al., 1999; Knobbe et al., 2002). The C2 domain mediates lipid binding of PTEN, thus enabling the interaction of PTEN with the cell membrane. The C-terminus also contains two PEST sequences, which are important for controlling the stability and degradation of PTEN, and a PDZ-binding motif, which facilitates the interaction of PTEN with various PDZ domain-carrying proteins, also important for PTEN function and stability. Finally, PTEN contains various phosphorylation sites in its C-terminus. Phosphorylation of PTEN can

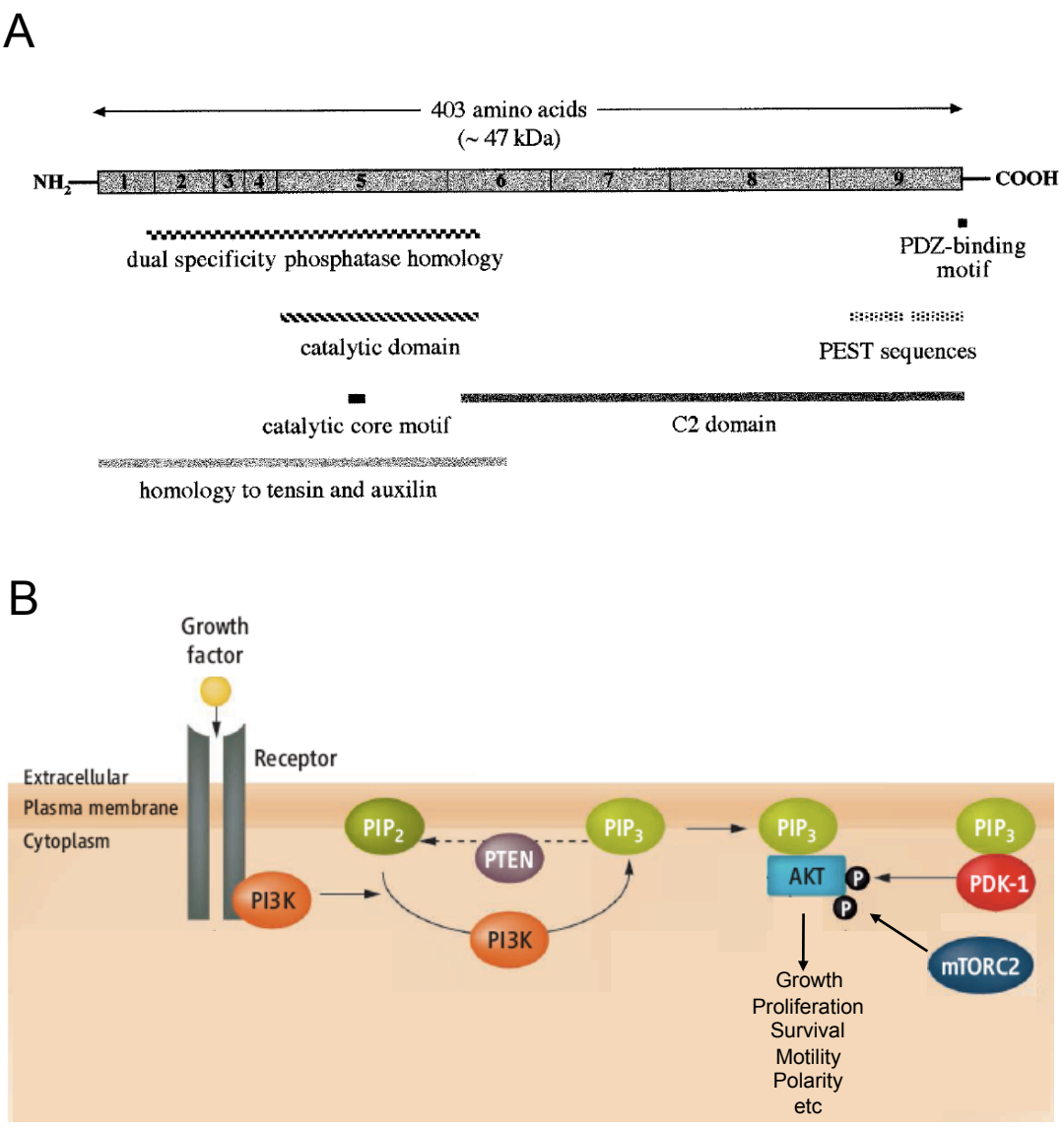

Figure 3.1. PTEN structure and function. A, A schematic of PTEN, showing the N-terminal catalytic domain and C-terminal regulatory domain. The N-terminal domain of PTEN contains its phosphatase function. The C-terminal domain includes several elements implicated in regulating PTEN stability and function. ***B,*** A model of PI3K/Akt regulation by PTEN is shown. While PI3K phosphorylates PIP2 to PIP3, PTEN antagonizes PI3K function by reversing that process and converting PIP3 to PIP2. One of the primary downstream targets of PI3K is Akt. Akt is recruited to PIP3 at the plasma membrane and is phosphorylated by several kinases including PDK-1 and mTORC2. Several biological processes are regulated by PI3K/Akt and thus by PTEN due to its crucial role as a negative regulator of PI3K signaling. Adapted from Knobbe et al. 2002 and Yang et al. 2009.

enhance or prevent its degradation by the proteasome, depending on which sites are phosphorylated (Torres and Pulido, 2001; Leslie et al., 2008)

The main function of PTEN is to antagonize the PI3K/Akt pathway (Leslie and Downes, 2002) (Figure 3.1b). Phosphatidylinositol-3-kinase (PI3K) is recruited to receptors, such as receptor tyrosine kinases in response to various growth factors. At the membrane, one of the most prominent roles of PI3K is the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3) (Engelman et al., 2006). One of the major downstream proteins activated by PI3K is Akt, a serine-threonine kinase that is recruited to PIP3 at the plasma membrane via its pleckstrin homology domain. Akt is then activated through phosphorylation by various kinases including phosphoinositide-dependent kinase 1 (PDK1) (also a PI3K target) (Alessi et al., 1997) and mTORC2 (Sarbassov et al., 2005). Akt is a critical regulator of many cellular functions including promotion of cell survival, increasing cell proliferation, and increasing cell size (Manning and Cantley, 2007; Keniry and Parsons, 2008). PTEN is a phosphatase for PIP3, converting it to PIP2 and thus down regulating Akt signaling (Maehama and Dixon, 1998). PTEN is crucial to controlling PI3K signaling and disruption of PTEN function, resulting in the deregulation of PI3K/Akt, has severe consequences *in vivo* as evidenced by emergence of tumors with *PTEN* mutations and the early embryonic lethality in PTEN knockout mice.

PTEN in neurite outgrowth inhibition

PTEN plays an important role in the development and maintenance of the nervous system. PTEN is ubiquitously and highly expressed in the brain during embryonic development and remains expressed in the adult (Luukko et al., 1999; Gimm et al., 2000). PTEN regulates neuron cell size, neuronal precursor proliferation, and process (dendrite and axon) branching (Groszer et al., 2001; Kwon et al., 2001; Marino et al., 2002; Kwon et al., 2006; van Diepen and Eickholt, 2008). A great deal of what we know about PTEN's function in the brain has come from studies with conditional *PTEN* knockout mice (Table 3.1). Deletion of *PTEN* in neural stem cells by crossing *PTEN* floxed mice with nestin promoter-driven Cre mice resulted in marked changes in brain morphology such as enlarged brains and layering defects throughout the brain, including the cortex and cerebellum (Groszer et al., 2001). These gross changes were attributed to increased neuronal size and number (due to decreased apoptosis and increased proliferation) and likely defects in migration. *PTEN* was deleted from differentiated cortical and hippocampal neurons by crossing *PTEN* floxed mice with *Neuron specific enolase-Cre* mice (Kwon et al., 2006). Similar to the above study, these mice exhibited macrocephaly as a result of neuronal hypertrophy. In this study, the effect of *PTEN* knockout on neurons with established neuronal processes was analyzed. Most notably, the neurons from these mice had thickened or elongated processes, ectopic processes and increased synapses. Perhaps as a result of these neuronal defects, *PTEN* null mice showed abnormalities in social interaction and learning similar to autism spectrum disorders.

Table 3.1

Effects of PTEN deletion on brain development

Cre promotor	Onset and cell type of *Pten* deletion	Brain morphology	Cellular effects	Phenotype	Reference
Nestin	Neural stem cells	Increased brain size; layering defects in brain stem, cortex, cerebellum and hippocampus	Increase in neuronal soma size; increase in cell number	Mice die after birth	Groszer et al., 2001
En2	*Pten* loss from E9.5 in neurons from the mid-hindbrain junction	Increased brain size; layering defects in cerebellum	Increase in neuronal soma size; increase in cell number; loss of Purkinje cells after birth	Mice develop seizures, ataxia, impaired balance and are less active; mice die prematurely	Marino et al., 2002
L7	*Pten* loss mainly after birth, selectively in Purkinje cells	Only minor irregularities in Purkinje cell layering	Increase in Purkinje cell soma size, thicker dendrites and axons; followed by degeneration of Purkinje cells	Not reported	Marino et al., 2002
Gfap	*Pten* loss from ~E13.5 mainly in neurons of the cerebellum and dentate gyrus	Increased brain size; layering defects in cerebellum and dentate gyrus	Increase in neuronal soma and nuclear size; loss of Purkinje cells and neurodegeneration in dentate gyrus after birth	Mice develop seizures and ataxia; mice die prematurely	Kwon et al., 2001 Backman et al., 2001
Nse	*Pten* loss 4 weeks after birth in differentiated neurons in hippocampus and cerebral cortex	Increased brain size; layering defect in hippocampus and cerebral cortex	Increased neuronal soma size, thicker dendrites and axons, higher spine density and thickness; formation of ectopic axons, increased and thickened axonal and dendritic branches	Learning defects, behavioral changes typical for autistic spectrum disorder	Kwon et al., 2006

Adapted from Read et al., 2009

There is growing evidence that both PI3K/Akt and PTEN play an important role in the regulation of neurite outgrowth, with the PI3K/Akt pathway promoting neurite outgrowth and PTEN negatively regulating it (Hou et al., 2008; Read and Gorman, 2009; Park et al., 2010b). The role of PI3K/Akt as a positive modulator of neurite outgrowth has been shown in various populations of PNS and CNS neurons. Axon outgrowth in response to numerous growth factors, for example, depends on PI3K and Akt signaling. Overexpression of constitutively active AKT in neurons increased dendritic lengths (Zheng et al., 2008) or axonal caliber (Markus et al., 2002). Interestingly, a function for Akt in axon regeneration *in vivo* was shown in motor axons, where constitutively active Akt overexpression in these neurons promoted axon regeneration following axotomy (Namikawa et al., 2000).

A role for PTEN has been demonstrated downstream of extrinsic cues in the regulation of neurite outgrowth. PTEN was identified downstream of Sema3A mediated growth cone collapse in sensory neurons. Stimulation of PI3K signaling by a peptide activator of the kinase or through expression of a dominant negative PTEN lacking its phosphatase function resulted in attenuation of Sema3A induced growth cone collapse (Chadborn et al., 2006). Sema3A reduced levels of phosphorylated Akt in neuroblastoma cells, a change that required functional PTEN, suggesting that Sema3A utilizes PTEN to modulate Akt activity in growth cone collapse. PTEN can also negatively regulate neuronal branching and arborization. Overexpression of PTEN in the developing retina resulted in a dramatic decrease in the quantity of branched axons as well as the number of branches (Drinjakovic et al., 2010). There is also evidence that PTEN is negatively regulated to promote axon outgrowth in response to NGF. In cultured hippocampal neurons, NGF activates CK2 to

phosphorylate and inactivate PTEN and thus promote neurite outgrowth (Arevalo and Rodriguez-Tebar, 2006). Furthermore, NGF induced differentiation of PC12 cells to a neuronal phenotype, which includes the development of neurites, was blocked with PTEN over-expression (Musatov et al., 2004).

Park et al. (2008) postulated that the growth impeding properties of PTEN might come into play in inhibition of axon regeneration. They demonstrated a role for PTEN as a negative regulator of CNS axon regeneration following injury *in vivo.* Deletion of *PTEN* in retinal ganglion cells resulted in significant axon outgrowth, past the injury site, following optic nerve injury (Park et al., 2008) (Figure 3.2). PTEN may be an intrinsic negative regulator of axon regeneration (Park et al., 2008; Park et al., 2010b). However, evidence for PTEN's function in response to positive and negative extracellular signals in axon outgrowth regulation (see above) suggest that PTEN may be modulated by inhibitory proteins in axonal injury to prevent regrowth. We postulated that PTEN is involved in MAG mediated inhibition of cortical neuron outgrowth. We found that knockdown of PTEN by shRNA or by neuronal specific knockout resulted in significant, although partial reversal of outgrowth inhibition by MAG. Overexpression of consitutively active Akt in cortical neurons also reduced their susceptibility to MAG inhibition. Furthermore, we found that MAG reduced levels of phosphorylated Akt in cortical neurons, suggesting that MAG may activate PTEN and thus inhibit Akt-mediated axonal outgrowth.

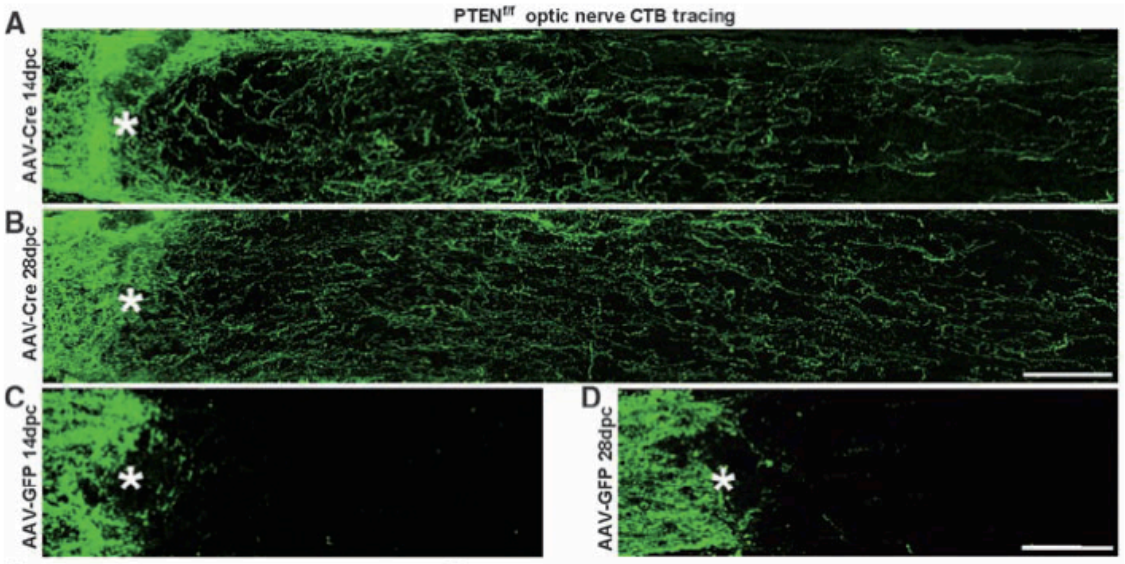

Figure 3.2. PTEN is a negative regulator of axon regeneration in vivo. PTEN floxed mice were injected in the eye with an adenovirus expressing Cre for ***A,*** 14 days or ***B,*** 28 days or a control GFP expressing adenovirus for ***C,*** 14 days or ***D,*** 28 days following optic nerve injury. Optic nerves were labeled with cholera toxin B (CTB). Significant growth of retinal ganglion cell axons was observed past the injury site (*) with PTEN knockout (A) and (B). (Park et al. 2008).

Experimental Procedures

Primary neuron cultures

Cortical neurons were isolated at E15-E17, dissociated in 0.06% trypsin (Worthington) for 30 min at room temperature, triturated and plated in Neurobasal media with B27 supplement on dishes coated with 2 μg/ml laminin (Invitrogen) or on CHO cells for neurite outgrowth assays.

Immunostaining

Cells were fixed in 3.7% formalin, blocked with 10% normal goat serum in PBS containing 0.2% triton X-100, incubated with primary antibody overnight and visualized with fluorescently labeled secondary antibodies and mounted with medium containing DAPI (Vectashield, Vector Labs). Primary antibodies used: anti-PTEN (1:100, Cell Signaling), anti-TuJ1 (1:500, Covance) and chicken anti-GFP for enhancement of GFP fluorescence (1:500, Ambion). Secondary antibodies used: Rhodamine conjugated donkey anti-rabbit (1:300, Jackson ImmunoResearch), goat anti-mouse Alexa 488 (1:500, Invitrogen), Rhodamine conjugated donkey anti-mouse (1:400, Jackson ImmunoResearch) and Cy2 conjugated donkey anti-chicken (1:200, Jackson ImmunoResearch).

Neurite outgrowth assays

Neurite outgrowth assays on MAG transfected CHO cells have been previously described (Mukhopadhyay et al., 1994). Cortical neurons or CGNs (40,000/well) were plated on confluent monolayers of MAG-CHO or control CHO cells in 8 well tissue culture slides (Lab-Tek, Nalge Nunc International). After incubation for ~20 hrs, co-cultures were fixed and immunostained with anti-TuJ1, as described above. Where indicated, cell permeable C3 exoenzyme (1μg/ml, Cytoskeleton, CT04) was added at the time of neuronal plating. The percent of neurons with neurites was quantified in a blinded manner using a Zeiss fluorescence microscope at 40x magnification. A neurite was defined as a process at least twice the length of the cell body. For each experiment, 2-4 wells were used for each condition and at least 250 neurons were counted per condition. Neurite lengths were determined using ImageJ software analysis of images taken with a Zeiss fluorescence microscope at 40x magnification. The longest neurite ≥1 cell body was measured for each neuron, with at least 145 neurites evaluated for each experimental condition.

Mice

CD1 mice (Charles River), EMX1-Cre mice (Iwasato et al., 2000) or PTEN floxed mice (Jackson Laboratory) (Groszer et al., 2001) were used, as indicated. All experiments with animals were approved by the Animal Care and Use Committee at Vanderbilt University.

Neuronal transfections

Cortical neurons were co-transfected with GFP (pEGFP, Clontech) (1 μg) and control shRNA (5 μg) or PTEN shRNA (5 μg; in pSUPER.retro.puro; generously provided by C. Arteaga, Vanderbilt University, Nashville, TN (Miller et al., 2009)), or with GFP and myristoylated Akt (Myr-Akt; in pUSE; kindly provided by W. Pao, Vanderbilt University, Nashville, TN) or control vector by electroporation with an Amaxa Nucleofactor device according to the manufacturer's protocol. Cells were plated on laminin-coated dishes and re-fed at 24 hrs post-transfection. The neurons were incubated 48 hrs to allow for PTEN knockdown or Myr-Akt expression, then they were incubated in trypsin and dispase and gently lifted with a cell lifter. Neurons were spun down and resuspended in Neurobasal/B27. 10,000 neurons/well were plated on 8-well slides containing confluent monolayers of CHO or MAG-CHO cells in Neurobasal/B27. Twenty hours after plating, cells were fixed and immunostained for TuJ1 and GFP and analyzed for neurite outgrowth. The remaining neurons were collected for Western blot analysis or replated on a laminin coated 8-well slide for immunostaining to verify PTEN knockdown. Myr-Akt expression was confirmed by Western blot. For analysis of the percent of neurons with neurites, 3-4 wells were counted per condition in a blinded manner for each experiment and a minimum of 800 neurons total were counted per experimental condition. Replicate wells, with at least 145 total neurites measured per experimental condition, were evaluated in a blinded fashion for neurite length.

FACS isolation of neurons

Cortical neurons isolated from E15 to E17 mice were labeled with calcein (2 nM PBS) (Invitrogen, Molecular Probes) for 30 min with gentle rotation at room temperature, washed with PBS and incubated in PBS at room temperature for an additional 30 min. The labeled neurons (5 x 10^6) were resuspended in Neurobasal/B27 and plated on a confluent monolayer of MAG-CHO or control CHO cells in 6 cm dishes. After 2-4 hrs, the cells were washed twice with ice cold PBS to remove non-adhered neurons and then harvested by gentle scraping. The cells were re-suspended in PBS and passed through a cell strainer (BD Falcon, 40μm nylon) to remove cell clumps. The calcein labeled neurons were collected by FACS using a BD FACS Aria cell sorter. Reanalysis of sorted cells verified that only calcein labeled cells were collected. The sorted neurons were lysed and subjected to Western blot analysis as indicated. For control experiments involving BDNF treatment, the cells were treated with 100 ng/ml BDNF for 10 min prior to harvesting for FACS analysis. Recombinant BDNF was generously provided by Regeneron, Inc.

Western blot analysis

Cells were lysed in NP40 lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol) containing a protease inhibitor cocktail (Complete Mini, Roche). For experiments analyzing phosphorylated proteins, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM β-glycerophosphate were added to the lysis buffer. Cell lysates were subjected to SDS-PAGE and Western blotting

using anti-Akt (1:1000, Cell Signaling), anti-phospho-Akt (Ser483) (1:1000, Cell Signaling), anti-PTEN (1:1000, Cell Signaling), anti-TuJ1 (1:1000, Covance) or mouse anti-GFP (1:1000, Roche). Secondary antibodies conjugated to horse radish peroxidase included anti-rabbit (1:3000, Thermo Scientific) and anti-mouse (1:3000, Promega). For FACS collected neurons, the Odyssey infrared imaging system (Licor) was used for Western blot analysis and quantification according to the manufacturer's protocol. Secondary fluorescent antibodies used for Odyssey were IRDye goat anti-mouse 800 and goat anti-rabbit 680 (Licor).

Results

Knockdown or genetic deletion of PTEN partially reverses neurite outgrowth inhibition by MAG

Numerous signal transduction pathways have been implicated in the regulation of neurite outgrowth (Hou et al., 2008). We tested inhibitors of several of these pathways for their ability to reverse the effects of MAG: SP600125, a c-Jun N-terminal kinase inhibitor, BIO/Inhibitor IX, an inhibitor of GSK3, Roscovitine, which targets cdk5, and the Src-family kinase inhibitor PP2. However, we did not observe any notable rescue (data not shown). We were also interested in evaluating a possible role for PTEN (Phosphatase and tensin homologue deleted on chromosome 10), since it was recently reported that deletion of this phosphatase resulted in considerable regeneration of retinal ganglion cell axons following optic nerve injury (Park et al., 2008). To determine whether MAG signals through PTEN to

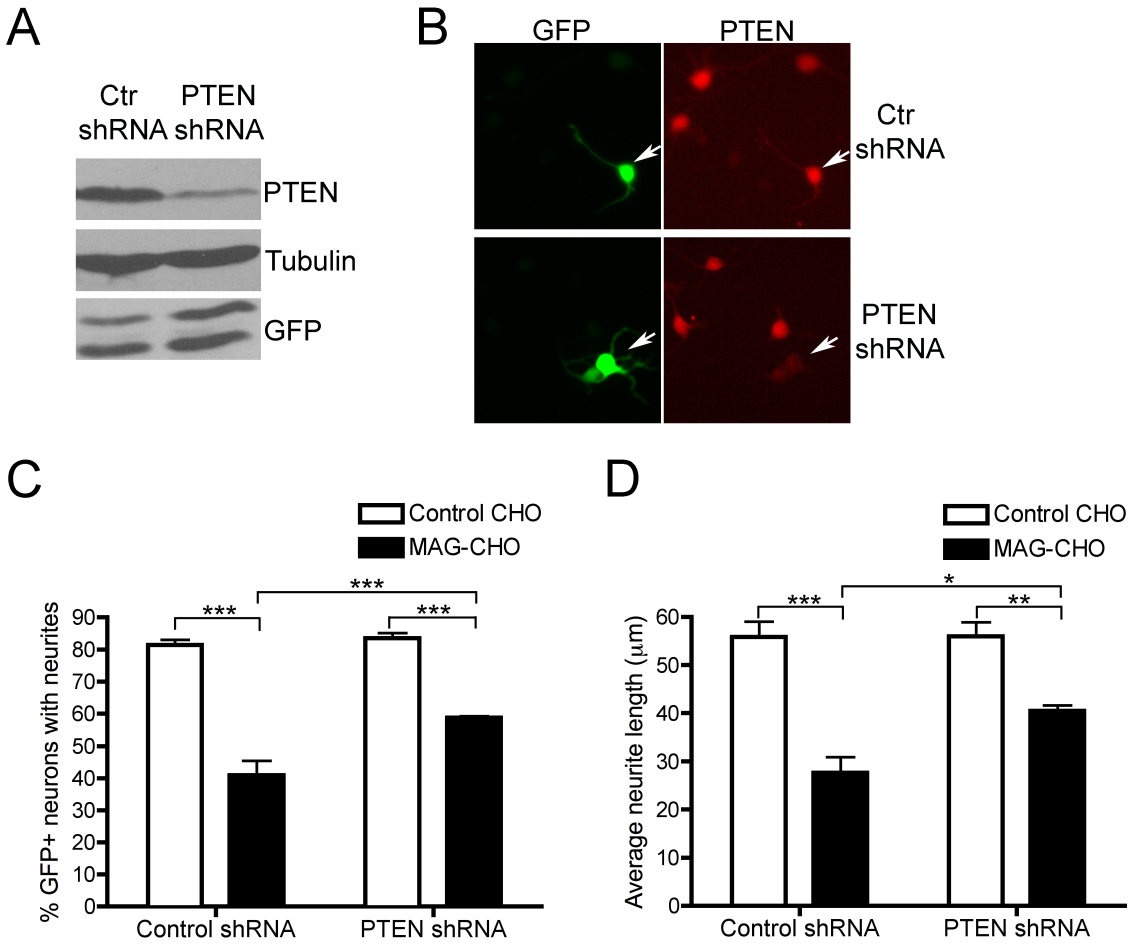

Figure 3.3. PTEN knockdown in cortical neurons partially reverses neurite outgrowth inhibition by MAG. Cortical neurons were electroporated with GFP and control shRNA or PTEN shRNA and plated on laminin-coated dishes for 72 hrs to allow for optimal PTEN knockdown. ***A,*** At 72 hrs, neurons were lysed and analyzed by Western blot or, ***B,*** replated and 20 hrs later fixed and immunostained for PTEN to verify PTEN knockdown or, ***C and D,*** replated on control CHO or MAG-CHO cells for neurite outgrowth analysis. The cells were fixed 20 hrs after plating and immunostained for Tuj1. Neurite outgrowth was analyzed by counting the percentage of GFP+ neurons with neurites (C) (n=4) or measuring neurite lengths of GFP+ neurons (D) (n=3). Data represent mean ± SEM (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ by two way ANOVA).

negatively regulate neurite outgrowth we first evaluated the effect of PTEN knockdown. Cortical neurons were co-transfected with GFP and a control shRNA or PTEN shRNA and plated on laminin for 72 hours to allow for efficient PTEN knockdown, at which point they were replated onto MAG-CHO cells or control CHO cells. PTEN knockdown was confirmed by Western blot analysis of neurons collected after 72 hours (Figure 3.3a) and immunostaining of replated neurons (Figure 3.3b). Approximately 98% of GFP+ neurons co-transfected with PTEN shRNA had decreased PTEN staining. Interestingly, knocking down PTEN significantly restored neurite outgrowth in neurons plated on the MAG-CHO cells (a 20.2% increase in the percent of neurons with neurites and a 22.7% increase in the average neurite length relative to neurons on control CHO cells), although there was not a complete recovery to control levels (Figure 3.3c and Figure 3.3d). It is important to note that reducing PTEN did not affect neurite outgrowth when the neurons were plated on control CHO cells (Figure 3.3c and Figure 3.3d). These results suggested that PTEN contributes to the growth inhibitory signal elicited by MAG.

To further establish a role for PTEN in MAG mediated inhibition of neurite outgrowth, we evaluated the effect of genetically deleting *pten* in cortical neurons. Mice expressing Cre recombinase under an EMX1 promoter (Iwasato et al., 2000) were crossed with *pten* floxed mice (Groszer et al., 2001). PTEN knockout was confirmed in cortical neurons by Western blot analysis of cultured neurons (Figure 3.4a). Embryonic EMX1-Cre;$pten^{+/+}$, WT;$pten^{fl/fl}$ or EMX1-Cre; $pten^{fl/fl}$ cortical neurons were plated on control CHO or MAG-CHO cells for 20 hours. A significant increase in the

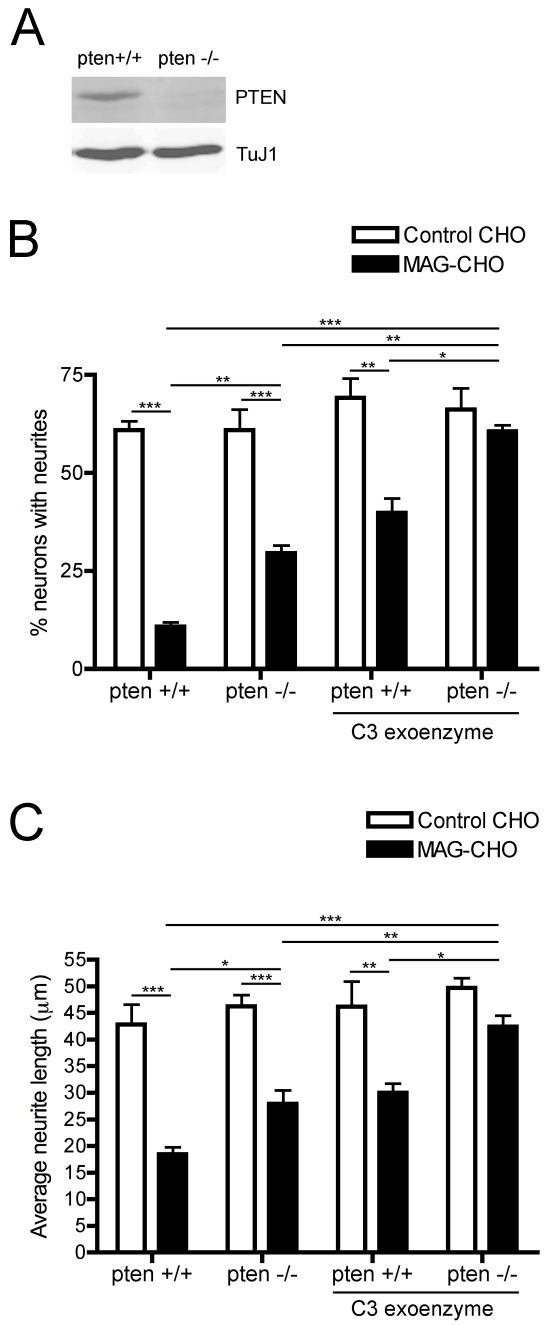

Figure 3.4. PTEN knockout cortical neurons are less susceptible to inhibition by MAG. A, Lack of PTEN expression in EMX1-Cre;*pten*$^{fl/fl}$ neurons was confirmed by Western blot analysis of cortical neuron culture lysates. ***B and C,*** EMX1-Cre;*pten*$^{+/+}$, wildtype;*pten*$^{fl/fl}$ or EMX1-Cre;*pten*$^{fl/fl}$ (pten-/-) embryonic neurons (E17) were plated on control CHO or MAG-CHO cells in the presence or absence of C3 exoenzyme (1μg/ml) and subsequently fixed and immunostained for Tuj1. No difference was observed between the EMX1-Cre;*pten*$^{+/+}$ and the wildtype;*pten*$^{fl/fl}$neurons; therefore the data were combined and are labeled as pten+/+. Neurite outgrowth was evaluated by determining the percent of neurons with neurites (B) or measuring neurite lengths (C). Data represent mean ± SEM (n=3, *, p<0.05, **, p<0.01, ***, p<0.001 by two way ANOVA).

neurons on MAG-CHO cells compared to EMX1-Cre;*pten*$^{+/+}$ or WT;pten$^{fl/fl}$ neurons (Figure 3.4b and Figure 3.4c). Interestingly, when *pten* null cortical neurons were treated with C3 exoenzyme, neurite outgrowth on MAG-CHO cells was not significantly different from that on control CHO cells (Figure 3.4b and Figure 3.4c). The additive effect of PTEN knockout and Rho inactivation suggests that both molecules contribute to inhibition of cortical neurite outgrowth by MAG, likely acting through different pathways.

Overexpression of constitutively active Akt in cortical neurons significantly reverses MAG inhibition of neurite outgrowth

One of the primary biological functions of PTEN is to counteract phosphatidylinositol 3-kinase (PI3K) by de-phosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3), thereby inhibiting activation of the downstream kinase Akt. As expected, we observed an increase in phosphorylated Akt in neurons transfected with PTEN shRNA (Figure 3.5a). To further establish a role for PTEN downstream of MAG, we assessed the effect of expressing an activated form of Akt, by-passing the effects of PTEN. A myristoylated form of Akt (myr-Akt) was transfected into cortical neurons and neurite outgrowth on MAG-CHO cells was compared to that on control CHO cells. The activation of Akt by the expression of myr-Akt was confirmed by an increase in the phosphorylated form of the enzyme (Figure 3.5b). Cortical neurons were co-transfected with GFP and myr-Akt or a control vector and 72 hours later, the neurons were replated on control CHO or MAG-CHO cells for twenty hours. When transfected with myr-Akt, there was a significant, 24.4% increase in the percent of

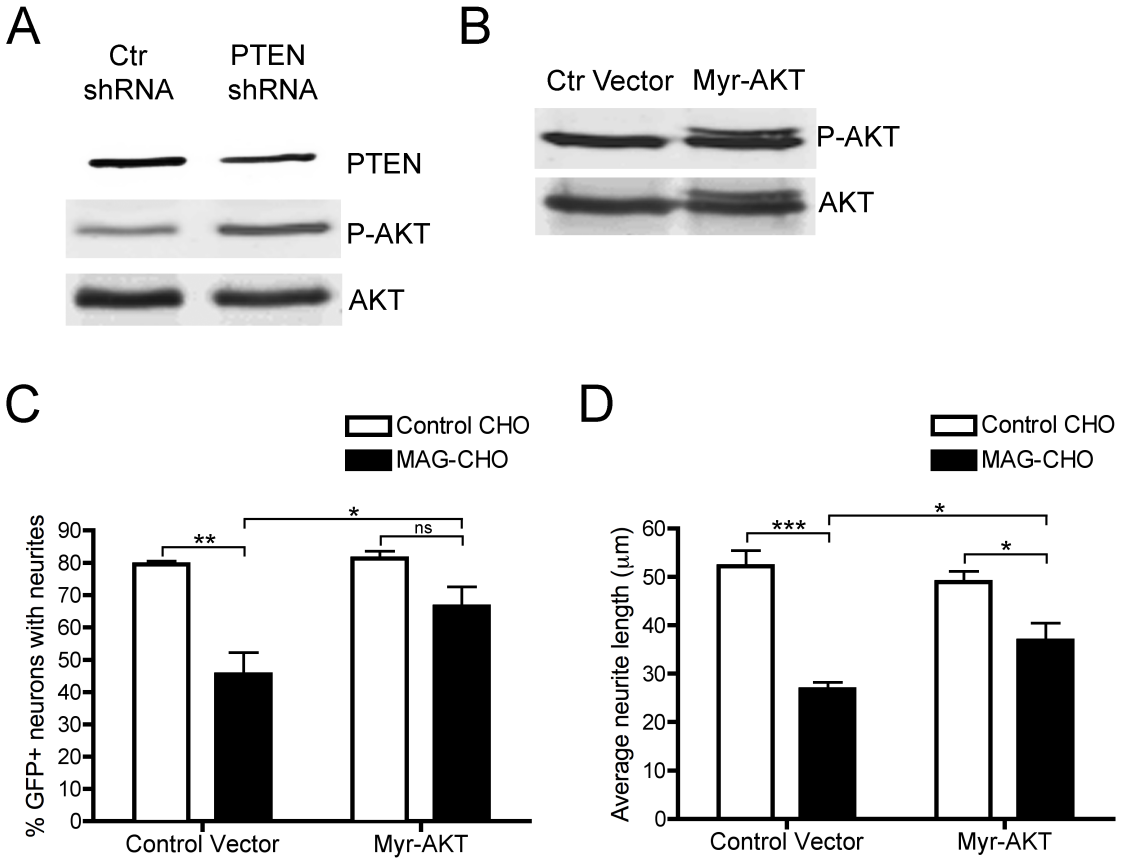

Figure 3.5. Expression of constitutively active AKT in cortical neurons reverses the inhibition of neurite outgrowth by MAG. ***A,*** Cortical neurons were electroporated with control shRNA or PTEN shRNA, cultured for 72 hrs, then subjected to Western blot analysis for levels of PTEN, phospho-AKT and AKT. ***B,*** Cortical neurons were electroporated with control vector or myr-AKT and 72 hrs later lysed and the level of phospho-AKT and total AKT analyzed by Western blot. ***C and D,*** Cortical neurons were electroporated with GFP and control vector or myr-AKT and plated on laminin-coated dishes for 72 hrs at which point they were replated on control CHO or MAG-CHO cells. The neurons were fixed 20 hr after plating, immunostained for Tuj1 and neurite outgrowth was analyzed by counting the percentage of GFP+ neurons with neurites (C) or determining neurite lengths of GFP+ neurons (D). Data represents mean ± SEM (n=3, *, $p < 0.05$, **, $p<0.01$, ***, $p<0.001$ by two way ANOVA).

neurons with neurites on MAG-CHO cells relative to neurons transfected with a control vector (Figure 3.5c). A similar 23.8% increase in average neurite length was observed for neurons expressing myr-Akt on MAG-CHO cells compared to control transfected cells (Figure 3.5d). These results indicate that preventing activation of PTEN by expressing active Akt can reverse the inhibition of neurite outgrowth by MAG.

MAG reduces levels of phosphorylated Akt in cortical neurons

Finally, to demonstrate that PTEN is regulated by MAG, we assessed the activity of Akt in neurons plated on MAG-CHO cells. If MAG activates PTEN, then the level of phospho-Akt should be reduced. We were specifically interested in the response of the neurons to membrane-bound MAG; therefore, the neurons were plated on MAG-CHO cells or control CHO cells for 2 - 4 hours, then isolated by fluorescence-activated cell sorting (FACS) and the level of phospho-Akt measured by Western blot. To separate neurons from CHO cells, the neurons were labeled with calcein dye prior to plating. Reanalysis of collected cells confirmed that only calcein positive cells were collected (Figure 3.6a). Sorted cells were lysed and subjected to Western blot analysis for TuJ1 expression and levels of phosphorylated Akt. To verify that this method reliably detected changes in Akt phosphorylation and that the phosphorylation was not reversed during the sorting protocol, the neurons on control CHO cells were treated with BDNF, which is known to increase Akt phosphorylation in cortical neurons (Hetman et al., 1999). We consistently detected an increase in phosphorylated Akt following BDNF treatment (Figure 3.6b and Figure 3.6c).

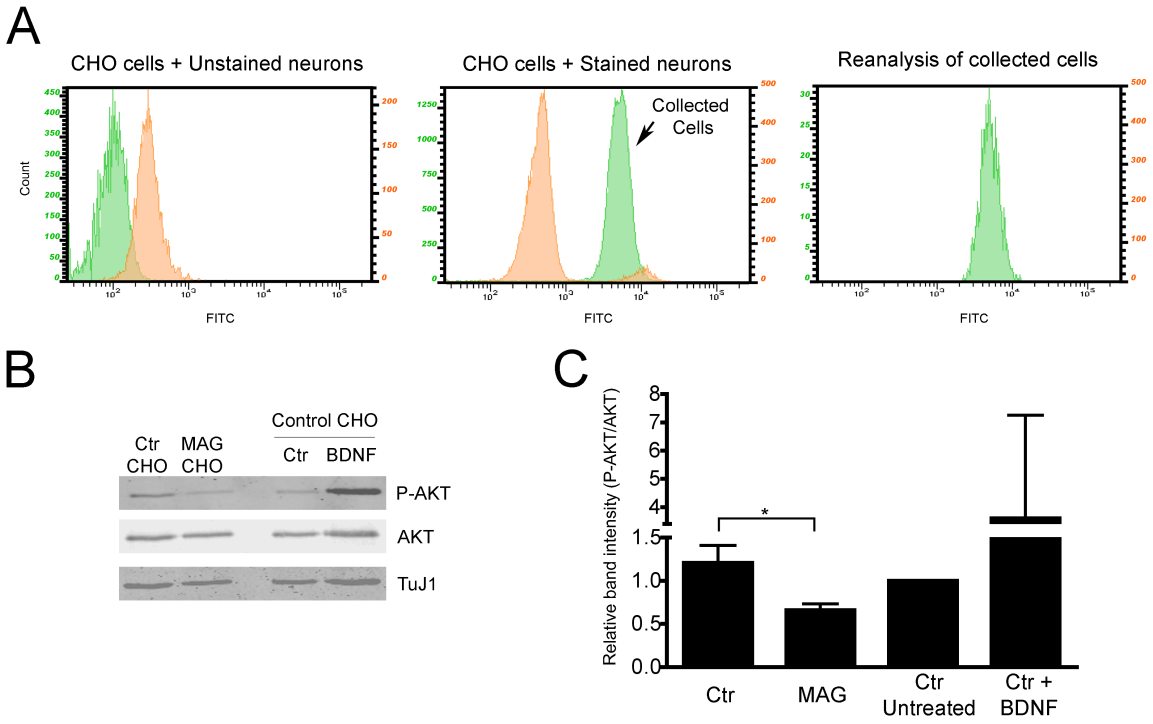

Figure 3.6. MAG reduces AKT phosphorylation in cortical neurons. Cortical neurons were labeled with calcein, plated on control or MAG expressing CHO cells for 2-4 hr, and then subjected to FACS to isolate the neurons from the CHO cells. ***A,*** The isolation of neurons from CHO cells using calcein labeling was confirmed by reanalysis of the sorted cells. The left panel depicts unlabeled neurons (green) plated on CHO cells (orange), which can be roughly differentiated from each other based on side scatter and forward scatter differences. The central panel shows calcein-labeled neurons plated on CHO cells. The small peak for CHO cells at higher fluorescence intensity in the central panel is likely neuron doublets. The right panel depicts fluorescent cells that were collected by FACS, then reanalyzed to verify that fluorescent negative cells were excluded. ***B,*** Following FACS, neurons that had been plated on control CHO or MAG-CHO were lysed and analyzed by Western blot for phospho-AKT, total AKT, and TuJ1 expression (to further confirm neuronal isolation and as a loading control). BDNF treated or untreated neurons plated on control CHO cells and isolated by FACS served as a control for the experimental method. ***C,*** Quantitative analysis of the ratio of phospho-AKT to total AKT. Values were normalized to untreated neurons plated on control CHO cells. Data represents mean ± SEM (n=5, * p = 0.0005, based on Student's *t*-test).

Importantly, we also found that neurons plated on MAG-CHO cells had significantly reduced levels of phosphorylated Akt compared to those plated on control CHO cells (Figure 3.6b and Figure 3.6c). Taken together, these results reveal a novel mechanism by which MAG inhibits cortical neurite outgrowth through inhibition of the PI3K/Akt pathway.

Discussion

We identified PTEN as an essential component of MAG's inhibitory effects on neurite outgrowth in cortical neurons. PTEN is a lipid phosphatase that indirectly inactivates the kinase Akt by reducing the levels of phosphatidylinositol 3,4,5 trisphosphate, which is required for Akt activation. A previous study suggested that the PI3K/Akt/mTOR pathway promotes axon extension and demonstrated that deletion of PTEN or the mTOR repressor, Tuberous sclerosis complex 1 (TSC1), significantly improved regeneration in the optic nerve following a crush injury (Park et al., 2008). Here, we demonstrated that membrane-bound MAG actively reduced Akt phosphorylation, thereby implicating activation of PTEN by this inhibitor. Suppression of Akt activity would decrease activation of Rac, a GTP binding protein that promotes neurite extension through regulation of actin polymerization, and increase the activation of Glycogen synthase kinase 3 (GSK3), a kinase that regulates microtubule dynamics (Park et al., 2010b). Similar to our findings, PTEN was identified as a target of Sema3A in triggering growth cone collapse of sensory neurons (Chadborn et al., 2006). Thus, PTEN appears to be a key component in

another signal transduction pathway, like the Rho/ROCK cascade, that is activated by inhibitors of axon growth.

The mechanisms by which PTEN is regulated are not well understood. It can be serine/threonine phosphorylated by CK2 and GSK3, a modification that inhibits its activity, reduces membrane localization and/or causes destabilization of the protein (Leslie et al., 2008). NGF increases PTEN phosphorylation by CK2 in hippocampal neurons, thereby promoting axonal growth (Arevalo and Rodriguez-Tebar, 2006). Reactive oxygen species generated in response to various growth factors can also suppress PTEN activity by causing the formation of disulfide bonds (Lee et al., 2002; Leslie et al., 2003; Kwon et al., 2004; Seo et al., 2005). In contrast, the recruitment to membranes by increasing the local concentration of acidic lipids, such as phosphatidylinositol 3,4 bisphosphate, can activate PTEN (Campbell et al., 2003; Iijima et al., 2004; Redfern et al., 2008). How MAG or Sema3A modulate PTEN activity is not known, but Sema3A induced a local accumulation of PTEN at the growth cone, leading to a depletion of phosphatidylinositol 3,4,5 trisphosphate (Chadborn et al., 2006). Determining the mechanisms by which MAG activates PTEN will be an interesting topic for future studies.

CHAPTER IV

TRAF6 IS REQUIRED FOR NEURITE OUTGROWTH INHIBITION BY SOLUBLE MAG

Introduction

We demonstrated in Chapter 2 that p75 is not involved in mediating signaling by membrane bound MAG. However, p75 is a key mediator of neurite inhibition in response to soluble MAG (Wong et al., 2002; Yamashita et al., 2002; Yamashita and Tohyama, 2003). Pathways downstream of NgR-p75/TROY-LINGO1 that result in neurite outgrowth inhibition and Rho GTPase regulation remain poorly understood. There is evidence for p75 cleavage in MAG mediated outgrowth inhibition and Rho activation (Domeniconi et al., 2005); however, how receptor processing influences Rho activity is not clear. An interaction between p75 and RhoGDI has been implicated in MAI signaling (Yamashita and Tohyama, 2003). By binding to Rho-GDI, p75 causes the release and activation of Rho. There is also evidence that Kalirin9, a Rho guanine nucleotide exchange factor (RhoGEF), is released from p75 upon addition of MAG to allow Rho GDI binding and to enhance Rho activation (Harrington et al., 2008).

Much of what is known about p75 signaling arises from studies of the role of p75 in neurotrophin mediated neuronal apoptosis or survival. The intracellular domain of p75 contains no intrinsic enzymatic activity (Bradley and Pober, 2001) and as such

requires adaptor proteins to transduce ligand mediated signaling intracellularly. Tumor necrosis factor Receptor Associated Factor 6 (TRAF6) has emerged as an important signaling molecule downstream of p75 and other TNFR family members (Ha et al., 2009). TRAF6 is a 60kDa E3 ligase and adaptor protein that participates in various physiological processes including immunity, bone morphogenesis, and neuronal survival/apoptosis (Chung et al., 2002; Wu and Arron, 2003). As an E3 ubiquitin ligase, TRAF6 attaches an ubiquitin chain through lysine 63 to its substrates and to itself. This modification does not target substrates for degradation but functions as a protein-protein interaction motif that can direct the formation of multi-protein complexes involved in the regulation of various kinase cascades, including IkB, MAPK, and c-Src (Wong et al., 1999; Wang et al., 2001; Wu and Arron, 2003). As an adaptor protein, TRAF6 plays an important role as a proximal signal transducer mediating the activation of signaling pathways initiated by receptor engagement. As an example, TRAF6 was recently identified as an E3 ligase for Akt. In mouse embryonic fibroblasts treated with insulin-like growth factor 1 (IGF-1), TRAF6 ubiquitinated Akt and mediated its membrane recruitment and subsequent phosphorylation (Yang et al., 2009). As a signaling intermediate for p75, TRAF6 was recruited to the receptor upon neurotrophin binding (Khursigara et al., 1999) and was required for the subsequent activation of NF-kB and JNK, which play an important role in neuronal survival and apoptosis respectively (Yeiser et al., 2004).

We hypothesized that TRAF6 is also an important mediator of signaling events downstream of NgR-p75/TROY-LINGO1 in the process of neurite outgrowth regulation. TRAF6 is involved in p75 and TROY signaling in other contexts (Kojima et al., 2000; Naito et al., 2002; Ohazama et al., 2004; Yeiser et al., 2004), making it

an attractive candidate as a signal transducing intermediary for the classic NgR complex. Furthermore, TRAF6 has been implicated in cytoskeletal regulation. During bone resorption, TRAF6 and c-Src function together to regulate the cytoskeletal changes required for osteoclast differentiation (Armstrong et al., 2002; Nakamura et al., 2002; Wang et al., 2006). We postulated that TRAF6 might also play a role in the cytoskeletal changes necessary for axon outgrowth inhibition downstream of MAIs. We found that TRAF6 is required for neurite outgrowth inhibition by soluble MAG. We also identified an interaction between TRAF6 and p21-activated kinase (PAK), a protein known to regulate the cytoskeleton. Furthermore, we found that overexpression of NgR decreased the interaction between p75 and TRAF6, which might be an important step in the role of TRAF6 in neurite outgrowth regulation.

Experimental procedures

Primary neuron cultures

Cerebellar granule neuron (CGN) cultures were prepared from postnatal day 4-7 mice. Cerebella were isolated, dissociated in 0.125% trypsin (Worthington) at 37^{o}C for 15 min, washed in PBS, triturated and subsequently plated in Neurobasal media (Gibco) containing B27 supplement (Gibco), 25 mM KCl, 33 mM dextrose, 2 mM glutamine, and 100 U/ml penicillin / 100 μg/ml streptomycin (Gibco). CGNs were plated on CHO cells or poly-L-ornithine (Sigma) coated dishes for neurite outgrowth assays. Cortical neurons were isolated at P1-P3, dissociated in 0.06% trypsin

(Worthington) for 30 min at room temperature, triturated and plated in Neurobasal media with B27 supplement on CHO cells for neurite outgrowth assays.

Neurite outgrowth assays

Neurite outgrowth assays on MAG transfected CHO cells have been previously described (Mukhopadhyay et al., 1994). Cortical neurons or CGNs (40,000/well) were plated on confluent monolayers of MAG-CHO or control CHO cells in 8 well tissue culture slides (Lab-Tek, Nalge Nunc International). Neurite outgrowth assays with soluble MAG were conducted with CGNs. 40,000 CGNs were plated on poly-L-ornithine coated 8 well tissue culture slides in the presence of MAG-Fc (25 μg/ml) (provided by Marie Filbin) or control IgG (25 μg/ml). After incubation for ~20 hrs, co-cultures were fixed and immunostained with anti-TuJ1 (1:500, Covance). The percent of neurons with neurites was quantified in a blinded manner using a Zeiss fluorescence microscope at 40x magnification. A neurite was defined as a process at least twice the length of the cell body.

Mice

CD1 mice (Charles River) and 129 mice with the *Traf6* gene deleted (Naito et al., 1999) were used, as indicated. All experiments with animals were approved by the Animal Care and Use Committee at Vanderbilt University.

Co-immunoprecipitation experiments

HEK293 cells, maintained in DMEM with 10% fetal bovine serum, were transfected with constructs expressing FLAG (in CMV-1), FLAG-TRAF6 (in pRK5), myc-PAK (in CMV-6) or FLAG-NgR1 using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells were harvested 48 hrs after transfection and lysed in NP40 lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol) for TRAF6 and PAK co-immunoprecipitation or RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS) for p75 and TRAF6 co-immunoprecipitation, both containing a protease inhibitor cocktail (Complete Mini, Roche). 2-4 mg of cleared lysates were utilized for immunoprecipitation experiments. 2 μg of anti-FLAG or 5 μl of anti-p75 (generated from a GST fusion protein with the p75NTR intracellular domain) were added to lysates, which were incubated overnight with rotation at 4°C. 30 μl of Protein A Sepharose slurry (Invitrogen) was added to lysates and samples were incubated with rotation at 4°C for 1 hr. Beads were centrifuged at 1,000 rpm for 1 min and washed 3 times with their respective lysis buffers. 20 ml of 6x SDS loading buffer was added to each sample and samples subjected to SDS-PAGE and Western analysis with anti-p75NTR (1:1000), anti-TRAF6 (1:500, Santa Cruz), anti-myc (1:500, Santa Cruz) or anti-FLAG (1:1000, Sigma). Secondary antibodies conjugated to horseradish peroxidase included anti-rabbit (1:3000, Thermo Scientific) and anti-mouse (1:3000, Promega).

Postnatal (P5-P7) mouse cerebella were collected in NP40 lysis buffer, homogenized with a dounce homogenizer and cleared at 12,000 rpm for 8 min. Anti-PAK1 (2.5 μl, Santa Cruz) or control rabbit gamma globulin (Jackson Laboratories)

were added to 4 mg of total protein and samples incubated overnight with rotation at 4°C. 30 μl of Protein A Sepharose slurry (Invitrogen) was added to each sample followed by rotation for 1 hr at 4°C. Beads were centrifuged at 3,000 rpm for 1 min at 4°C and washed three times with NP40 lysis buffer. 20 ml of 6x SDS loading buffer was added to each sample and samples subjected to SDS-PAGE and Western analysis with anti-TRAF6 (1:500, Santa Cruz).

GST-PAK pull-down assays

HEK293 cells were transfected by Lipofectamine with FLAG or FLAG-TRAF6. After 48 hrs, cells were washed with cold TBS on ice, harvested and lysed with Lysis Buffer (50 mM Tris pH7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$ and protease inhibitor cocktail). Lysates were centrifuged at 12,000 rpm for 4 min at 4°C and the supernatant utilized for pull-down assays. 40 μg of GST-PAK (attached to glutathione agarose beads) were added to the supernatant and samples incubated for 45 min with rotation in the cold room. Beads were centrifuged at 2,500 rpm at 4°C for 1 min and washed three times with Wash Buffer (50 mM Tris pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$ and protease inhibitor cocktail). Beads were then subjected to SDS-PAGE and analyzed by Western blot with anti-TRAF6 (1:500, Santa Cruz).

Results

TRAF6 is required for inhibition of neurite outgrowth by soluble but not membrane bound MAG

To investigate a role for TRAF6 in neurite outgrowth inhibition by MAG we compared outgrowth of *traf6+/+* and *traf6-/-* neurons in the presence of soluble MAG. Primary wild-type or *traf6* null cerebellar granule neurons (CGNs) were grown in the presence of MAG-Fc or control IgG for approximately 20 hours. Neurons were then fixed, immunostained for TuJ1 and neurite outgrowth analyzed by scoring the percent of neurons with neurites. MAG-Fc treatment significantly inhibited neurite outgrowth by decreasing the percent neurons with neurites (Figure 4.1a). In contrast, *traf6* null cells were not susceptible to the inhibitory effects of MAG-Fc on neurite outgrowth. There were no differences in neurite outgrowth between *traf6* null and wild-type neurons treated with control IgG (Figure 4.1b). These results indicate that TRAF6 is required for the inhibitory effects of MAG and provide the first evidence implicating TRAF6 in neurite outgrowth inhibition.

In order to further characterize the role of TRAF6 in the inhibition of neurite outgrowth by MAG signaling, we utilized CHO cells expressing MAG on the cell surface (MAG-CHO cells). Surprisingly, analysis of wild-type and *traf6-/-* CGNs or cortical neurons seeded on MAG expressing CHO cells revealed no difference in inhibition of process outgrowth, with MAG-CHO cells inducing a similar reduction in the percent of neurons with neurites in both *traf6+/+* and *traf6-/-* neurons (Figure 4.2a and 4.2b). Together, these findings suggest that TRAF6 is not required for inhibition of neurite outgrowth by surface bound MAG, but is required for the effect of

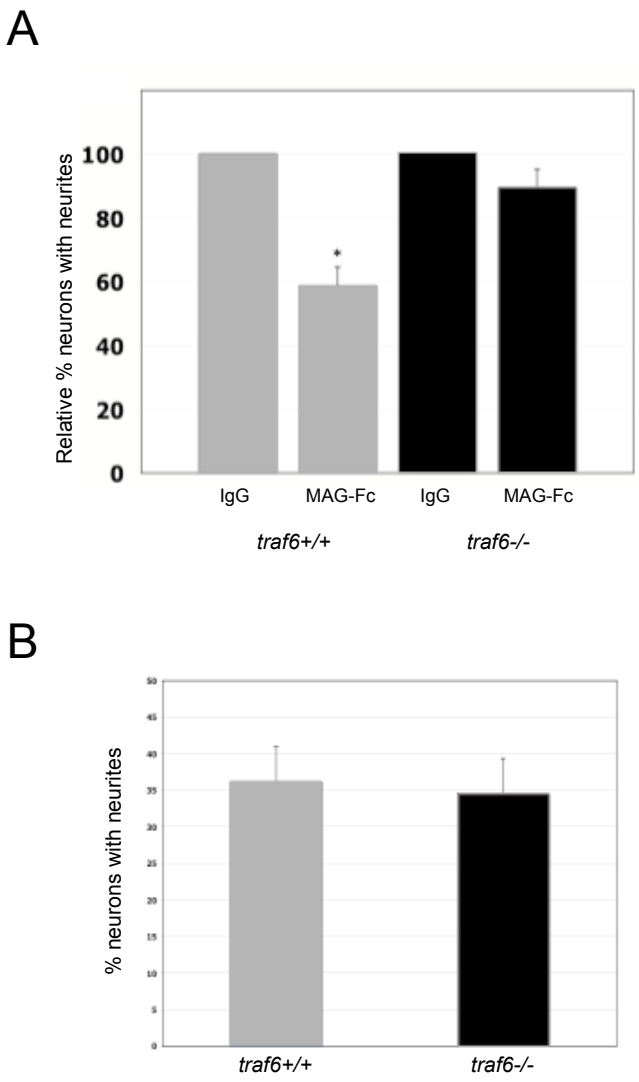

Figure 4.1. TRAF6 is required for neurite outgrowth inhibition by soluble MAG. ***A,*** CGNs from wild-type and *traf6-/-* mice were grown in the presence of MAG-Fc or control IgG for ~20 hours and assessed for neurite outgrowth. Neurite outgrowth is shown as percent of neurons with neurites relative to control treated neurons. A significant decrease in neurite outgrowth is observed for wild-type but not *traf6* null neurons with MAG-Fc treatment. (N=7) ***B,*** There is no difference in basal neurite outgrowth between wild-type and knockout control treated neurons.

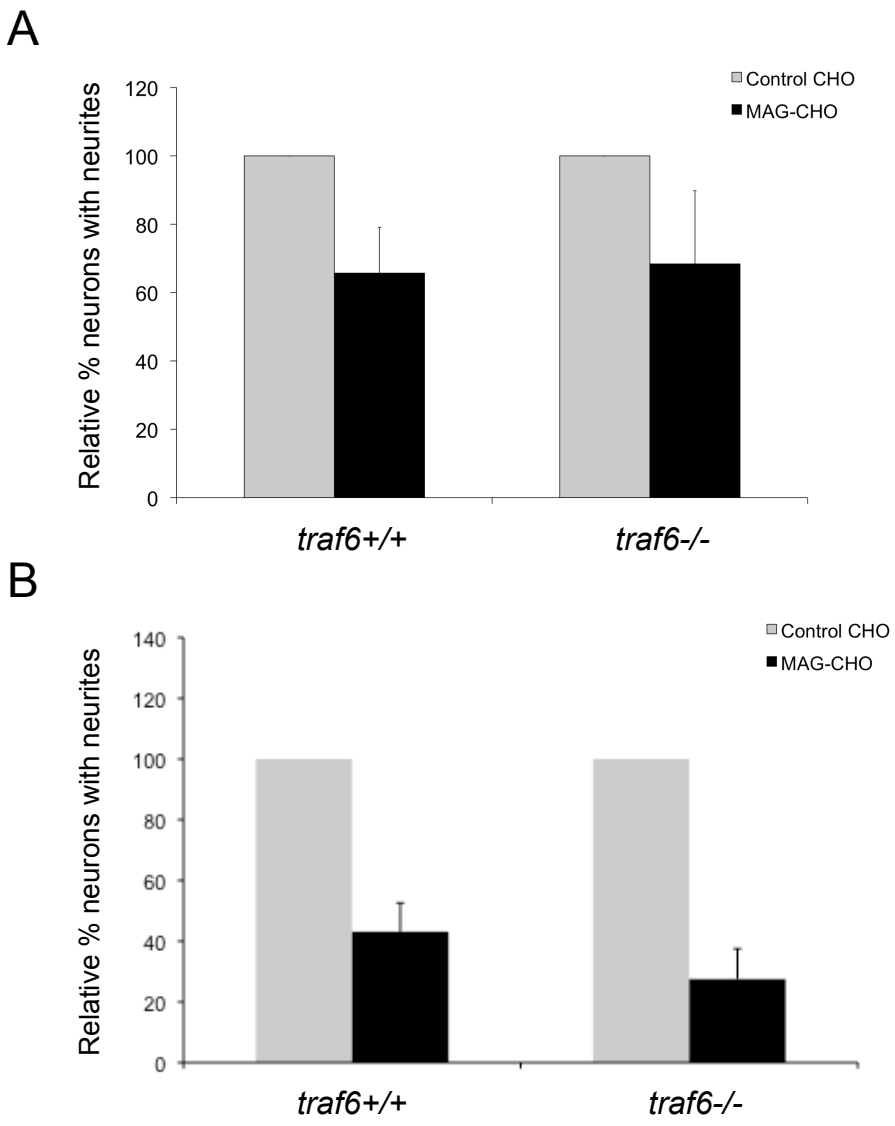

Figure 4.2. Neurite outgrowth inhibition by MAG-CHO cells does not require TRAF6. Wild-type or *traf6-/-* postnatal ***A,*** CGNs or ***B,*** cortical neurons were plated on control CHO or MAG-CHO cells for ~20 hrs, at which point the cells were fixed and stained for TuJ1. Neurite outgrowth was measured as the percent of neurons with neurites. Data is shown relative to outgrowth on control CHO cells. No differences were seen in outgrowth between *traf6+/+* or *traf6-/-* neurons on control CHO cells (not shown).

soluble MAG. Moreover, they indicate that soluble and membrane bound MAG may activate different signaling mechanisms to inhibit neurite outgrowth.

TRAF6 interacts with PAK

We hypothesized TRAF6 regulates Rho GTPAse signaling to mediate neurite outgrowth inhibition by soluble MAG. Rho GTPAse pull-down assays were conducted in 293 cells over-expressing TRAF6 in an effort to detect TRAF6 mediated changes in Rho or Rac activities. Although those studies proved inconclusive (data not shown), Rac pull-down assays with GST-PAK (p21 binding domain from PAK) revealed that TRAF6 itself was precipitated by GST-PAK. 293T cells expressing FLAG or FLAG-TRAF6 were subjected to pull-down assays with GST-PAK and the level of TRAF6 associated with PAK analyzed by Western blot analysis (Figure 4.3a). An interaction between TRAF6 and PAK was confirmed by co-immunoprecipitation of the two proteins from HEK293 cells (Figure 4.3b). Furthermore, we detected an interaction between TRAF6 and PAK endogenously by co-immunoprecipitation of the two proteins from cerebellar lysates (Figure 4.3c). The association of TRAF6 with PAK is particularly interesting since PAK is known to regulate several proteins that reorganize the actin-cytoskeleton, such as cofilin (Kreis and Barnier, 2009).

The interaction between TRAF6 and p75 is decreased with NgR overexpression

As an adaptor protein, TRAF6 is recruited to cell surface receptors upon engagement of the receptor with its ligand. An interaction between p75 and TRAF6

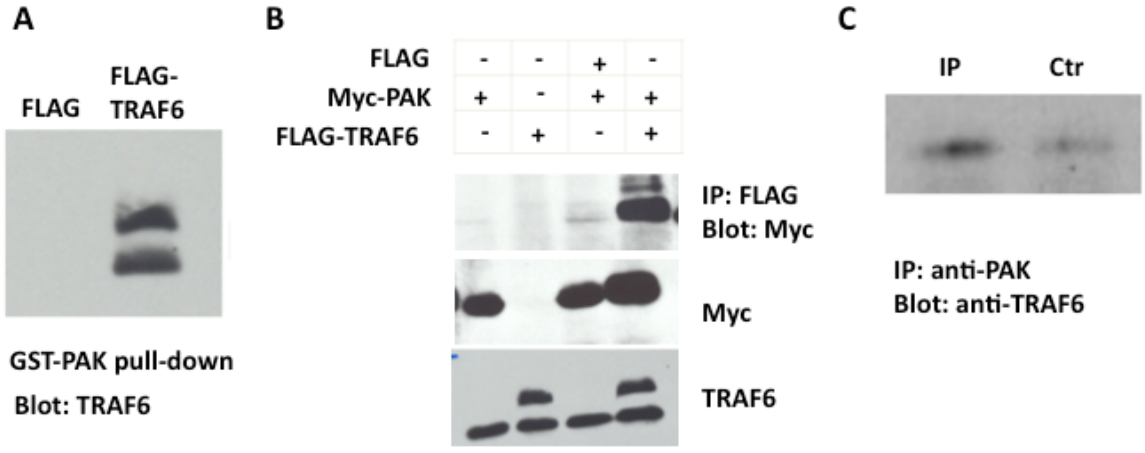

Figure 4.3. TRAF6 interacts with PAK. ***A,*** Pull-down assays with GST-PAK from TRAF6 expressing HEK293 cells followed by Western blot analysis with anti-TRAF6 reveals a possible interaction between TRAF6 and PAK. ***B,*** The interaction was confirmed by co-immunoprecipitation experiments in HEK293 cells co-expressing PAK and TRAF6. TRAF6 was immunoprecipitated with anti-FLAG and associating PAK detected with anti-Myc. ***C,*** Co-immunoprecipitation of PAK and TRAF6 from cerebellar lysates. PAK was immunoprecipitated with anti-PAK and associating TRAF6 analyzed by immunoblot analysis with anti-TRAF6. Ctr = IP with gamma globulin.

has been identified in 293T cells ectopically expressing both proteins. NGF treatment resulted in a significantly enhanced interaction between TRAF6 and p75 that was dose dependent (Khursigara et al., 1999). We co-immunoprecipitated p75 and TRAF6 in 293 cells over-expressing these proteins as well as NgR (Figure 4.4). Interestingly, co-expression of the NgR consistently (N=4) caused a decrease in the association of TRAF6 and p75 compared to cells expressing p75 and TRAF6 alone (Figure 4.4). One interpretation of this result would be that overexpression of NgR may mimic ligand activation and cause release of TRAF6 from the receptor, thereby allowing it to affect other interactors. Unfortunately, due to the limited availability of active MAG-Fc we were not able to investigate the effect of MAG on the association of TRAF6 and p75, a question that merits future investigation.

Discussion

In an effort to elucidate signaling downstream of NgR-p75/TROY-LINGO1, we considered TRAF6, a signaling protein for many TNFR family members. Our results indicate that TRAF6 is required for the inhibitory effects of soluble, but not membrane bound MAG on neurite outgrowth. Furthermore, we identified an interaction between TRAF6 and PAK, a known modulator of neurite outgrowth in mammalian cells. We also detected a reduction in the interaction between TRAF6 and p75 in the presence of NgR. These findings suggest that TRAF6 plays a role in the NgR-p75 mediated pathway leading to axon outgrowth inhibition, possibly through a functional interaction with PAK. Due to the limited availability of active, functional MAG-Fc we were not able to pursue these studies further and thus were

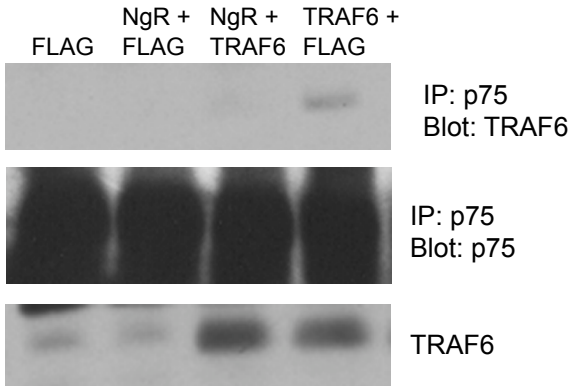

Figure 4.4. The interaction between TRAF6 and p75 is decreased in the presence of NgR. HEK293 cells stably expressing p75 were transfected with TRAF6 in the presence and absence of NgR. p75 was immunoprecipitated with anti-p75 and associating TRAF6 detected with anti-TRAF6 by Western blot analysis. Overexpression of NgR reduced the interaction between TRAF6 and p75.

not able to identify the mechanisms by which TRAF6 regulates neurite outgrowth downstream of MAG. However, we feel that our findings provide important insights as to how TRAF6 may be functioning.

We showed that TRAF6 is required for the inhibitory effect of soluble MAG. However neurons lacking TRAF6 were inhibited by membrane bound MAG similarly to wild-type neurons. We were initially surprised by these findings, but along with results presented in Chapter 2 and recent studies described in Chapter 1, these results are consistent with a role for NgR-p75 and TRAF6 in signaling by soluble MAG but not membrane bound MAG. Our results and those of others (Chivatakarn et al., 2007; Mehta et al., 2007) suggest that NgR and p75 do not play an essential role in inhibition of neurite outgrowth by MAG-CHO cells, at least in CGNs and cortical neurons. Many of the early studies demonstrating a role of p75 in neurite outgrowth inhibition and Rho activation by MAG (Wong et al., 2002; Yamashita et al., 2002; Yamashita and Tohyama, 2003) were conducted with soluble MAG. A role for TRAF6 downstream of soluble MAG is consistent with these findings and suggests that soluble MAG and membrane bound MAG inhibit neurite outgrowth by different mechanisms.

We also demonstrated that TRAF6 interacts with PAK, a serine/threonine kinase downstream of GTP bound Rac. Although the regulation of PAK by MAIs has not yet been analyzed, the activity of its upstream activator Rac and downstream target Cofilin are regulated by MAIs (Niederost et al., 2002; Hsieh et al., 2006). PAK is an important effector of actin reorganization in mammalian cells (Sells et al., 1997; Eby et al., 1998; Bokoch, 2003). In neurons, PAK has been linked to axon guidance and

the promotion of neurite outgrowth (Daniels et al., 1998; Hayashi et al., 2002; Robles et al., 2005; Kreis and Barnier, 2009). PAK regulates dendritic spine actin dynamics through its modulation of LIMK and Cofilin (Zhao et al., 2006). TRAF6 affects the activities of several kinases in various cellular contexts (Ha et al., 2009). Therefore, we postulate that TRAF6 regulates neurite outgrowth via its interaction with PAK, possibly binding to the kinase to suppress its activity. It will be interesting to evaluate the activity of PAK downstream of MAIs, particularly MAG, and analyze whether a functional link between PAK and TRAF6 exists in neurite outgrowth regulation.

As an adaptor protein for p75, TRAF6 is recruited to the receptor with neurotrophin stimulation (Khursigara et al., 1999). It is not yet known whether the interaction between TRAF6 and p75 is affected by other MAIs. We show that overexpression of NgR, a receptor for Nogo, MAG, and OMgp, disrupts binding of p75 and TRAF6. It is possible that NgR overexpression is similar to MAI binding, which enhances the interaction between NgR and p75. To further assess the role of MAI signaling on binding of TRAF6 to p75, future studies will need to be conducted in neuronal cells in response to inhibitory ligands. Release of TRAF6 from p75 may be an important step in its regulation of neurite outgrowth. Release of TRAF6 could free it to complex with other proteins in order to regulate outgrowth. I propose that upon its displacement, TRAF6 interacts with PAK to modulate its activity and inhibit outgrowth.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Damage to the CNS such as occurs in spinal cord injuries, results in permanent damage and disability due to the limited capacity of CNS axons to regenerate. CNS myelin has emerged as one of the main barriers to axon regeneration. Following injury, myelin is a major source of growth-inhibitory ligands that interact with the axon surface to regulate axonal capacity for growth. In the work described in the previous chapters, we investigated the molecular mechanisms involved in the inhibition of neurite outgrowth by one of these myelin inhibitors, Myelin-associated glycoprotein (MAG) (Figure 5.1).

Diverse axonal tracts respond uniquely to spinal cord injury and it is speculated that different strategies may be required to achieve regeneration of various neuronal populations. Surprisingly, very few studies to date have investigated the response of cortical neurons to MAIs and the mechanisms of cortical neuron outgrowth inhibition by these proteins. We found that cortical neurons were particularly vulnerable to neurite outgrowth inhibition by MAG. Interfering with the function of known MAG receptors did not reverse outgrowth of cortical neurons on membrane bound MAG, indicating that other receptors exist to mediate the substantial outgrowth inhibition observed. These results confirm and extend recent findings showing that the previously identified MAG receptors were not required for the inhibitory effects of MAG in some neuronal populations. Thus far the receptors for the classic MAIs,

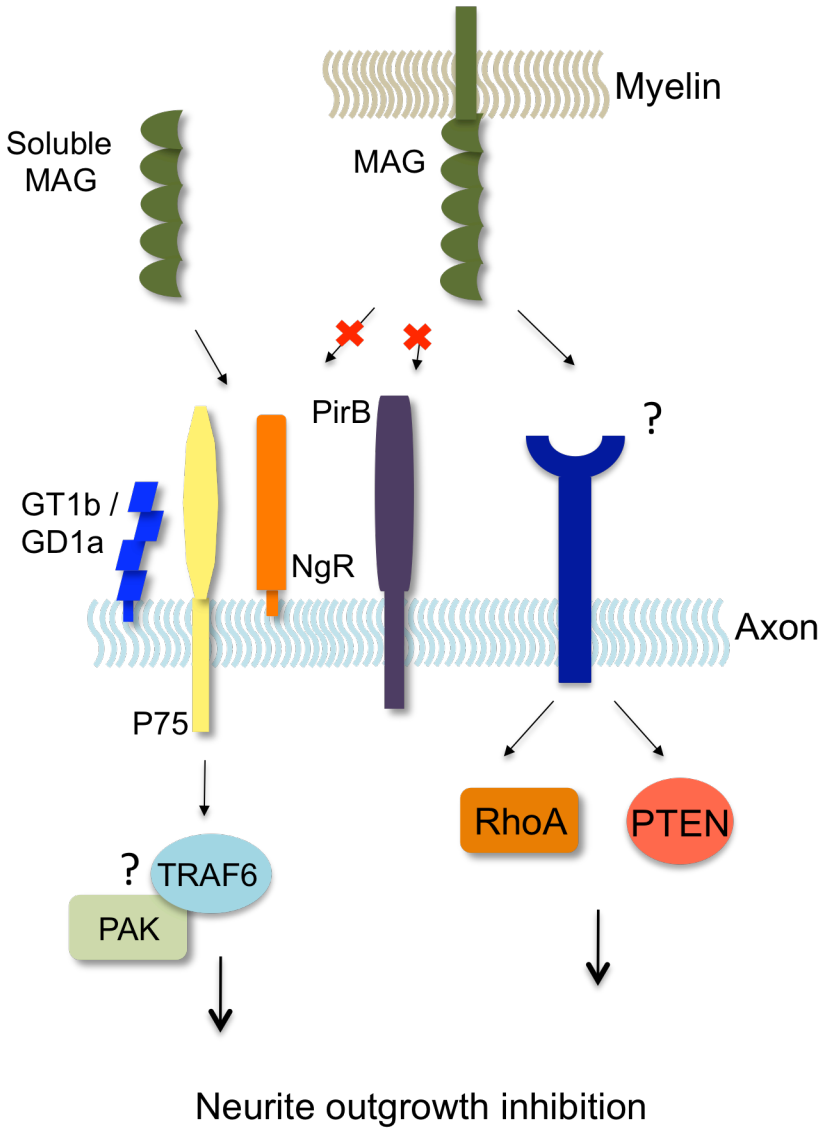

Figure 5.1. Schematic illustrating novel mechanisms of neurite outgrowth inhibition by MAG. The work described in the previous chapters is summarized here. We demonstrated that membrane bound MAG signals through Rho and PTEN to inhibit cortical neurite outgrowth. We found that membrane bound MAG does not require its known receptors in cortical neurite outgrowth inhibition and likely utilizes an unidentified receptor to mediate its inhibitory effect. TRAF6 is required for neurite outgrowth inhibition by soluble, but not membrane bound MAG. We identified an interaction between TRAF6 and PAK and hypothesize that this association may play a functional role in neurite outgrowth inhibition by soluble MAG.

such as NgR and PirB, have been identified in screens for Nogo-66 binding partners (Fournier et al., 2001; Atwal et al., 2008). Future studies should focus on identifying receptors for MAG, particularly the membrane bound form. One approach would be an expression screen with a CNS cDNA library for MAG binding partners. Another approach that would allow us to identify receptors for MAG with a function in neurite outgrowth inhibition would be a siRNA screen in cortical neurons. siRNA transfected neurons would be plated on MAG-CHO or control CHO cells and examined for reversal of inhibition by MAG. Our finding that p75 was not required for neurite outgrowth inhibition by MAG in cortical neurons or CGNs, but Rho/ROCK signaling was, suggests that other receptors exist to activate Rho downstream of MAG. To identify Rho activating receptors involved in MAG signaling I suggest a future study utilizing a siRNA library targeting known receptors combined with *in situ* visualization of Rho activation in neurons to detect lack of GTPase activation by MAG. Identifying additional receptors, unique to MAG, will benefit not only the regeneration field, but may shed some light on other MAG functions, since the axonal receptors important for maintenance of myelin stability, structuring of Nodes of Ranvier, and regulation of neurofilaments have yet to be identified.

It is likely that various inhibitors and receptors contribute to inhibition of corticospinal tract regeneration and our work highlights the importance of future efforts to characterize mechanisms of cortical axon outgrowth regulation. A recent study demonstrated that knockout of the three major MAIs, MAG, Nogo, and OMgp, did not result in significant regrowth of the corticospinal tract following injury (Lee et al., 2010), suggesting the presence of other inhibitory cues. To investigate additional inhibitory signals and neuronal receptors involved in preventing corticospinal tract

regrowth, gene expression profiling of the injury site may prove useful. Comparison of genes altered in corticospinal tract injury to uninjured spinal cord and injured peripheral nerve would allow us to identify CNS specific genes regulated by injury. Such a study will undoubtedly yield a vast number of genes with altered expression, not all involved in outgrowth inhibition. In order to identify potential inhibitors of outgrowth, I would focus on genes with known cytoskeletal functions. Another approach would be to limit the search to proteins known to regulate axonal outgrowth during development. During development, positive and negative extracellular cues ensure that axons reach their appropriate targets. Semaphorins and ephrins, known regulators of axonal pathfinding during development, were identified as inhibitors present on myelin capable of inhibiting outgrowth (Moreau-Fauvarque et al., 2003; Benson et al., 2005). Developmental cues may resurface following injury to prevent outgrowth. I would also focus on less acute phases following injury when the injury site becomes more stable, while neurons are still alive but after the peak of inflammation and glial cell death.

One of the main signaling pathways that mediates inhibition of neurite outgrowth by MAG, Nogo and OMgp is Rho GTPase and its effector ROCK. Our results show that Rho and ROCK play an important role in cortical neurite outgrowth inhibition by MAG. However, inhibition of Rho or ROCK did not fully reverse neurite outgrowth inhibition by MAG. This finding is important because it is the first observation that inhibition of Rho or ROCK does not fully reverse inhibition by MAG and prompted us to search for other signals in cortical neurons employed by MAG to mediate its effect on neurite outgrowth. Rho GTPAse has been one of the key targets *in vivo* to promote regeneration following injury (Lehmann et al., 1999; Dergham et al., 2002;

Fournier et al., 2003). It is important to identify and understand other potential pathways that can be targeted to promote axonal outgrowth *in vivo* in order to obtain optimal regeneration and functional recovery.

The work presented here identifies PTEN as a novel mediator of neurite outgrowth inhibition by membrane bound MAG. Our work also suggests that MAG (or other extracellular signals) may contribute to activating PTEN following injury. It will be interesting to assess whether PTEN or Akt activities are affected at injury sites. Future studies evaluating regeneration of the corticospinal tract following injury in PTEN conditional knockout mice will reveal whether PTEN also negatively regulates cortical axon outgrowth *in vivo*. In fact, one study was just published showing that adult cortical axons with *PTEN* deleted regrew to form synapses in the spinal cord following injury, supporting a role for PTEN in preventing regeneration of cortical neurons (Liu et al., 2010). The findings presented here also indicate that manipulation of Rho and PTEN function may have an additive effect on corticospinal tract regrowth *in vivo*.

The finding that MAG may contribute to the regulation of PTEN and its role as a negative regulator of axon outgrowth raises the question of whether there are other extrinsic cues that regulate PTEN signaling after injury. Other MAIs or other extracellular signals such as those produced by the glial scar may regulate PTEN to exert their growth inhibitory effect. In fact, the levels of regeneration attained *in vivo* with PTEN conditional knockout mice, indicates that PTEN may be a major convergence point for multiple inhibitory signals since the axonal outgrowth observed was so significantly above that seen with deletions of various inhibitors or their receptors (Yiu and He, 2006; Park et al., 2008; Liu et al., 2010). Chadborn et

al. (2006) demonstrated that Sema3A, a known axon guidance molecule during development, engaged PTEN to induce growth cone collapse. Identification of extrinsic cues that like MAG and Sema3A regulate neurite outgrowth through PTEN could also enhance our understanding of PTEN function during neuronal development.

Although our work indicates that PTEN is regulated by MAG, we have not directly shown this. In fact, other PIP3 phosphatases, such as SHIP1 and SHIP2, can also modulate levels of PIP3 in the cell (Leslie 2002). Future studies investigating effects of MAG on PTEN activity or localization are required. PTEN phosphatase assays following immunoprecipitation of PTEN from cortical neurons exposed to MAG can be conducted to evaluate the effect of MAG on PTEN activity. We can investigate the requirement for PTEN's phosphatase function by conducting neurite outgrowth assays with cortical neurons over-expressing a PTEN mutant lacking its lipid phosphatase function. One of the ways by which PTEN is activated in the cell is by recruitment to the plasma membrane (Leslie et al., 2008). Sema3A induced membrane localization of PTEN in growth cone collapse (Chadborn et al., 2006). The localization of PTEN in response to MAG can be analyzed by comparing the localization of GFP tagged PTEN in neurons encountering MAG-CHO cells or control CHO cells. In an effort to investigate changes in PIP3 levels at the plasma membrane in response to MAG we imaged neurons expressing a GFP tagged pleckstrin homology domain of Akt (PH-GFP) on MAG-CHO and control CHO cells by confocal microscopy. However, we did not observe any reproducible differences in PH-GFP membrane localization between neurons on control and MAG expressing cells. PTEN regulation is still not well understood, but in addition to membrane

recruitment, PTEN is also regulated by reactive oxygen species, phosphorylation, and ubiquitination (See Chapter 3 Discussion) (Drinjakovic et al., 2010). Future studies will need to be carried out to elucidate how PTEN is affected by MAG.

Our results suggest that MAG can modulate Akt signaling, likely by employing PTEN to reduce Akt activity. To confirm that changes in Akt phosphorylation by MAG require PTEN, we can evaluate whether Akt phosphorylation is decreased in response to MAG in PTEN knockout neurons. Although PTEN's main function is the reduction of PI3K/Akt activity, other functions are emerging for PTEN as both a lipid and protein phosphatase (Knobbe et al., 2002; Keniry and Parsons, 2008); hence, PTEN may have additional targets that contribute to inhibition of neurite outgrowth by MAG.

It will also be interesting to characterize Akt downstream pathways affected by MAG. Several pathways have been implicated in Akt mediated effects on neurite outgrowth including other kinases, cytoskeletal proteins, and transcription factors. GSK3β plays a role in modulating neurite outgrowth downstream of PI3K/Akt (Yoshimura et al., 2006). The Akt pathway can also influence the cytoskeleton by direct phosphorylation of several cytoskeletal proteins such as tau, Girdin and peripherin or by influencing Rac1, a major cytoskeletal regulator (Read and Gorman, 2009). Transcription factors downstream of Akt implicated in the effects of Akt on neurite outgrowth include CREB and NF-kB (Du and Montminy, 1998; Ozes et al., 1999; Jessen et al., 2001; Gutierrez et al., 2005). Regulation of mTOR signaling may also be involved downstream of Akt. In their characterization of PTEN as a negative regulator of axon outgrowth in optic nerve injury, Park et al. (2008) proposed mTOR dependent and independent mechanism of regeneration. Furthermore, although the

role of Akt in neurite outgrowth has been studied in various neuron populations, little is known about the role of Akt in cortical neuron development or regeneration of cortical pathways following injury, both important areas for future study.

In addition to evaluating mechanisms of neurite outgrowth inhibition by membrane bound MAG, we were also interested in investigating signaling by soluble MAG. The functional significance of soluble MAG *in vivo* is unknown. However, disease related changes in soluble MAG levels have been demonstrated. Increased soluble MAG was detected in brains of individuals with multiple sclerosis for instance (Moller et al., 1987). It is possible that MAG release is stimulated at the injury site. Matrix metalloproteinases (MMPs) have been shown to promote release of the extracellular domain of MAG from MAG expressing CHO cells (Milward et al., 2008). Furthermore, several MMPs are upregulated at the injury site (Rosenberg, 1995). Future studies on soluble MAG should focus on investigating its relevance in neurite outgrowth inhibition following injury. We have obtained frozen spinal cords from rats subjected to spinal cord crush or transection from Mark Tuszynski (UCSD). We will conduct Western blot analysis of these injured spinal cords to detect the presence of soluble and membrane bound MAG as well as compare differences between injured and uninjured spinal cords. A comparison of soluble MAG levels in injured CNS tissue versus PNS tissue may also provide insight regarding the growth inhibitory role of soluble MAG. I postulate that soluble MAG and membrane bound MAG both contribute to inhibition of axonal growth following injury.

Work presented here indicates that soluble and membrane bound MAG utilize differential mechanisms to exert their effect on outgrowth inhibition. One possible explanation for the differences observed between soluble and membrane bound

MAG is that the soluble form mediates a more acute effect at the growth cone, such as growth cone collapse, while signaling by membrane bound MAG provides a more continuous inhibitory signal preventing forward movement of the growth cone. Schwab (2009) demonstrated that soluble MAG can be internalized by the axon and retrogradely transported to the cell body. Perhaps soluble MAG binding results in acute collapse followed by internalization of the ligand, which may further inhibit neurite outgrowth or mediate other functions. It is possible that p75 is retrogradely trafficked in response to soluble MAIs. Proteolytic cleavage of p75 and release of its intracellular domain has been suggested as an important step in neurite outgrowth inhibition by soluble MAG (Domeniconi et al., 2005). There is also evidence for retrograde trafficking of the intracellular domain of p75 in response to neurotrophins (Lalli and Schiavo, 2002; Deinhardt et al., 2006), with a possible translocation of p75 to the nucleus (Parkhurst et al., 2010). I hypothesize that MAG retrograde trafficking, possibly with p75, may be involved in regulating genes involved in mediating and maintaining neurite outgrowth inhibition. In addition to activating signaling cascades that ultimately regulate the cytoskeleton, it is likely that myelin proteins also induce changes in gene expression to exert more long-lasting inhibition of outgrowth. The observation of MAG retrograde trafficking by MAG (Schwab 2009) backs future studies investigating changes in neuronal gene expression by MAG.

We found that the absence of TRAF6 in CGNs resulted in a neuronal phenotype that was resistant to neurite outgrowth inhibition by soluble MAG. It will be interesting to evaluate whether TRAF6 is also required for neurite outgrowth inhibition by Nogo or OMgp. A role for TRAF6 downstream of other MAIs would establish TRAF6 as an important mediator of myelin signaling. To elucidate the physiological importance of

TRAF6 in axon regeneration an *in vivo* mouse injury model can be evaluated for the ability of *traf6* null axons to regenerate. I expect that if TRAF6 does indeed play a role in axon outgrowth inhibition in the CNS, *traf6* null animals will display enhanced regeneration compared to wild-type.

I postulate that TRAF6 is activated by MAIs, which can be evaluated by increased ubiquitination of the protein, to regulate downstream signaling events leading to inhibition of outgrowth. We identified an interaction between TRAF6 and PAK and propose that this interaction plays a role in regulation of neurite outgrowth by MAG. Future studies should be conducted to evaluate the effect of MAG on PAK activity as well as evaluating the requirement for PAK in MAG outgrowth inhibition. To investigate a role for TRAF6 in PAK regulation downstream of MAIs, any effect of MAG on PAK activity should be assessed in *traf6* null neurons. I expect that if TRAF6 regulates PAK function or is part of a complex that does, its interaction with PAK may be inducible by MAG. I anticipate that TRAF6 acts to modulate PAK, likely by ubiquitinating the kinase to inhibit its activity and thus negatively regulate neurite outgrowth. We may find that TRAF6 does not interact with PAK to regulate its activity, but instead binds to PAK and modulates the ability of PAK to regulate its downstream targets. Alternatively, PAK may modulate the ability of TRAF6 to activate its downstream targets, such as NFkB, JNK, or Src (Ha et al., 2009).

Perhaps even more importantly, the interaction between TRAF6 and PAK may have significant implications for other cellular processes regulated by these proteins. TRAF6 is known to regulate several kinases in a variety of contexts. TRAF6 may function to ubiquitinate PAK and modulate its function in processes such as cell motility, survival and cell cycle regulation. If so, this interaction may be important

during development and in various cancers and neuronal disorders in which PAK regulation is implicated (Molli et al., 2009). Investigating a role for PAK in known TRAF6 functions could also reveal novel signaling pathways in TNFR family signaling.

Lastly, the findings described in this dissertation may have implications for other processes mediated by MAG including emerging novel functions for myelin proteins. During development of the nervous system, establishment of appropriate connections involves degeneration and pruning of axons (Carter, 2010). Recent findings suggest that this process also occurs in the mature nervous system in order to eliminate aberrant sprouting and maintain axons on a narrow path (Park et al., 2010a). Myelin is a negative regulator of irregular sprouting through its role in triggering degeneration of neuronal processes (Park et al., 2010a). Moreover, the pathways involved in degeneration of axons by myelin were distinct from those involved in inhibition of outgrowth. It will be exciting to investigate whether MAIs, including MAG, also function normally to keep axons on a straight path. A role for OMgp in preventing axonal sprouting at Nodes of Ranvier has been shown (Huang et al., 2005). Whether other MAIs function in this capacity will be an interesting area for future study. The end of plasticity in the cortex occurs at a time that corresponds with completion of myelination, suggesting that myelin may contribute to limiting refinement of neuronal circuitry. Interestingly, one study found that mice lacking NgR exhibited an extended period of plasticity in the visual cortex, suggesting that MAIs and their associated signaling pathways may also play a role in this process (McGee et al., 2005).

Gaining a more complete understanding of the different mechanisms employed by myelin-associated inhibitors in specific neuron types could help in developing therapeutics that will be effective for specific fiber tracts and brain regions following injury. Our results with cortical neurons suggest that in addition to Rho/ROCK, PTEN is another therapeutic target that may enhance regeneration of these neurons. Furthermore, the identification of PTEN and TRAF6 as downstream effectors of MAG in preventing neurite outgrowth may have important implications for other processes mediated by MAG.

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