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Identification of the Receptor Complex Required for Inhibition of Axonal Regeneration by Myelin-Associated Glycoprotein

by

Marco Domeniconi

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2003

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Abstract

Identification of the Receptor Complex Required for Inhibition of Axonal Regeneration by Myelin-Associated Glycoprotein.

By

Marco Domeniconi

Adviser: Marie T. Filbin, Ph.D.

The lack of axonal growth after injury in the adult central nervous system (CNS) is due to the formation of a glial scar, the absence of neurotrophic factors, and the presence of growth-inhibitory molecules associated with myelin, rather than an intrinsic inability of neurons to regenerate. Three inhibitors have been identified in myelin: Myelin-Associated Glycoprotein (MAG), Nogo-A, and Oligodendrocyte-Myelin glycoprotein (OMgp). In this study we show that MAG inhibits regeneration by high affinity interaction (K_D 8 nM) with the glycosylphosphatidylinositol (GPI)-linked Nogo66 receptor (NgR) and activation of a p75 neurotrophin receptor (p75NTR)-mediated signaling

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pathway. Treatment of neurons with PI-PLC to remove GPI-linked proteins results in the loss of both MAG binding and growth inhibition. NgR-AP binds MAG-expressing CHO cells and Nogo66 competes with MAG for binding to NgR-expressing cells. MAG precipitates NgR from cerebellar neurons (CN), dorsal root ganglia (DRG), and NgR-expressing CHO cells. Importantly, MAG-induced inhibition of neurite outgrowth in CN and DRG neurons is blocked by the addition of an anti-NgR antibody or soluble NgR-AP, and by expression of a dominant negative NgR construct.

As NgR is a GPI-linked protein it cannot itself transduce the signal for inhibition. Thus, it must be part of a larger receptor complex containing a transmembrane protein. We demonstrate that MAG-Fc, Nogo66-AP and NgR-AP precipitate p75NTR from cerebellar granule neurons (CN). Neurons that are sensitive to the activity of MAG express both NgR and p75NTR. Notably, MAG does not inhibit neurite outgrowth from the DRG subpopulation that binds isolectin-B4. This subpopulation expresses NgR but not p75NTR. Further, we find that p75NTR is required for the inhibition of axonal regeneration by both MAG and myelin. DRG from p75NTRexonIII -/- mice, which lack the p75NTR ligand binding domain, are not sensitive to either MAG or myelin inhibitory activity. Finally, neurite outgrowth inhibition by MAG, Nogo66 and myelin is abolished by expression of a truncated p75NTR construct lacking the intracellular domain. Because MAG and Nogo66 use a

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common receptor complex, these results point to a redundancy in myelin inhibitors of regeneration and suggest possible targets for the development of therapies to encourage regeneration after spinal cord and CNS injury.

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CHAPTER 1 - INTRODUCTION

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The central nervous system (CNS) of adult mammals recovers very poorly from injury. Mature central neurons, such as those in the spinal cord, respond to injury with an initial period of growth but their growth cones soon collapse and their axons fail to regenerate to any significant degree (Ramon y Cayal 1928; Johnson 1993; Schwab and Bartholdi 1996). There is no a priori reason for this failure, since lower vertebrates can regenerate a severed spinal cord (Yin and Selzer 1983). Even in mammals, the inability to regrow axonal tracts is limited to the mature central nervous system as peripheral nerves can regenerate in adult animals (Fawcett and Keynes 1990) and the immature CNS, as the one of neonatal rats, easily regenerates after injury (Bates and Stelzner 1993). Aguayo and his colleagues used peripheral nerve segments to make bridges between the medulla and spinal cord to demonstrate that central neurons could grow in a peripheral environment (David and Aguayo 1981). When the optic nerve of adult rats was replaced with segments of peripheral nerve, the axons from retinal ganglion cells could grow and form new functional synapses in the superior colliculi (David and Aguayo 1981; Bray et al. 1987). Others have shown that isolated CNS neurons can extend long processes *in vitro* (Doherty and Walsh 1989; Bixby and Harris 1991). These observations led to the hypothesis that the failure of CNS neurons to regenerate is not due to an intrinsic inability to grow new axons, but rather to their growth status and to the lack of a permissive growth

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environment (Johnson 1993; Keynes and Cook 1995). For example, dorsal root ganglia (DRG) extend branches in both the central and peripheral nervous systems. But whereas the DRG peripheral branches will regenerate after injury the central branches will not. However, DRG central branches will regenerate after transection if a conditioning lesion is first produced in their peripheral branches (Figure 1.1) (Neumann and Woolf 1999). Embryonic neurons transplanted into adult spinal cord will extend long axons (Li and Raisman 1993). Adult CNS neurons have been shown to regenerate *in vivo* either by modification of the local environment with embryonic tissue transplants (Bregman 1998) or by mimicking a conditioning lesion through modulation of the endogenous cAMP levels (Qiu et al. 2002). Overall, inhibition of neurite outgrowth can be accredited to a number of factors: lack

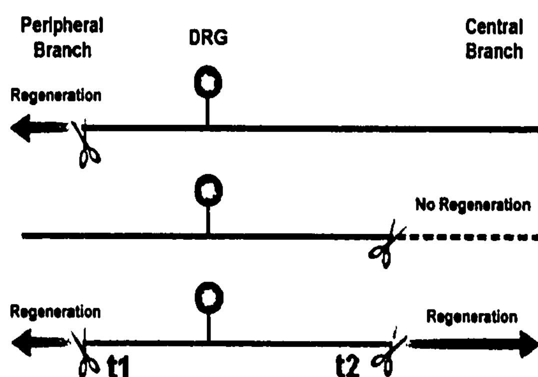

Figure 1.1 – Schematic representation of a conditioning lesion.
The central axons of DRG neurons enter the spinal cord and run up the dorsal column to the brainstem. Though the peripheral axons of these neurons can regenerate after an injury, the central axons do not regenerate when the spinal cord is injured. However, dorsal column fibers will regenerate if a 'conditioning' peripheral nerve injury (t1) is produced prior to the spinal cord lesion (t2).

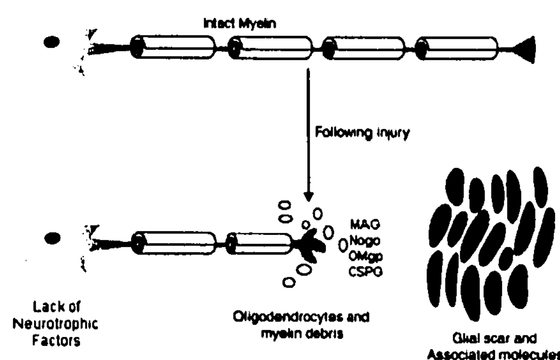

Figure 1.2 – CNS environment after injury.
After spinal cord injury neurons lose neurotrophic support and many undergo apoptosis. The severed axons that attempt to regenerate encounter inhibitory influences in their environment. At the lesion site, myelin debris and oligodendrocytes express MAG, Nogo and OMgp. At the glial scar, growth cones meet a physical barrier as well as molecules such as chondroitin sulfate proteoglycans (CSPG) and tenascin.

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of neurotrophic factors, formation of the glial scar, and molecular components of the myelin sheath (Figure 1.2) (McKerracher et al. 1994; Mukhopadhyay et al. 1994; Bregman et al. 1995).

Myelin-Associated Glycoprotein

In 1994, we (Mukhopadhyay et al. 1994), and others (McKerracher et al. 1994) identified Myelin-Associated Glycoprotein (MAG) as a major inhibitor of axonal growth *in vitro*. MAG expression is limited to myelin forming cells, oligodendrocytes in the CNS and Schwann cells in the PNS. In the CNS MAG comprises 1% of the total myelin protein and it is localized solely to the periaxonal membrane in the internodal segments of the myelin sheath. In the PNS MAG is expressed in paranodal, Schmidt-Lanterman incisures, and outer mesaxon segments though it only represents 0.1% of total PNS myelin protein (Trapp et al. 1989; Trapp 1990). MAG (Figure 1.3) is a member of the sialic acid binding Ig-like lectin (siglec) family of adhesion molecules (Crocker et al. 1998). It contains five Ig-like extracellular domains, a single transmembrane

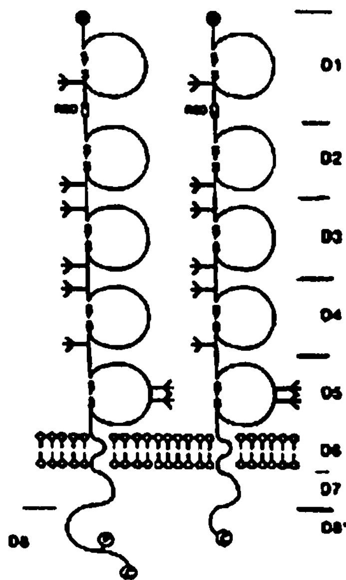

Figure 1.3 – A diagram of the structure of MAG.
MAG consists of five Ig-like extracellular domains (D1-D5), a transmembrane domain and a short cytoplasmic tail.

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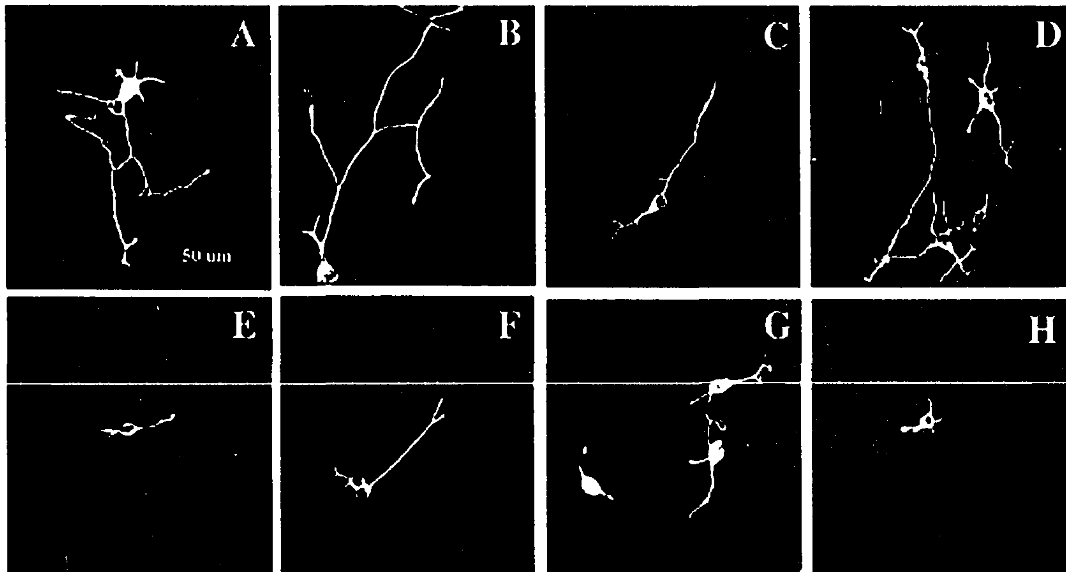

Figure 1.4 – MAG inhibits neurite outgrowth from a variety of neurons.
Retinal ganglia neurons (RG), hippocampal neurons (HN), spinal motor neurons (SMN) and superior cervical ganglia neurons (SCG) were plated on monolayers of control CHO cells (A-D) or MAG-expressing CHO cells (E-H). After 24 hr., the neurons were fixed and immunostained with anti-GAP43 antibody to visualize neurite outgrowth.

domain, and a short cytoplasmic tail (Lai et al. 1987; Salzer et al. 1987; Salzer et al. 1990). Because of its molecular structure and localization, MAG is hypothesized to play a role in the stability of the axon-glia interface (Quarles 1983; Salzer et al. 1990; Trapp 1990; Filbin 1995). This idea is supported by studies on older MAG-knockout mice, which express altered periaxonal architecture and axonal loss (Li et al. 1994; Lassmann et al. 1997). MAG inhibits neurite outgrowth *in vitro*. Primary CNS neurons cultured on monolayers of MAG-expressing cells display a drastic reduction in axonal growth as compared to neurons plated on control cells (Figure 1.4) (Mukhopadhyay et al. 1994). NG108 cells (a neuronal cell line) fail to extend neurites when plated on slides coated with myelin or MAG (McKerracher et al.

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1994). Further, a soluble, proteolytic fragment of MAG, consisting of the entire extracellular domain and found *in vivo* (Sato et al. 1984; Yim and Quarles 1992; Moller 1996), was shown to inhibit neurite outgrowth *in vitro* (Tang et al. 1997b).

Wallerian degeneration (Waller 1850; Ramon y Cayal 1928) applies to all nerve lesions disrupting the integrity of the axoplasm. It refers to a set sequence of molecular and cellular events that result in the disintegration of the axolemma and axoplasma (Stoll et al. 1989). In the PNS, Schwann cells de-differentiate, down-regulate myelin proteins, sequester myelin debris and divide to form guiding tubes for the regenerating fibers (Stoll et al. 1989). Macrophage recruitment also leads to the rapid removal of myelin debris and the creation of a more favorable growth environment. During Wallerian degeneration of the CNS axons disintegrate with a similar time course as in the PNS (George and Griffin 1994), but removal of myelin debris is delayed by months. Macrophages are virtually excluded from the distal stump (George and Griffin 1994) and microglia only exert minor phagocytic activity (Schroeter et al. 1997).

In the mutant C57BL/6WLD/OLA (Wld) mice, Wallerian degeneration following peripheral nerve injury is very slow and axonal regeneration is impaired (Brown et al. 1991). Martini and colleagues crossbred Wld mice with MAG-knockout mice and studied axonal regrowth *in vivo*. Following peripheral nerve injury, analysis of MAG-deficient/Wld mice revealed that the number of myelin sheets associated with regrowing axons doubled as compared to Wld

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mice (Schafer et al. 1996). These results suggest that MAG-deficient myelin is less inhibitory than the wild type myelin. Further support for MAG's role in the inhibition of regeneration *in vivo* comes from our studies on facial nerve regeneration. In response to injury, MAG is down-regulated by Schwann cells, making them permissive for growth (Fawcett and Keynes 1990), while the p75 neurotrophin receptor ($p75^{NTR}$) is up-regulated (Piehl et al. 1998). We created transgenic mice expressing exogenous MAG under the control of the $p75^{NTR}$ promoter, thus up-regulating MAG in Schwann cells in response to injury. In these mice recovery of whisker control following transection of the facial nerve was delayed by five days as compared to wild type mice. This retarded recovery indicates that MAG is acting *in vivo* to delay axonal regeneration (Filbin et al., Unpublished data).

Nogo

Oligodendrocytes were initially recognized as a poor substrate for axonal growth *in vitro* and, subsequently, myelin was proposed as an inhibitor of regeneration (Berry 1982). Schwab's team studied CNS myelin's inhibitory activity and found it to be membrane-bound and associated with the protein fraction of CNS myelin (Caroni and Schwab 1988b). The inhibitory components of myelin could be recovered after separation in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as two minor myelin-associated proteins with relative molecular masses (M_r) of 35 kDa and 250 kDa (now called neurite growth inhibitors NI-35 and NI-250, respectively)

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(Caroni and Schwab 1988b). When the same group generated a monoclonal antibody, IN-1, against the two proteins, they showed that the addition of IN-1 to cultures reduced the inhibitory activity of myelin (Caroni and Schwab 1988a). Notably, IN-1 injected into the injured spinal cord resulted in regeneration of 5% of the axons and functional improvement in injured adult rats (Schnell and Schwab 1990; 1993; Bregman et al. 1995).

Using peptide sequences derived from the bovine homologue of NI-250 (Spillmann et al. 1998), three groups independently identified the IN-1 antigen(s) as products of the *Nogo* gene (Chen et al. 2000; Goldberg and Barres 2000; GrandPre et al. 2000; Prinjha et al. 2000). Three Nogo isoforms, Nogo-A, -B, and –C, are encoded from a single gene by alternative splicing and/or promoter usage (Figure 1.5). In human and rat fetal tissue, Nogo-A mRNA is strongly expressed in the ventral spinal cord, DRG and autonomic ganglia. The same expression pattern was observed in the adult spinal cord

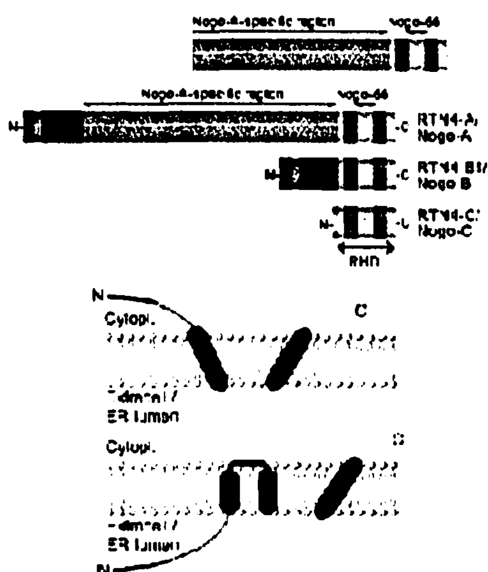

Figure 1.5 – A schematic diagram of the structure of Nogo proteins and the membrane topology of Nogo-A.
The three transcripts from the mammalian *nogo/rtn4* gene. The common C-terminus encodes the reticulon-homology domain (RHD), whereas the N-termini are specific for each variant and have no obvious sequence homologies. The 66-amino-acid region between the two putative transmembrane (TM) domains (called Nogo-66) and a stretch within the Nogo-A specific N-terminal region have been found to inhibit neurite outgrowth.
The two proposed membrane topologies for the C-terminal hydrophobic regions of Nogo proteins. The lengths of the hydrophobic stretches (35 amino acids) could allow them to span the membrane once or twice.

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and ganglia. High levels of Nogo-A message were present in oligodendrocytes, motor neurons and sensory ganglia neurons, but not in astrocytes or Schwann cells. Minor expression was also observed in developing muscle tissue (Josephson et al. 2001). Western blot analysis of adult tissue revealed Nogo-A presence in brain and spinal cord, and low levels in testis and heart (Huber et al. 2002). After spinal cord injury, Nogo-A is upregulated to a moderate degree (Wang et al. 2002c), whereas traumatic lesions to the cortex do not change Nogo-A expression (Huber et al. 2002). Nogo-B and Nogo-C have a wider expression profile in neurons, skeletal muscle and various peripheral tissues. The *nogo* products show a high degree of homology with the reticulon protein family (van de Velde et al. 1994) and contain a dilysine endoplasmic reticulum (ER) retention sequence. Nogo proteins are mostly localized to the ER with a small percentage present at the plasma membrane (GrandPre et al. 2000). Throughout the adult CNS, Nogo-A is detected by confocal and electronimmuno-microscopy on oligodendrocyte processes in the periaxonal and outermost myelin membranes (Huber et al. 2002; Wang et al. 2002c). It has been suggested that Nogo-A may have two different membrane topologies, one in which both the N- and C-terminus are oriented cytoplasmically, and a second in which both termini are oriented extracellularly (Figure 1.5) (Oertle et al. 2002).

All three Nogo isoforms contain a 66-amino acid extracellular region (Nogo-66) which displays neuron-specific growth inhibitory activity *in vitro* (GrandPre et al. 2000). An additional inhibitory domain, Amino-Nogo, has

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been localized to a 195-amino acid stretch near the N-terminus of Nogo-A (Fournier et al. 2001; Prinjha et al. 2002). Amino-Nogo inhibits neurite outgrowth as well as affecting 3T3 fibroblast spreading (Prinjha et al. 2000). Recently, Schwab's team generated a transgenic mouse expressing Nogo-A under the control of the Schwann cell-specific P0 promoter. P0 is strongly induced 7 days post peripheral nerve injury at the onset of re-myelination (Gupta et al. 1988). The transgenic mice displayed an impaired recovery from sciatic nerve crush injury suggesting a role for Nogo-A in the inhibition of neurite outgrowth *in vivo* (Pot et al. 2002).

Several groups using different strategies have created Nogo knockout mice but, at the present time, there are neither published results nor a consensus on the effects of the Nogo deletion. Both Tessier-Levigne's team and Schwab's teams created Nogo mutant mice though both groups preliminarily report a lack of a distinctive phenotype (Personal Communications, 2003). Meanwhile, Strittmatter's group used a retroviral gene trap method to generate Nogo-A/B$^{-/-}$ Loss-Of-Function (LOF) mice, which are viable and exhibit normal locomotion. The LOF mice produce myelin of normal thickness but the sheaths contain frequent gaps. Although the studies on older LOF mice are not concluded, the overall phenotype is described as similar to that of the MAG null mice. Inhibition of neurite outgrowth *in vitro* by Nogo-A$^{-/-}$ myelin is reduced and the residual effects are completely abolished by anti-MAG antibodies (Strittmatter 2003).

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Oligodendrocyte-Myelin Glycoprotein

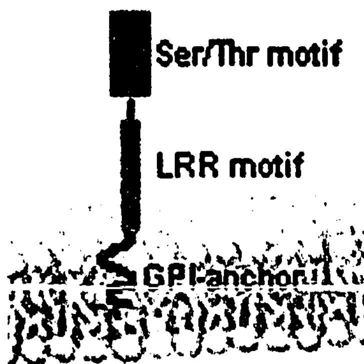

Figure 1.6 – A schematic diagram of the structure of OMgp.
OMgp consists of a series of tandem leucine-rich repeats (LRR), a serine/threonine-rich region (Ser/Thr) and a GPI anchorage site.

The latest addition to the group of inhibitors is Oligodendrocyte-Myelin Glycoprotein (OMgp). OMgp, originally identified in the late 1980s during studies of myelination, is a membrane glycoprotein anchored to the cell surface through a glycosylphosphatidylinositol (GPI) lipid intermediate (Figure 1.6) (Mikol and Stefansson 1988). OMgp represents only a minor percentage of total myelin protein in both CNS and PNS, where its expression during development parallels myelination. Its activity was thought to be limited to the mediation of axon-glia interactions and it was not until recently that OMgp properties as an inhibitor of axonal growth were discovered (Kottis et al. 2002; Wang et al. 2002b). Surprisingly, OMgp inhibits neurite outgrowth by binding the Nogo-66 receptor (NgR) (Wang et al. 2002b).

The ability of MAG to stabilize the axon-glia interface and to inhibit neurite outgrowth suggests the presence of a specific neuronal receptor or receptor complex. Similar to the other siglec molecules, MAG binds sialic acid through a conserved arginine residue (Collins et al. 1997), which was mapped to ARG118 (Tang et al. 1997a). Although MAG can bind isolated gangliosides

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(Yang et al. 1996), it was demonstrated that MAG binding to neurons is both sialic acid dependent and trypsin sensitive (Kelm et al. 1994; DeBellard et al. 1996) indicating that the binding partner is a sialo-glycoprotein rather than a sialo-glycolipid. Both neuronal desialylation and mutation of the ARG118 residue to Alanine (R118A) in a soluble MAG chimera (MAG-Fc) result in the loss of neuronal binding and the loss of inhibition of neurite outgrowth. In contrast, when expressed by transfected CHO or Schwann cells, the MAG(R118A) construct is still a strong inhibitor of axonal growth (Tang et al. 1997a). These results suggest that MAG contains two discrete neuron binding sites, one for sialic acid binding and one for inhibition, and that the role of the sialic binding is to potentiate a low affinity interaction.

Several teams have reported precipitating neuronal sialo-glycoproteins that are potential receptors for MAG (De Bellard and Filbin 1999; Strenge et al. 1999) but the functional MAG receptor transducing the inhibitory signal to neurons has yet to be identified. Some groups have concluded that sialylated glycans are the mediators of MAG inhibition of neurite outgrowth and that MAG inhibition is a result of carbohydrate recognition (Vinson et al. 2001; Vyas et al. 2002). It is true that MAG specifically binds gangliosides GT1b and GD1a, which are both expressed on the surface of MAG-responsive neurons. It is also true that multivalent IgM antibody cross-linking of cell surface GT1b mimics the effect of MAG, in that neurite outgrowth is inhibited through activation of Rho kinase. However, these studies do not address the sialidase-independent nature of MAG's inhibitory activity. Although they

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indicate a difference in mean neurite length when comparing MAG-Fc and MAG(R118A)-Fc at low concentrations, the growth inhibition by MAG(R118A)-Fc increases in a dose dependant manner. While this fact could be explained by residual sialic acid binding, it could just as easily indicate the presence of a discrete site responsible for inhibition of neurite outgrowth. Also, the addition of IgM Fab fragments generated from either anti-GT1b or anti-GD1a antibodies to outgrowth assays mimics MAG inhibitory activity indicating that this interaction is not specific to a MAG pathway (Filbin, unpublished results). These data suggest a model in which the recognition of sialylated glycans is not essential for inhibition of neurite outgrowth by MAG and where arginine 118 on MAG makes a key contact with sialic acid to enhance a low affinity interaction. A possible interpretation is that MAG has two discrete functions: first, to hinder aberrant sprouting and generate structural axon-glia stability via interaction with gangliosides, and, second, to strongly inhibit outgrowth via interaction with a functional high-affinity receptor.

Other studies on MAG binding partners indicate that MAG directly associates with microtubule-associated protein 1b (MAP1b) (Franzen et al. 2001), and that clustering of gangliosides can activate a p75NTR cascade resulting in Rho-A activation and subsequent inhibition of neurite outgrowth (Yamashita et al. 2002).

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A receptor for Nogo-66 (NgR) has been identified using an alkaline phosphatase (AP) fusion protein to screen a mouse brain cDNA library transfected into COS-7 cells (Fournier et al. 2001). NgR is a 473 amino acid protein containing a translocation signal sequence, eight leucine-rich repeat (LRR) motifs, one LRR C-terminal motif (LRRCT), a unique C-terminal region and a GPI anchoring site (Figure 1.7). The functional specificity of NgR was demonstrated by a gain-of-function experiment in chick early embryonic retinal ganglion cells (RGC), which are insensitive to Nogo66-induced growth cone collapse. Viral-mediated NgR expression in embryonic day 7 (E7) RGCs renders these neurons sensitive to the Nogo-66 activity. Also, a truncated, soluble NgR antagonizes Nogo66-dependent inhibition of neurite extension by E13 DRGs (Fournier et al. 2002).

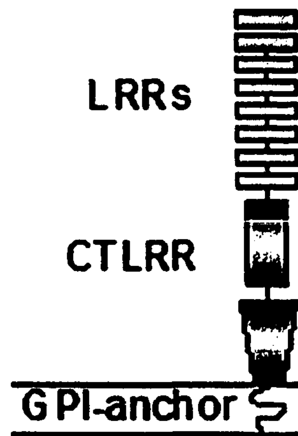

Figure 1.7 – A schematic diagram of the structure of NgR.
The NgR protein contains a signal peptide followed by eight leucine-rich repeats (LRR), a LRR carboxy-terminal flanking domain (LRRCT), a unique region and a GPI anchorage site.

The expression pattern of NgR is consistent with a role in the inhibition of axonal regeneration. Transcripts are present predominantly in the adult and maturing brain where the NgR protein is found in a wide variety of neurons but not in oligodendrocytes. Expression is localized to the axons and it is minimal prior to myelination (Wang et al. 2002c). After trauma, there are no detectable changes in NgR expression.

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The neurotrophins (NT) are a small family of growth factors that play roles in the development, maintenance, survival and death of the vertebrate nervous system (Bibel and Barde 2000; Huang and Reichardt 2001). Neurotrophins mediate their wide range of biological activities by binding two types of receptors: the p75 neurotrophin receptor (p75NTR), a member of the tumor necrosis factor receptor superfamily (TNFR), and the members of the tropomyosin-related kinase (Trk) receptor family (Kaplan and Miller 2000). TrkA, TrkB, and TrkC each bind a different subset of neurotrophins and transduce only positive signals related to survival (Figure 1.8). Meanwhile, p75NTR can bind all neurotrophins with equal affinity and transmit both positive and negative signals. Studies have shown that p75NTR can also bind non-neurotrophin ligands like prion protein fragment PrP(26-106) (Della-Bianca et al. 2001) and the Aβ-peptide of the amyloid precursor protein (APP) (Perini et al. 2002). Further, there is evidence that

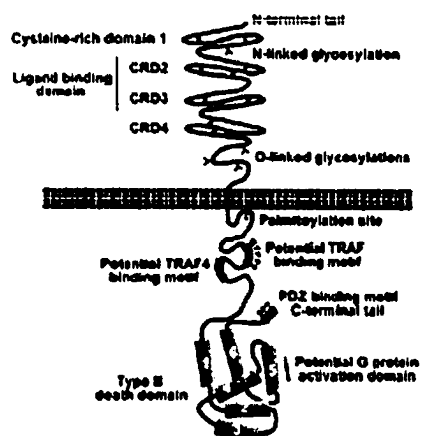

Figure 1.8 – A schematic diagram of the structure of p75NTR.
The p75NTR is a Type I transmembrane receptor with an extracellular domain consisting of four cysteine-rich domains (CRD) and multiple glycosilation sites. The intracellular domain contains a palmitoylation site, two TRAF-binding sites, a Type II death-domain, a potential G-protein activating domain and a PDZ domain binding motif.

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p75NTR and Trk receptors form complexes that result in increased ligand selectivity (Bibel et al. 1999).

The extracellular domain (ECD) of p75NTR contains four cysteine-rich motifs (Figure 1.8). The intracellular domain (ICD) does not have an intrinsic enzymatic activity and the signal transduction takes place through interaction with several adaptor proteins. p75NTR ICD contains a palmitoylation site, two TNFR-associated factor (TRAF) binding sites, a type II death domain, a G-protein activating domain, and a PDZ domain-binding motif (Roux and Barker 2002).

During early development p75NTR is expressed in a wide variety of cells within both CNS (Buck et al. 1987) and PNS (Heuer et al. 1990), as well as many non-neuronal tissues such as kidney, testis, lung and muscle (Wheeler et al. 1998). Postnatally, p75NTR levels are reduced in most tissues and restricted to a narrower range of cells. Trauma induces p57NTR expression in many cell types. Increased mRNA and protein levels have been documented in motor, corticospinal and hippocampal neurons, oligodendrocytes, Schwann and Purkinje cells, microglia and macrophages.

Because of its upregulation under pathological and inflammatory conditions, current views suggest that p75NTR functions as a stress receptor that modulates the balance between survival and apoptotic pathways. Several typical effectors associated with cell death, such as caspases, Jun N-terminal kinase (JNK) and p53, are activated by p75NTR (Yoon et al. 1998; Troy et al. 2002). Concurrently, p75NTR directly associates with TRAF proteins (Roux

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and Barker 2002) and was shown to activate the neural factor NF-κB *in vitro* (Kuner et al. 1998), both of which are important regulators of cell survival. Other adaptor proteins that bind to p75NTR have been reported. An ankyrin-rich transmembrane protein (ARMS) was shown to interact with p75NTR and, possibly, create a link to Trk receptors (Kong et al. 2001). At least three proteins, NRIF (Casademunt et al. 1999), NADE (Mukai et al. 2000) and NRAGE (Salehi et al. 2000), bind to independent motifs in the ICD of p75NTR and contribute to neurotrophin-dependent cell death pathways.

More recent studies have implicated p75NTR beyond cell death and survival pathways, including synaptic transmission, plasticity and axonal growth. Nerve growth factor (NGF) promotes neurite outgrowth in neurons that express p75NTR but not TrkA, the NGF-specific Trk receptor. These effects are the results of p75NTR modulation of RhoA (Yamashita et al. 1999). RhoA is a small GTPase that modulates actin filament assembly (Schmidt and Hall 2002). In its active, GTP-bound form RhoA rigidifies the actin cytoskeleton thus preventing axonal growth. Yamashita's group demonstrated that the fifth loop in the death domain of p75NTR directly binds RhoA in a yeast two-hybrid system. They indicate that p75NTR constitutively activates RhoA and that NGF quickly reduces the levels of GTP-bound RhoA in cultures. A second report by the same group indicates that p75NTR associates with gangliosides (Yamashita et al. 2002). As mentioned before, clustering of gangliosides by various methods can lead to p75NTR-dependent RhoA activation and inhibition of axonal growth.

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CHAPTER 2 - SCREENING OF RAT BRAIN cDNA LIBRARY BY TRANSIENT EXPRESSION, PANNING AND EPISOMAL RESCUE

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We attempted to enrich a rat brain library for the cDNA coding for MAG-Fc binding proteins by multiple rounds of panning and episomal rescue. The rat brain library was transfected into COS cells which were allowed to adhere overnight on plates coated with either MAG-Fc, MAG(R118A)-Fc or a control MUC18-Fc. The specifically bound cells were lysed in situ and episomal DNA recovered. The DNA was then transformed and used in protoplast fusion leading to a new population of transiently expressing COS cells. The enrichment was carried out for 3 successive rounds of panning and rescue. After the final round the plasmid DNA was transfected in COS cells and MAG-Fc binding was monitored by staining with a fluorescent anti-human Fc antibody. We observed very minimal adhesion from the transfected cells to the panning plates regardless of the chimera used to coat the plates. Further, the number of adhering cells did not seem to increase with successive rounds of panning and rescue. We obtained no positive clones following 4 individual experiments.

Since binding to the functional receptor is independent from sialic acid binding, our strategy focused on the identification of clones that would adhere to both the MAG-Fc plates as well as to the MAG(R118A)-Fc plates. We encountered a problem with the panning method: the binding of MAG-Fc to

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sialic acid on the cell surface was very strong and resulted in high background adhesion. Increasing the stringency of the washing steps reduced the background but also resulted in the loss of the weaker binding of MAG(R118A)-Fc to its receptor. Ultimately, we concluded that the difference in binding affinities among the protein used was too great to achieve the desired pool enrichment and we decided to change our approach.

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CHAPTER 3 - MAG INTERACTS WITH A G-LINKED PROTEIN

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While the addition of neurotrophins to cultures has no effect on MAG inhibition of neurite outgrowth, we found that priming neurons with neurotrophins prior to plating them on a monolayer of MAG-expressing CHO cells blocks MAG inhibition in a cAMP dependent manner (Cai et al. 1999). This priming requirement is removed if pertussis toxin (PTX), a $G_{i/o}$ inhibitor that alone has no effect on neurite outgrowth, is added along with brain-derived neurotrophic factor (BDNF) during the neurite outgrowth assay. We reasoned that BDNF treatment may require the priming time to reach a cAMP threshold necessary to block inhibition by MAG. Activation of G_i-linked cascades results in the inhibition of adenylate cyclase, the enzyme that catalyzes the formation of cAMP. Thus, the removal of adenylate cyclase inhibition by PTX could simply accelerate the accumulation of cAMP and remove the requirement for a longer BDNF treatment. We next hypothesized that the MAG receptor may be a multimeric complex containing a G-protein of the $G_{i/o}$ family that lowers cellular cAMP levels. Although the G-protein component is not required for inhibition, its activation could be monitored and possibly used to further elucidate the receptor complex identity.

Guanine-nucleotide-binding (G) proteins are molecular switches that regulate a variety of cellular processes. Heterotrimeric G proteins consist of α, β and γ subunits. In the inactive form, this complex is bound to a receptor on the cell surface (GPCR). When the receptor is stimulated, GDP is

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exchanged for GTP. The GTP-bound α subunit then dissociates from the $\beta\gamma$ heterodimer and targets downstream effectors. Binding of a ligand to a G_s or G_i-linked receptor will induce activation (G_s) or inhibition (G_i) of adenylate cyclase. Binding of a ligand to a G_q-linked receptor results in the dissociation on its α, β and γ subunits and the activation of the inositol-lipid pathway (Smrcka et al. 1991). The GTP-bound α_q subunit activates phospholipase C (PLC), which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG and IP_3 act as second messengers and activate further cascades. IP_3 undergoes a quick enzymatic breakdown to generate inositol, which is then recycled to the cell surface as PIP_2. Lithium chloride inactivates inositol monophosphatase (IMPase), the enzyme that catalyzes the hydrolysis of inositol monophosphates (IP) to inositol. Thus, activation of a G_q-linked receptor in the presence of lithium chloride will result in the accumulation of inositol monophoshates (IP). Loading cells with $_3$H-inositol can then help monitor IP accumulation and pathway activation.

Specific amino acid residues at the C-terminus of α subunits determine the abilities of individual G proteins to discriminate among specific subsets of receptors (Conklin et al. 1993). By replacing 5 C-terminal amino acids of α_q with the corresponding residues of α_i, Bourne's team created chimaeras that can mediate stimulation of PLC by receptors otherwise coupled exclusively to G_i proteins (Conklin et al. 1992).

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We first wanted to confirm the implication of a G_i protein in the MAG inhibition pathway. Pertussis toxin (PTX) catalyzes the ADP- ribosylation of the G_i regulatory component of adenylate cyclase (Bokoch et al. 1983). PTX consists of two subunits: the A protomer, which is responsible for the enzymatic activity of the toxin, and the B oligomer, responsible for binding the toxin to the cell surface (Tamura et al. 1982). To strengthen our previous findings, we decided to use an additional G_i inhibitor, NF023, which does not interfere with the interaction between α-subunits and βγ-dimer but competes for the effector's binding site. As a negative control we used the B-oligomer of

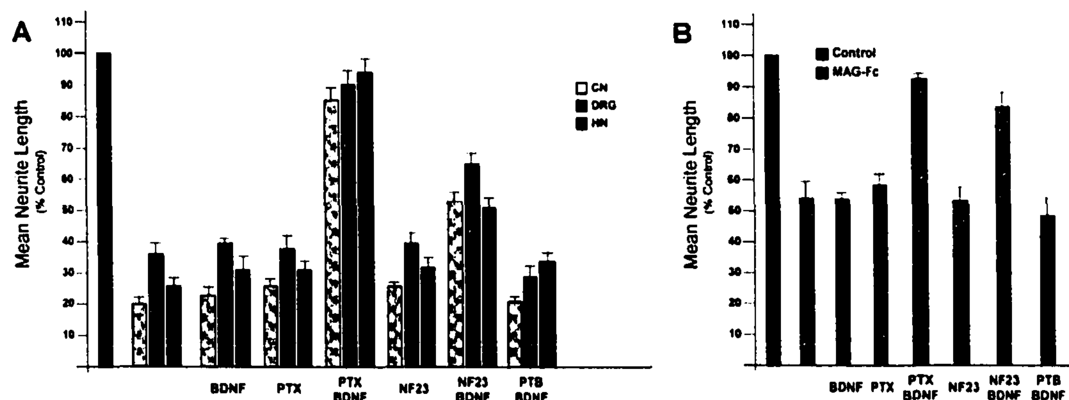

Figure 3.1 – The effect of G_i inhibition on the priming requirement for a BDNF-induced block of inhibition by MAG.
(A) Cerebellar (CN), dorsal root ganglia (DRG) and hippocampal (HN) neurons were incubated for 2 hr. in the presence of pertussis toxin (2 ng/ml, PTX), pertussis toxin B-oligomer (2 ng/ml, PTB) or NF023 (3.5 μg/ml, NF23) before being cultured overnight on a monolayer of either MAG-expressing (stippled bars) or control CHO cells (solid bars) and BDNF (200 ng/ml), as indicated, before being fixed and immunostained for GAP43.
(B) Cerebellar neurons were incubated for 2 hr. in the presence of pertussis toxin (2 ng/ml, PTX), pertussis toxin B-oligomer (2 ng/ml, PTB) or NF023 (3.5 μg/ml, NF23) before being cultured overnight on a substrate of L1-Fc in the presence of BDNF (200 ng/ml) and MAG-Fc (hatched bars) or not (black bar). Neurons were fixed and immunostained for GAP43.
Results show the mean length of the longest neurite per neuron (+/- sem) for 180–200 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells or in the absence of MAG-Fc.

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PTX. In Figure 3.1 we show results of neurite outgrowth experiments conducted using either soluble MAG-Fc or monolayers of MAG-expressing cells. When tested on CN, DRG and HN, both inhibitors removed the BDNF priming requirements. PTX completely abolished the priming effect while NF023 gave slightly weaker results. Either inhibitor alone or the PTX B-oligomer did not have any effects on axonal growth.

We acquired plasmid vectors encoding for the mutant G protein (Gq-i5) and proceeded to test our hypothesis by the phosphatidylinositol turnover method. To test the ability of the chimeras to induce an IP accumulation in response to a G_i-linked stimulus, we co-transfected COS-7 cells with vectors encoding for the wild-type M2 muscarinic receptor (M2Rwt), a known G_i-linked receptor, and for the G proteins constructs (Gq-wt, Gi-wt or Gq-i5). The transfected cells were incubated in inositol-free medium for 24 hrs and loaded for 3 hrs with $_3$H-inositol prior to stimulation with carbachol, an agonist of M2Rwt. The cell lysates were centrifuged and the supernatant applied to an anion-

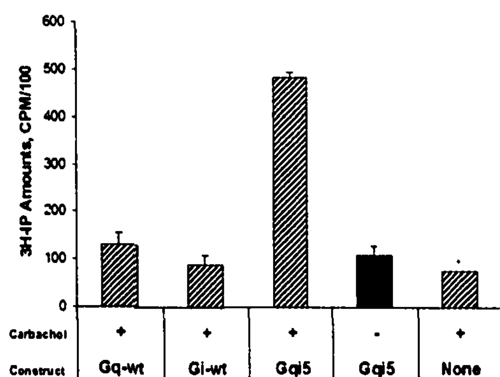

Figure 3.2 – PLC activation mediated by wild type m2 muscarinic receptor after coexpression with mutant α_q subunits.
COS cells were transfected with expression plasmids coding for the wild type m2 muscarinic receptor (m2R) and wild type α_q (Gq-wt), wild type α_i (Gi-wt) or a mutant α_q subunit in which the C-terminal 5 aa of Gq-wt were replaced with the corresponding sequence from α_i (Gqi5). About 48 hr after transfection, cells were stimulated (hatched bars) with the muscarinic agonist carbachol (1 mM), and increases in intracellular IP levels were determined. IP levels in stimulated cells transfected with m2R alone or m2R and Gqwt were not significantly different from those found in unstimulated cells (solid bars).

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exchange column to isolate inositol monophosphates (IP). The IP fraction was then analyzed with a scintillation counter. Figure 3.2 shows the mean results of 2 turnover experiments. Scintillation counts from cells transfected with both M2Rwt and Gq-i5 vectors indicate a dramatic accumulation (4-5x) of $_3$H-IP as compared to unstimulated cells and cells transfected with control Gq-wt, Gi-wt vectors.

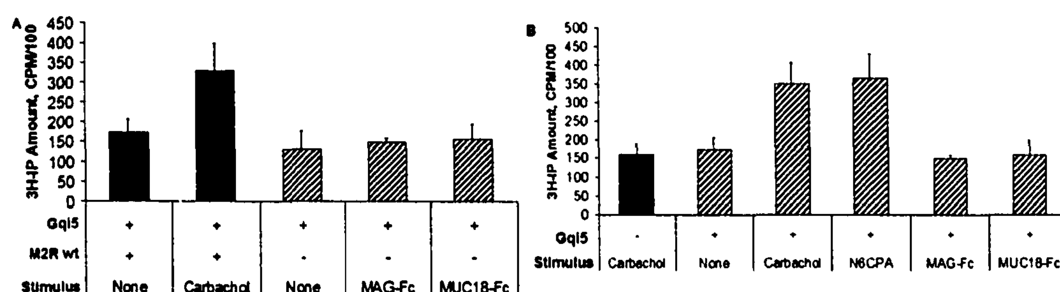

Figure 3.3 – PLC activation in response to MAG-Fc stimulation.
A) NG108 cells were cotransfected with expression plasmids coding for the mutant α_q subunit Gqi5 and m2R (solid bars) or the empty vector (hatched bars). About 24 hr post transfection, cells were incubated with db cAMP (1mM) for 24 hr to induce differentiation to the neuronal phenotype. The differentiated cells were stimulated for 1 hr with carbachol (1mM), MAG-Fc (20 μg/ml) or MUC18-Fc (20 μg/ml), and the increases in intracellular IP levels were determined.
B) Cerebellar neurons from PND8 rats were transfected with expression plasmids coding for the mutant α_q subunit Gqi5 (hatched bars) or the empty vector (solid bars). About 24 hr post transfection, neurons were stimulated for 1 hr with carbachol (1mM), N6CPA (100 nM), MAG-Fc (20 μg/ml) or MUC18-Fc (20 μg/ml), and the increases in intracellular IP levels were determined.
Data are expressed as means ±SD of triplicate determinations in a single experiment; two additional experiments gave similar results.

We then proceeded to test the method using NG108 cells, a neuronal cell line that is inhibited by MAG and that expresses M2R endogenously. Transfection with Gq-i5 resulted in a modest 1.3x carbachol-induced IP accumulation while stimulation of the same transfectants with MAG-Fc did not induce IP accumulation (Figure 3.3). Transfection of rat primary cerebellar

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neurons with Gq-i5 yielded a 2x IP accumulation when the cells were stimulated with carbachol or N6CPA, a selective adenosine receptor (A1AR) agonist, but no MAG-Fc induced accumulation could be detected (Figure 3.3).

We reasoned that, if the activation of a G_i protein in the MAG receptor complex causes the priming requirement, stimulation of a known G_i-linked pathway during priming would abolish the BDNF effects. When CN were primed with BDNF and either carbachol or N6CPA, the reversal of MAG inhibition was abolished (Figure 3.4).

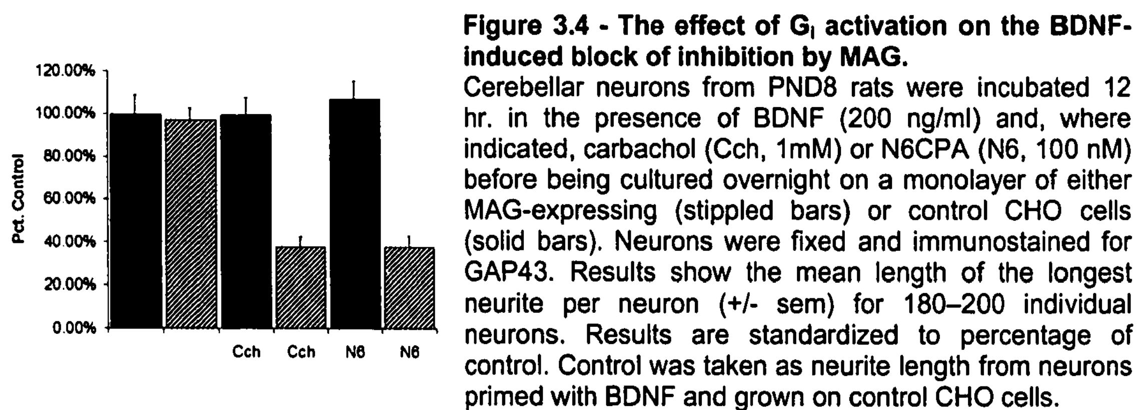

Figure 3.4 - The effect of G_i activation on the BDNF-induced block of inhibition by MAG.
Cerebellar neurons from PND8 rats were incubated 12 hr. in the presence of BDNF (200 ng/ml) and, where indicated, carbachol (Cch, 1mM) or N6CPA (N6, 100 nM) before being cultured overnight on a monolayer of either MAG-expressing (stippled bars) or control CHO cells (solid bars). Neurons were fixed and immunostained for GAP43. Results show the mean length of the longest neurite per neuron (+/- sem) for 180–200 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed with BDNF and grown on control CHO cells.

The fact that two G_i inhibitors with different modes of activity can both interfere with the BDNF priming pathway supports our hypothesis concerning the MAG receptor complex. The logical follow up was to try to detect the activation of the receptor using the G-protein chimeras. The IP-turnover assay proved successful in detecting the activation of known G_i-linked receptor, adenosine and muscarinic receptors, in all the cells tested. We expected to detect lower IP accumulation levels in neuronal cells (mostly due to the

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difficulties related to their transfection), but we were still able to detect significant changes in response to known stimuli. MAG-Fc failure to induce an IP accumulation could be due to either the absence or the very low expression levels of a putative G_i-linked MAG receptor complex. Although we decided not to pursue this approach any further, we obtained valuable information on the pathway leading to BDNF priming of neurons. Since the G_i activation by carbachol and N6CPA blocked the BDNF priming effect on cerebellar neurons, we believe that we are observing a threshold effect. Without priming, BDNF elevates cAMP to barely sub-threshold levels and the modification of another, even unrelated, cAMP pathway may be sufficient to push the cells into a growth state and overcome MAG inhibition.

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CHAPTER 4 - INCREASING RECEPTOR-LIGAND AFFINITY: MULTIMERIC MAG COMPLEXES

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Generation of MAG-biotin chimaeras

We have tried to find an assay that can discriminate between binding by MAG to the functional receptor and MAG binding to molecules bearing sialic acid residues. The MAG(R118A)-Fc chimera, which does not bind sialic acid, can still inhibit neurite outgrowth under some conditions (Tang et al. 1997a). However, we have thus far not been able to identify the MAG receptor using this chimera. We speculated that binding to the functional receptor is weak and that it can be potentiated by other interactions, i.e. binding sialic acid-bearing molecules on the cell surface. Therefore, we reasoned that increasing MAG(R118A)-Fc binding avidity could generate a new, more effective tool to screen a cDNA library.

Interactions between leukocyte cell surface molecules are often weak and transient, making them difficult to identify (van der Merwe and Barclay 1994). When recombinant forms of extracellular regions do not express the avidity necessary to bind to cells, they are made multivalent by attaching them in an oriented manner to fluorescent beads (Brown et al. 1995). Brown and colleagues described an assay in which an enzymatically biotinylated peptide was engineered onto CD4d3 (domains 3 and 4 of rat CD4) fusion proteins and coupled to streptavidin-coated beads (Figure 4.1). The fusion protein-coated beads were then used as a tool to find additional, low affinity ligands for CD48

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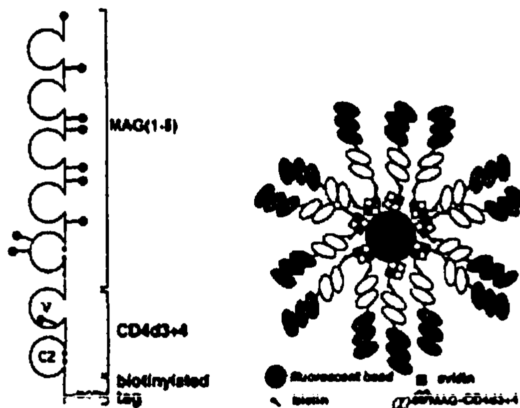

Figure 4.1 – Schematic representation of the chimeric proteins.
Recombinant chimeric MAG(1-5)-CD4d3+4-biotin protein (left). The chimeric protein bound to fluorescent beads (right).

(Brown et al. 1998). We reasoned that the same approach could be used to enhance MAG(R118)-Fc avidity for the receptor.

We obtained constructs from Dr. Brown and proceeded to create MAG fusion proteins. The original vector (cd4L34biotin-pEF-BOS) encodes for a CD4 fragment fused to a linker region (L34) and a peptide sequence (Bio) recognized by BirA, a biotinylating enzyme found in *E. coli*. Since we were only interested in the L34-Bio segment, we proceeded to digest the vector with restriction endonucleases and purify the fragment. First, we digested with *HindIII* and *SalI* to obtain an intermediate fragment, which was blunt ended using the Klenow enzyme. The blunt ends strategy was necessary due to a lack of sufficient or suitable restriction sites within the various sequences. The intermediate fragment was run on an agarose gel and purified using a Qiagen kit. Further digestion with *BamHI* yielded a sequence, BLUNT/L34-Bio/*BamHI*, which was suitable for our final step. Second, we proceeded to extract the sequence coding for the five extracellular domains of MAG from the MAG-Fc vector. The same strategy was also used for the MAG(R118A) sequence, which is identical to MAG in the region of interest, and only MAG will be referenced in the next steps. MAG-Fc pIg vector was digested using *BamHI* and *SnaBI*, purified and blunt

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ended with Klenow. The fragment was digested further with *HindIII* to yield the sequence *HindIII*/MAG(1-5)/BLUNT. Last, we ligated the two blunt end cDNA sequences to create a *HindIII*/MAG-L34-Bio/*BamHI* fragment that could be inserted into a pcDNA3.1 mammalian expression vector. We confirmed that the construct was correctly inserted by with multiple restriction endonuclease digests of the vector (Figure 4.2). Further, the plasmid was sequenced using primers designed to amplify the region containing the hinge between MAG domain 5 and the linker. Sequencing results confirmed that the constructs did not contain mutations within the target sequence.

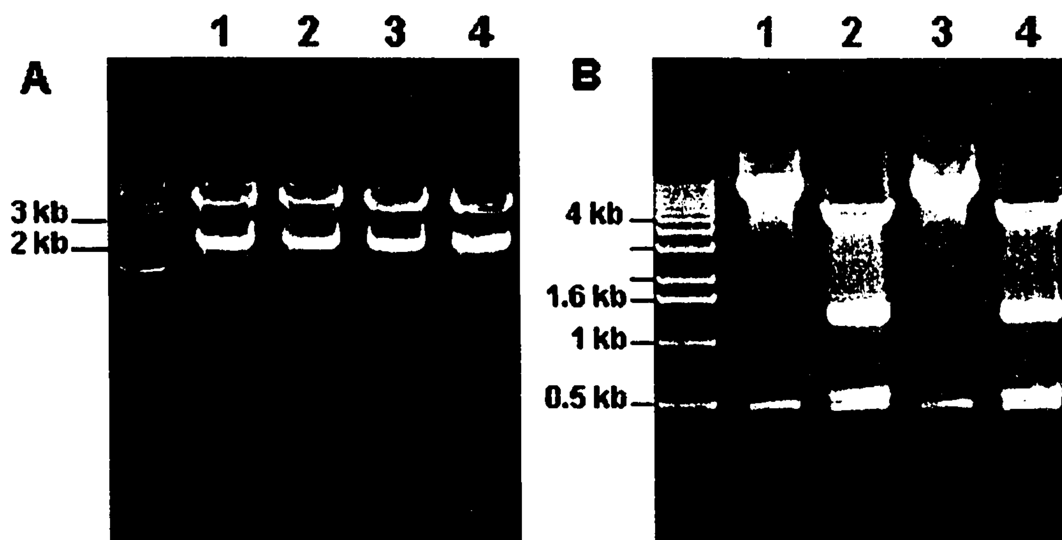

Figure 4.2 – MAG-L34-biotin restriction analysis.
(A) The plasmid was digested with HindIII and BamHI and run on a 1% agarose gel to verify the size of the insert. Both the MAG(1-5)-L34-biotin (1 and 2) and the MAG(R118A)-L34-biotin (3 and 4) sequences are expected to be 2.3 kb.
(B) SacI (1 and 3) and PstI (2 and 4) digests were performed to verify the orientation of the insert. Correct fragments from SacI digest: 0.3 and 7.3 kb (4 fragments if incorrect). Correct fragments from PstI digest: 0.4, 0.5, 1.3 and 5.2 kb (3 fragments above 1 kb if incorrect).

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Transiently transfected COS-7 cells produced recombinant proteins that were subsequently biotinylated using a BirA kit from Avidity, Denver. The expeL34-biotin protein, which we used as control, is 47 kDa. Protein samples were subjected to non-denaturing PAGE and western blot analysis with a conformation-dependent anti-MAG monoclonal antibody (513) or streptavidin. We show in Figure 4.3 the correct M_r of the fusion proteins as well as demonstrate the presence of the biotin tag.

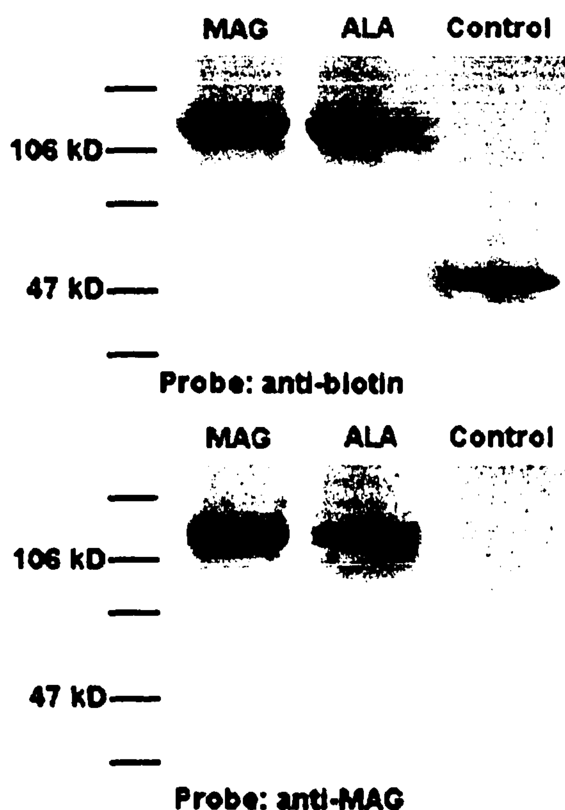

Figure 4.3 – Western blot of biotin constructs.
Recombinant MAG(1-5)-L34-biotin (MAG), MAG(R118A)-L34-biotin (ALA) and CD4d3+4-biotin (Control) proteins were enzymatically biotinylated prior to PAGE and Western blotting with anti-MAG and anti-biotin antibodies.

Multivalent MAG complexes

We wanted to assess if the newly constructed fusion proteins would exhibit binding of neurons comparable to that of the MAG-Fc chimeras. Cerebellar and DRG neurons were cultured overnight in 96-well plates coated with laminin. To generate multivalent complexes we incubated the biotinylated proteins with streptavidin-HRP. Because the HRP molecules are chemically coupled to sites on streptavidin other than the biotin

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binding sites this approach allowed the creation of tetravalent complexes. The complexes were added to the neurons and allowed to bind before incubation with an HRP substrate. Detection of color changes was done using an ELISA plate reader. We could not detect colorimetric changes with any of the fusion proteins.

We reasoned that binding of the proteins to streptavidin might have altered the protein conformation and subsequently blocked the neuron binding sites. To verify this hypothesis we incubated cultured neurons with either MAG-L34-biotin or MAG-Fc, then fixed the cells and immunostained them with anti-MAG antibody. Although the neurons were prominently labeled by MAG-Fc, the MAG-L34-biotin did not seem to bind to any degree (Figure 4.4). These results pointed to a problem with the fusion protein itself. We decided

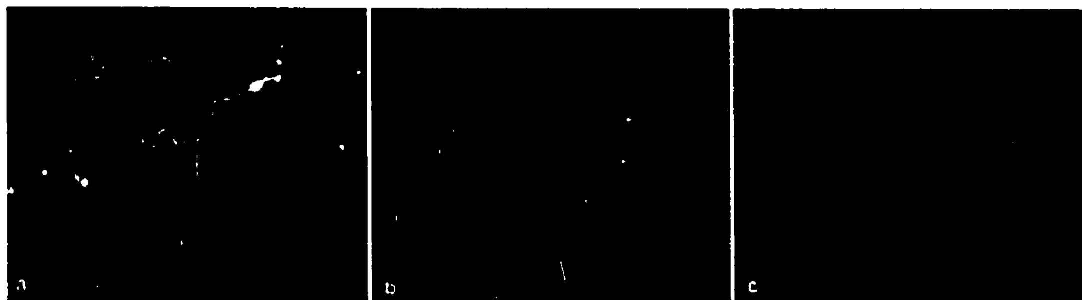

Figure 4.4 – DRG staining using MAG-Fc or MAG-biotin chimeras.
DRG neurons (PND7) were plated onto laminin-coated wells and cultured overnight before MAG-Fc (20 μg/ml) (a), MAG(1-5)-L34-biotin (b) or MAG(R118A)-L34-biotin (c) were added and incubated for 2 hr. The cultures were fixed and immunostained with anti-human Fc (a) or anti-biotin (b and c) antibodies and FITC-conjugated secondary antibodies.

to test this issue further by measuring the binding of calcein AM-labeled neurons to 96-well plates coated with the fusion protein. This type of assay is routinely used in our lab to measure the quality of MAG-Fc preparations. We ensured the correct protein orientation by using streptavidin-coated plates

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(SIGMA) that were incubated with MAG-L34-biotin at various concentrations. The plates were scanned with a Fluoroimager and the data analyzed with ImageQuant software. The results confirmed that the fusion proteins could not bind to neurons (Figure 4.5).

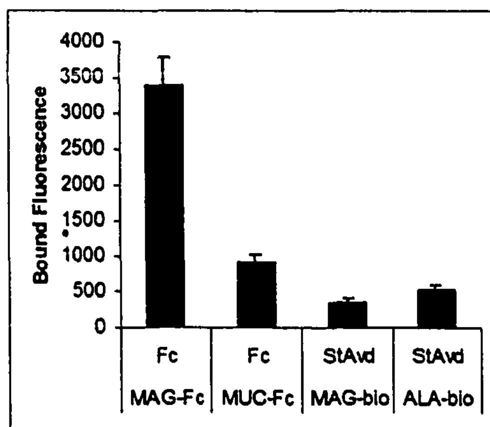

Figure 4.5 – Binding of CN to immobilized MAG constructs. Recombinant MAG(1-5)-L34-biotin (MAG-bio), MAG(R118A)-L34-biotin (ALA-bio), MAG-Fc or MUC18-Fc were immobilized onto 96-well microtiter plates coated with either Streptavidin (StAvd) or anti-human Fc (αFc). After 4 hr the binding of labeled neurons was measured. Results are the mean of three discrete assays run in quadruplicate, ± sem.

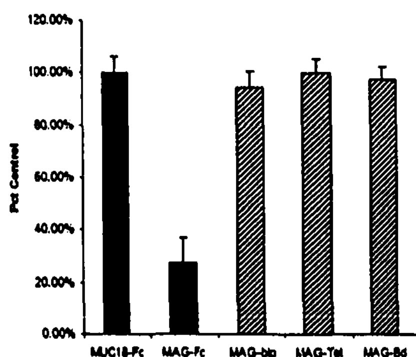

Figure 4.6 – Soluble neurite outgrowth from cerebellar neurons. Cerebellar neurons (P8) onto L1-Fc as a substrate, and MUC18-Fc (black bar), MAG-Fc (gray bar) or MAG(1-5)-L34-biotin (hatched bars) with different valences (MAG-bio monomer, -Tet tetramer and –bd coated bead) were added. After overnight incubation, the cultures were fixed and stained for GAP43 and the longest neurite for 180–200 neurons was measured. Results represent % of control, which is neurons in the presence of MUC18-Fc, +/- SEM. Results are from two experiments.

As we could not detect binding of our chimeras to neurons, we did not expect them to inhibit neurite outgrowth. Nevertheless, we tested the monomer, the tetramer and the coated beads in a soluble outgrowth assay from cerebellar neurons. Although the MAG-Fc controls strongly inhibited axonal growth, MAG-L34-biotin under the same conditions failed to inhibit regardless of the valence of the complexes (Figure 4.6).

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We can only speculate on the reasons for the failure of the fusion proteins to bind to neurons. We are confident the sequences used in the construction of the chimeras were correct. Sequencing and restriction analysis confirmed that constructs were inserted correctly into the vector. Neither production of the proteins nor their subsequent biotinylation appeared to be hindered. Monoclonal antibody 513, which detected the fusion proteins in Figure 4.3, recognizes MAG in its native state and it is considered to be conformation dependent. Yet, the cumulative data suggests a problem with the folding of the chimeric protein.

We believe the problem may have arisen from the linker used. Domains 3 and 4 of CD4 are Ig-like domains. Although there is no evidence demonstrating an interaction between MAG and CD4, it is possible that the presence of two additional Ig-like domains may have influenced the folding of the MAG domains. Monoclonal antibody 513 (mAb 513) is indeed conformation dependent, but it recognizes an epitope spanning MAG domains 1, 2, and 3 (Meyer-Franke et al. 1995). MAG inhibitory activity is localized to domain 5 of the protein (Filbin, unpublished data). Because there are no crystallography data showing MAG's structure, we cannot predict if the linker region is proximal to the functional binding site (d5) or the sialic acid binding site (R118). The Ig-like domains of the linker region may block the binding sites or, perhaps, induce conformational changes that hinder the protein's ability to bind neurons while preserving the mAb 513 epitope.

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CHAPTER 5 - MAG INTERACTS WITH THE NOGO-66 RECEPTOR TO INHIBIT NEURITE OUTGROWTH

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We have previously shown that MAG-Fc can precipitate a number of proteins from CN, DRG and PC12 cells (De Bellard and Filbin 1999). While others have recognized some of these proteins and discarded them as the functional receptor, one protein with a relative molecular mass of approximately 85 KDa was yet to be identified (Figure 5.1). In light of the recent identification of the Nogo-66 receptor (Fournier et al. 2001), an 85 kDa protein, we decided to verify if there was redundancy in the receptors for myelin inhibitors.

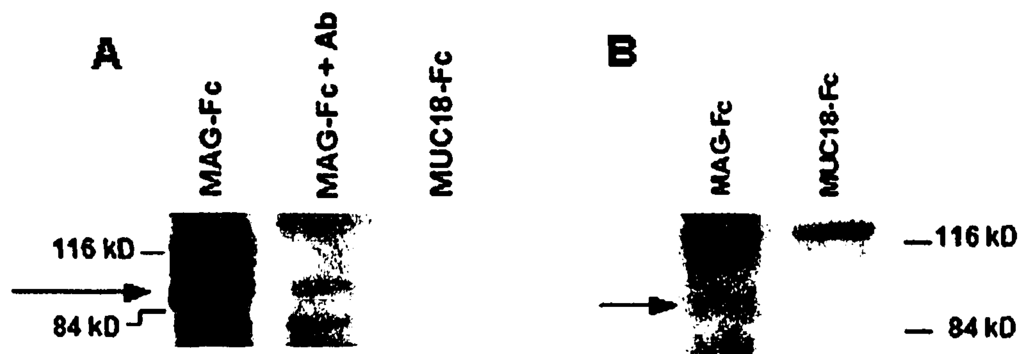

Figure 5.1 - MAG-Fc specifically precipitates two surface neuronal proteins.
Intact DRG (A) or cerebellar (B) neurons were surface biotinylated before being lysed with NP40-containing buffer, incubated with pre-aggregated MAG-Fc overnight at 4°C. After precipitation with protein A Sepharose beads, dissociated proteins were separated by SDS PAGE, transferred to PVDF membranes and detected by avidin:HRP-chemoluminescence. Where indicated, MUC18-Fc (MUC18-Fc) and MAG monoclonal antibody 513 (Ab) were used. Arrows indicate the 85 kD protein.

A GPI-Linked Protein Is Required For MAG Binding and Inhibition of Neurite Outgrowth

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We first checked that the commercially available antibodies could detect NgR in western blots. Figure 5.2 shows that the SC-16708 antibody recognizes an 85 kDa protein expressed by cerebellar neurons and NgR-expressing CHO cells. The antibody could also recognize a recombinant protein consisting of the NgR extracellular domain fused to alkaline phosphatase (NgR-AP, M_r 150 kDa). The same band is detected using an anti-alkaline phosphatase antibody. Further, the antibody can be used for NgR immunostaining of primary cerebellar neurons (Figure 5.3).

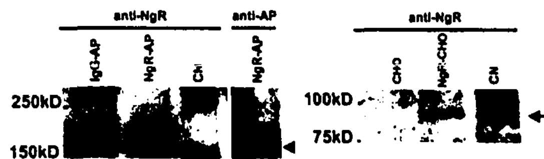

Figure 5.2 – An anti-NgR antibody specifically detects NgR.
Proteins from lysates of NgR-expressing CHO cells (NgR-CHO), CHO cells and cerebellar neurons (CN) or IgG-AP and a preparation of NgR-AP were separated by PAGE and transferred to a membrane before being stained for NgR or AP, as indicated. Arrow points to NgR and arrowhead points to NgR-AP. Molecular weight standards are on the left.

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment to remove NgR and other GPI-linked proteins from the cell surface abolishes anti-NgR staining of cultured cerebellar neurons (Figure 5.3). The discovery that PI-PLC treatment, which does not remove sialic acid, also completely abolishes binding by MAG-Fc (Figure 5.3) was the first indication that a GPI-linked protein may mediate MAG activity. Replacement of cell surface proteins restores both anti-NgR and MAG-Fc binding within 8 hours of treatment. The speed of the replacement does not permit the monitoring of neurite outgrowth using standard methods, which require 18-24 hours incubation. To circumvent the problem, we modified our soluble assay: following PI-PLC treatment,

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neurons were cultured for four hours after which the percentage of neurons bearing neurites was determined. Figure 5.4 shows that MAG-Fc inhibition of neurite outgrowth is abolished by the removal of GPI-linked proteins.

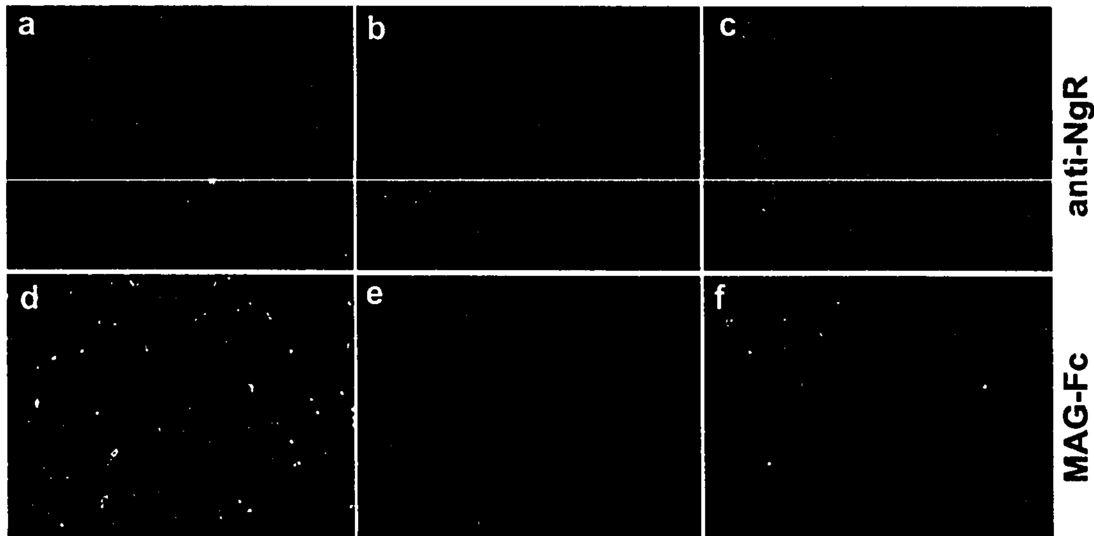

Figure 5.3 – binding of MAG-Fc requires GPI-linked neuronal proteins.
Cerebellar neurons (PND4) were plated onto laminin-coated wells and cultured overnight before NgR antibody (1:1000) (a–c) or MAG-Fc (25 μg/ml) (d–f) were added and incubated for 2 hr. The cultures were fixed and NgR detected with a secondary antibody conjugated to rhodamine. MAG-Fc was detected with a MAG monoclonal antibody, followed by a R-phycoerthrin-conjugated secondary antibody. Where indicated, neurons were treated with PI-PLC for 30 min and then cultured for 1 hr (b and e) or 8 hr (c and f) before addition of the NgR antibody or MAG-Fc.

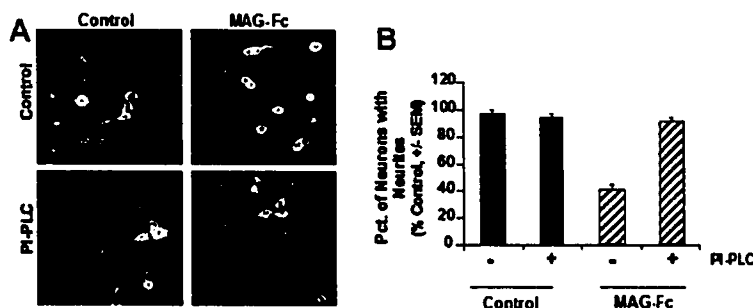

Figure 5.4 - Inhibition of Neurite outgrowth by MAG requires GPI-linked proteins.
(A) Cerebellar neurons (P4), either control or treated with PI-PLC, were plated onto a substrate of immobilized L1-Fc, and MAG-Fc (20 μg/ml) was added. After 4 hr incubation, cultures were fixed and stained for GAP43.
(B) Quantification of neurite outgrowth from cerebellar neurons, either treated (+) or not (-) with PI-PLC. Where indicated MAG-Fc was added (hatched bars). Results are the number of neurons with neurites, expressed as a percentage of untreated neurons in the absence of MAG-Fc (black bars). The results are the mean of four experiments, +/- SEM.

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MAG Binds NgR with High Affinity

To demonstrate that the GPI-linked protein mediating MAG inhibitory activity was NgR we tested the ability of MAG-Fc to bind NgR-expressing CHO cells (NgR-CHO). While MAG-Fc did not bind to either control cells or PI-PLC treated NgR-CHO, we observed a very strong staining on NgR-CHO (Figure 5.5). It is important to note that sialidase treatment did not reduce MAG-Fc binding to NgR-CHO, thus confirming that sialic acid residues on

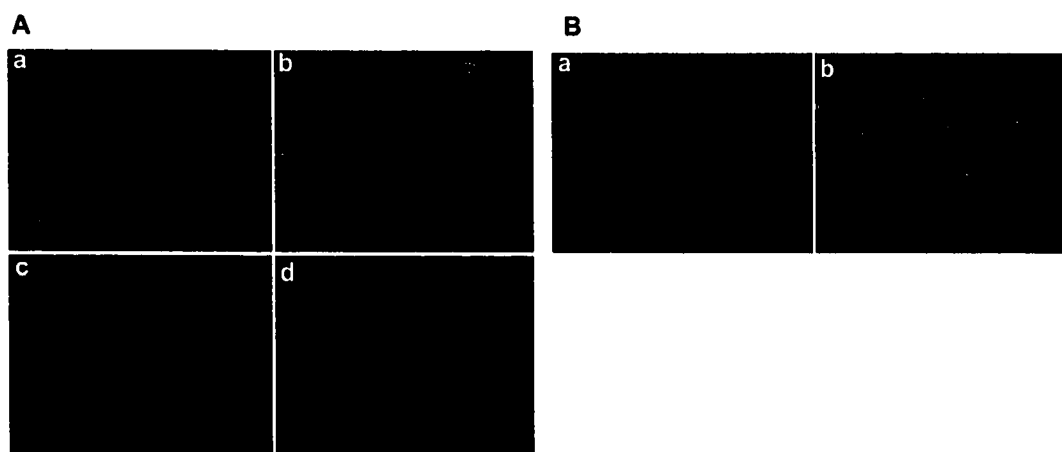

Figure 5.5 – MAG interacts with NgR.
(A) Confluent monolayers of CHO cells expressing NgR (b–d) or control CHO cells (a) were incubated with MAG-Fc (20 μg/ml) for 2 hr before being fixed and immunostained for MAG. The monolayer was treated with PI-PLC (c) or with sialidase (d) before addition of MAG-Fc.
(B) Confluent monolayers of MAG-expressing (b) or control (a) CHO cells were incubated with NgR-AP for 2 hr before being fixed and immunostained for NgR.

gangliosides do not mediate MAG binding to NgR. The reciprocal experiment, where we monitored NgR-AP binding to MAG-expressing cells (MAG21), provided complementary results (Figure 5.5) and confirmed the direct interaction between MAG and NgR.

We performed a co-precipitation assay from lysates of NgR-CHO, which express a FLAG-NgR construct, using MAG-Fc, MAG(1-3)-Fc or an

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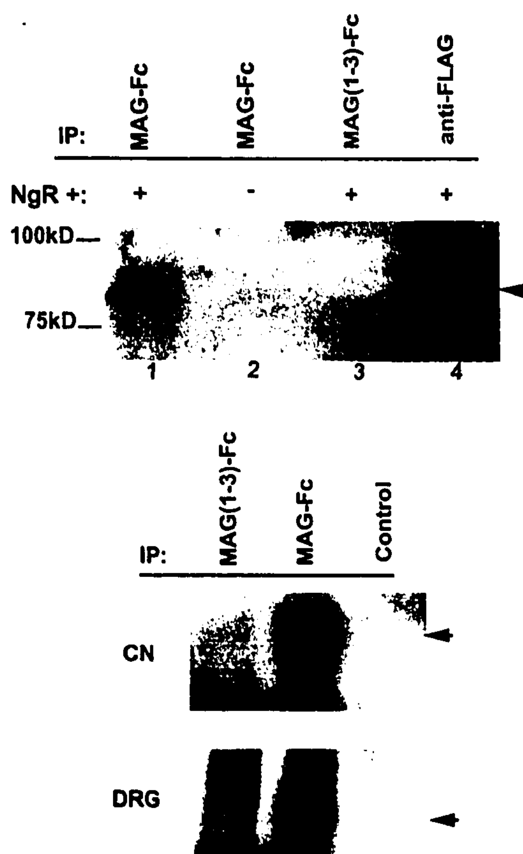
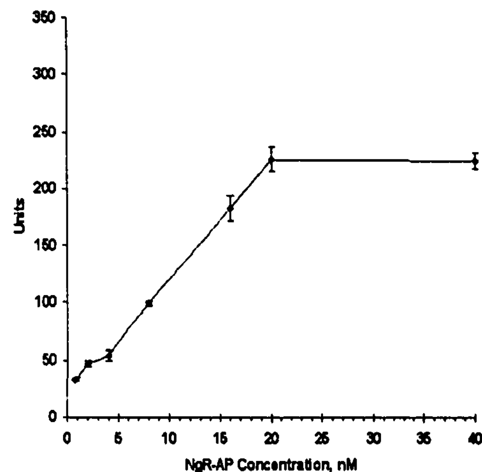


Figure 5.6 – MAG-Fc co-precipitates NgR. Lysates of NgR-expressing or control CHO cells, cerebellar neurons (CN), or DRG neurons (DRG) were incubated with MAG-Fc, MAG (d1-3)-Fc, or an anti-flag antibody as indicated. Precipitated proteins were dissociated, separated in a 12% PAGE, transferred to PVDF membranes, and immunostained for NgR.

For (CN) and (DRG), controls were without MAG-Fc. Arrows indicate NgR.



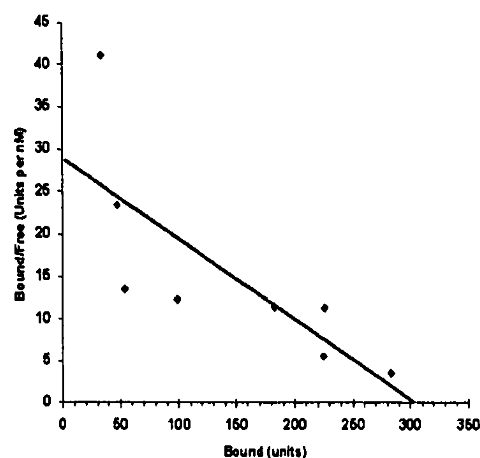


Figure 5.7 – MAG binds NgR with high affinity.
NgR-AP binding, at various concentrations, to immobilized MAG-Fc was measured. The Kd of NgR-MAG binding was calculated from bound NgR/free NgR plotted against bound NgR and calculated to be 8 nM.

anti-FLAG antibody (Figure 5.6). MAG-Fc was able to precipitate NgR while MAG(1-3)-Fc, a fusion protein that binds neurons but does not inhibit outgrowth, was not. The same results were obtained in precipitation experiments from primary cerebellar and DRG neurons (Figure 5.6). Since the data confirmed that MAG is a ligand for NgR, we wanted to know what is the

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strength of their interaction. To measure the dissociation constant (K_D) for the interaction, we monitored NgR-AP binding to MAG-Fc absorbed to micro titer plates and plotted bound NgR/free NgR over bound NgR. The K_D was found to be 8 nM (Figure 5.7). This high affinity interaction is of the same magnitude measured for the binding of Nogo-66 to NgR (Fournier et al. 2001).

NgR is the Functional MAG Receptor

If NgR is indeed a functional receptor for MAG, NgR blockade by either soluble NgR-AP competition or bath application of an anti-NgR antibody should result in a block of MAG inhibition of neurite outgrowth. Addition of soluble NgR

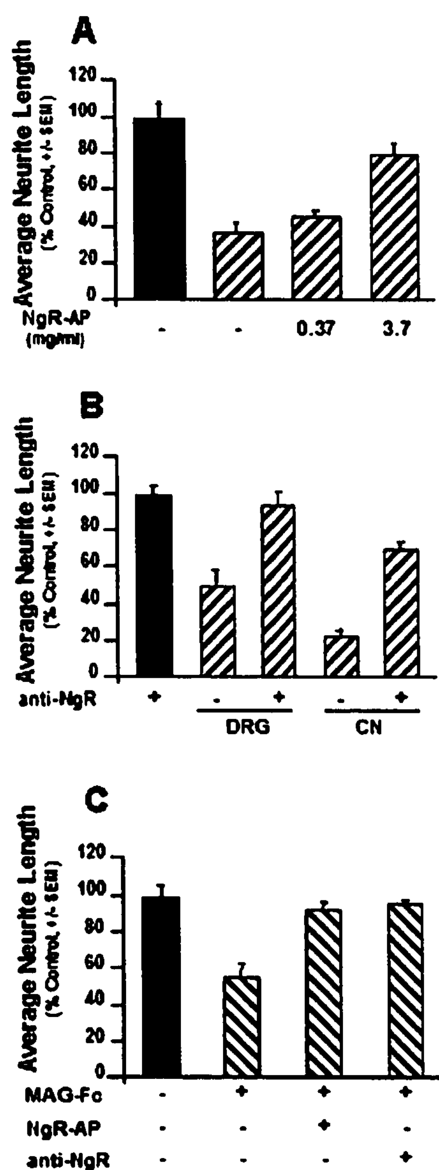

Figure 5.8 - Soluble NgR and a NgR antibody each block MAG's inhibition of neurite outgrowth.
DRG (A–C) or cerebellar (B) neurons were plated on confluent monolayers of MAG-expressing or control CHO cells (A and B) or onto L1-Fc as a substrate, and MAG-Fc (20 μg/ml) was added (C). Where indicated, the monolayers were incubated with soluble NgR-AP at either 0.37 or 3.7 μg/μl or NgR antibody (1:100) for 30 min prior to addition of neurons. For MAG-Fc, NgR-AP (3.7 μg/μl) or NgR antibody were added simultaneously to MAG-Fc (C). After overnight incubation, the cultures were fixed and stained for GAP43 and the longest neurite for 180–200 neurons was measured. Results represent % of control, which for (A) and (B) is neurons on control CHO cells and for (C) is in the absence of MAG-Fc, +/- SEM. Results are from at least three experiments, each in at least duplicate. Black bars, neurons grown on control CHO cells; hatched bars, neurons on MAG-expressing CHO cells or neurons with soluble MAG-Fc as indicated.

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to DRG cultures prevented inhibition of neurite outgrowth by MAG-expressing cells in a dose dependent manner (Figure 5.8). A receptor blockade using anti-NgR also abolished MAG21 inhibition in both cerebellar and DRG neurons. The same results were obtained when NgR-AP or anti-NgR were added simultaneously to MAG-Fc to DRG growing on a growth promoting L1 substrate (Figure 5.8).

NgR is a GPI-linked receptor, thus it is unlikely that it can transduce the inhibitory signal into the cell. Since both leucine-rich regions of NgR, LRR and CTLRR, have been shown to be necessary for the binding of Nogo-66 and OMgp (Wang et al. 2002b), we hypothesized that the unique C-terminal domain may interact with a required, signal-transducing element. Expression of a truncated NgR, lacking the region spanning from CTLRR to the GPI anchor, could therefore act in a dominant negative fashion and block NgR signaling. We overexpressed full-length NgR (FL-NgR), the C-terminal region (C-NgR) or the N-terminal region (N-NgR), which contains the ligand binding sites alone, into NG108, a neuronal cell line that is responsive to MAG and that can be transfected more easily than primary neurons. We cultured transfected NG108 cells on poly-L-lysine (PLL) or recombinant MAG (rMAG) substrates and measured their ability to generate neurites (Figure 5.9). The expression of the putative dominant negative N-NgR construct reversed the growth inhibition by rMAG to control levels (PLL). In contrast, the FL- and C-NgR constructs did not have any effect on neurite outgrowth either on PLL or

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rMAG substrates. These results indicate that the dominant negative effect is specific for MAG and confirm NgR as the functional MAG receptor.

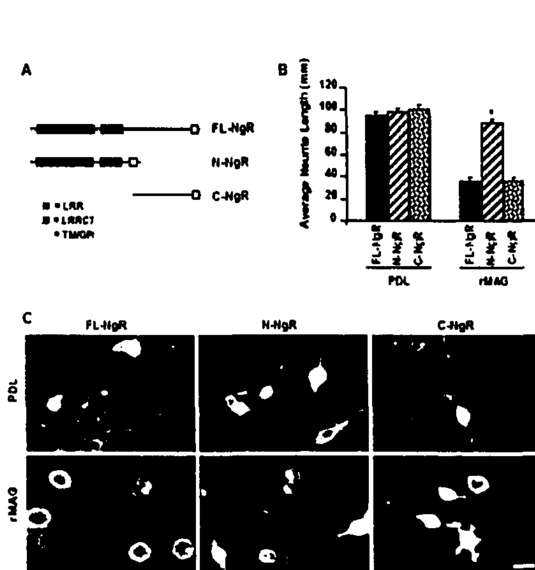

Figure 5.9 - A dominant-negative NgR blocks the inhibitory activity of MAG. (A) Schematic diagram showing the NgR constructs.
(B) The longest neurite from individual transfected neurons for each condition was measured for 180–200 neurons. Statistical analysis was done by one-way ANOVA ($p < 0.0001$). *Star indicates N-NgR-expressing cells on MAG have significantly longer neurites than cells expressing FL-NgR or C-NgR on MAG.
(C) Differentiated NG108 cells expressing full-length (FL-NgR), N-terminal (dominant-negative) (N-NgR), or C-terminal (C-NgR) NgR were grown 24 hr on either PDL or immobilized MAG substrates. Scale bar: 12 μm.

MAG and Nogo-66 Competition

If all three myelin inhibitors act through the same receptor and require the same region for binding, MAG, OMgp and Nogo66 may compete for the same site. We addressed this question by measuring the binding of MAG-Fc to NgR-CHO in the presence Nogo66-AP. When Nogo66-AP was added in two-fold excess the binding of MAG-Fc, as visualized by anti-MAG immunostaining, was greatly reduced (Figure 5.10).

When two ligands compete for a single binding site, the competitive binding curve will have a shape determined by the law of mass action. In this case, the curve will descend from 90% specific binding to 10% specific binding over an 81-fold increase in the concentration of the unlabeled ligand.

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More simply, virtually the entire curve will cover a 100-fold change in concentration. The slope factor (Hill factor) is used to quantify the steepness of a competitive binding curve. A standard competitive binding curve that follows the law of mass action has a Hill slope of -1.0. If the slope factor is far from -1.0 (shallower curve) then the binding does not follow the law of mass action with a single site. We derived the competition-binding curve of MAG-Fc and Nogo66-AP for NgR as a function of MAG-Fc binding to cerebellar neurons in the presence of increasing concentrations of Nogo66-AP. From the competition-binding curve (Figure 5.10) we calculated the IC_{50} (inhibitory concentration 50%) to be approximately 120 nM. The computed Hill factor is –1.0 (Standard Error = $7.1x10^{-8}$, R_2=1) supporting a model in which Nogo66 and MAG compete for binding to a single receptor site.

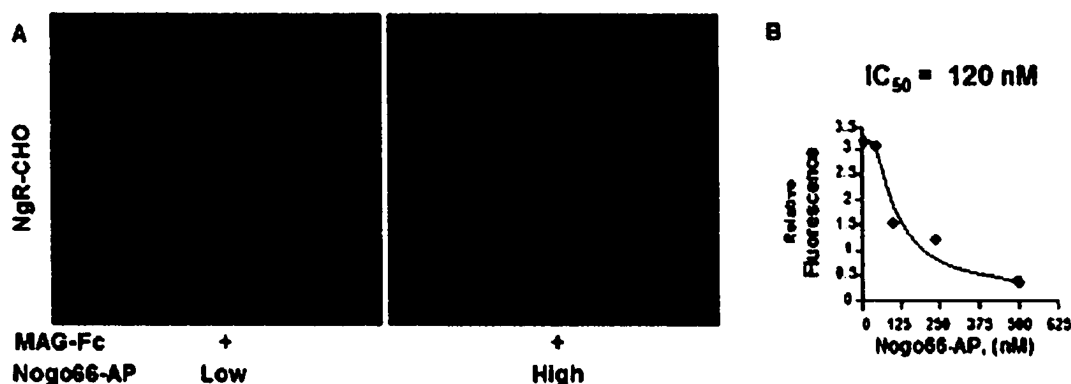

Figure 5.10 - Nogo66 competes with MAG for binding to NgR.
(A) Confluent monolayers of CHO cells expressing NgR were incubated with MAG-Fc (20 μg/ml) along with Nogo-66-AP at 3.5 μg/ml (Low) or 7 μg/ml (High) for 2 hr before being fixed and immunostained for MAG.
(B) The IC_{50} of the Nogo66-MAG competition for the NgR was determined by measuring binding of labeled neurons to immobilized MAG-Fc in the presence of various concentrations of Nogo66-AP (5–500 nM). The IC_{50} was calculated to be 120 nM. The Hill factor was calculated to be –1.0. Results are the mean of sextuplets and standard errors are less than 0.5% of the measured fluorescence and are therefore smaller than the symbols.

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Consensus Sequence Generated for MAG, Nogo66 and OMgp

Nogo66, MAG and OMgp competition for the same receptor site raises questions about their structural similarity and about the distinctness of their binding sites. In the absence of NMR data for any of the three proteins, we used the AlignX component of VectorNTI to search for sequence and predicted structural similarities. We obtained a consensus sequence between the myelin-associated inhibitors of axonal growth (Figure 5.11).

```
   Nogo66  .......... .......... .......... .......... ...RIYKGVI
   MAG_d5  .......... .......... .......... .......... ..........
     OMgp  LNLSSNKLWT VPTNMPSKLH IVDLSNNSLT QILPGTLINL TNLTHLYLHN

   Nogo66  QAIQKSDEGH PFRAYLESEV AISEELVQKY SNSALGHVNS TIKELRRLFL
   MAG_d5  CVVKSNPEPS VAFELPSRNV TVNETEREFV YSERSGLLLT SILTLRGQAQ
     OMgp  NKFTFIPEQS FDQLLQLQEI TLHNNRWSCD HKQNITYLLK WVMETKAHVI
Consensus  N I   PE S     L   EV TI E       H   IGHLLS SILELRA  I

   Nogo66  VDDLVDSLK. .......... .......... .......... ..........
   MAG_d5  APPRVICTSR NL........ .......... .......... ..........
     OMgp  GTPCSKQVSS LKEQSMYPTP PGFTSSLFTM SEMQTVDTIN SLSMVTQPKV
Consensus  A P V  LS
```

Figure 5.11 – Alignment of MAG, Nogo-A and OMgp sequences shows a consensus sequence.

The consensus sequence is located within the fifth Ig-like domain of MAG and it covers residues that were previously identified by Zixuan Cao (Filbin, unpublished data) as necessary for MAG inhibitory activity. Within the consensus region, similarity between Nogo66, MAG and OMgp is maximal in two distinct peaks, E439 and S466-G478 of MAG. These amino acid residues correspond to E208 and L235-R247 in OMgp and E15 and L42-R54 of Nogo-

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66. The results correlate with data from Strittmatter's lab identifying two peptides within the Nogo-66 sequence: N1-31, which is necessary for high affinity binding to NgR, and N31-55, which is responsible for the inhibitory activity (GrandPre et al. 2002). We used the consensus sequence to generate MAG-derived peptides that might hinder receptor-ligand interactions without receptor activation. Other members of the Filbin group are currently testing the peptides.

We demonstrated that MAG is a ligand for the Nogo-66 receptor and that NgR is necessary for transducing MAG's inhibitory signal to responsive neurons. OMgp, another myelin inhibitor, has also recently been identified as a ligand competing with Nogo-66 for NgR (Wang et al. 2002b). In the past few years, results from different studies have been contradictory. Although there was consensus that MAG, OMgp and Nogo were potent inhibitors of axonal growth *in vitro*, neutralizing any one of them yielded poor results *in vivo.* Following spinal cord trauma, treatment with the IN-1 antibody, a monoclonal antibody to Nogo proteins, induces the regeneration of only a small percentage of the injured axons (Schnell and Schwab 1993; Bareyre et al. 2002). As mentioned previously, experiments on the MAG null mice also yielded poor results (Montag et al. 1994). Our results provide a reasonable explanation for the lack of regeneration induced by blocking any single molecule, i.e. Nogo, since the residual activity by MAG and OMgp would be

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sufficient to still inhibit neurite outgrowth dramatically. Support for this explanation is also provided by the fact that treatment of injured rats with NEP(1-40), a Nogo66-derived peptide that partially antagonizes NgR, results in better regeneration than treatment with the IN-1 antibody (GrandPre et al. 2002). The NEP(1-40) peptide still shows a weak inhibitory activity that may explain its failure to induce complete regeneration. The presence of two sites within the consensus sequence suggests that it may be possible to generate a peptide that blocks receptor-ligand interactions maximally without triggering the signaling cascade. Such a peptide, especially if small enough to cross the blood-brain barrier, could have significant effects in the treatment of spinal cord injury.

Our data also confirm previous results (Tang et al. 1997a; Liu et al. 2002), which demonstrated that MAG binding to the functional receptor is independent of sialic acid recognition. Like the other members of the siglec family, MAG binds sialic acid bearing gangliosides, notably GT1b and GD1a, and this may induce clustering of receptors and transduction of a signal. However, when we consider that binding of MAG to NgR is independent of removal of sialic acid residues (Figure 5.4; Liu et al. 2002), that axonal growth inhibition by MAG is not affected by sialidase treatment (Tang et al. 1997a), and that MAG inhibitory activity is NgR-dependent (Figures 5.7 and 5.8) we conclude that the MAG-gangliosides interaction is not necessary for MAG's inhibition of neurite outgrowth. Our model does not exclude that MAG binding to the gangliosides may have a significant role in the stabilization of the axon-

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glia interface and the prevention of aberrant sprouting. However, our data suggest that NgR is the functional MAG receptor that is necessary and sufficient for inhibition of axonal regeneration.

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CHAPTER 6 - MYELIN INHIBITION IS MEDIATED BY A NGR-P75NTR RECEPTOR COMPLEX

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Recent evidence has suggested that p75NTR is necessary for MAG inhibition of axonal growth (Yamashita et al. 2002). This report also suggests that p75NTR activation is mediated by gangliosides on the neuronal surface. Since we have already demonstrated that sialylated glycans are not required for MAG activity (Tang et al. 1997a), we questioned if NgR, the functional receptor for MAG, interacted with p75NTR to inhibit axonal growth.

Both MAG and Nogo66 Precipitate p75NTR from Primary Cerebellar Neurons

It was shown that MAG associates with endogenous p75NTR from postnatal cerebellar neurons (Yamashita et al. 2002). We reasoned that if NgR mediated MAG association with p75NTR, then MAG, Nogo-66 and, possibly, NgR could co-precipitate p75NTR from neuronal lysates. We examined these interactions using lysates from P13 rat cerebellar neurons. In the MAG-Fc precipitates, the presence of p75NTR was confirmed by anti-p75NTR western blot (Figure 6.1). Further, both Nogo66-AP and NgR-AP precipitates also contained p75NTR. Thus, p75NTR may be the signal-transducing component of an NgR-p75NTR receptor complex that mediates the activity of MAG and Nogo-66. As NgR-AP can co-precipitate with p75NTR in a ligand independent manner, the data suggest a constitutive association between NgR and p75NTR.

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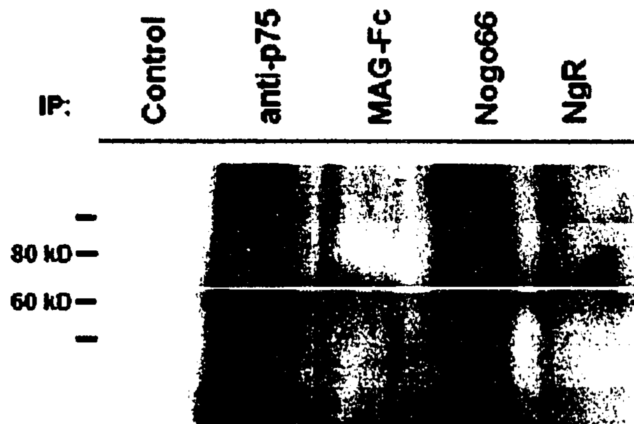
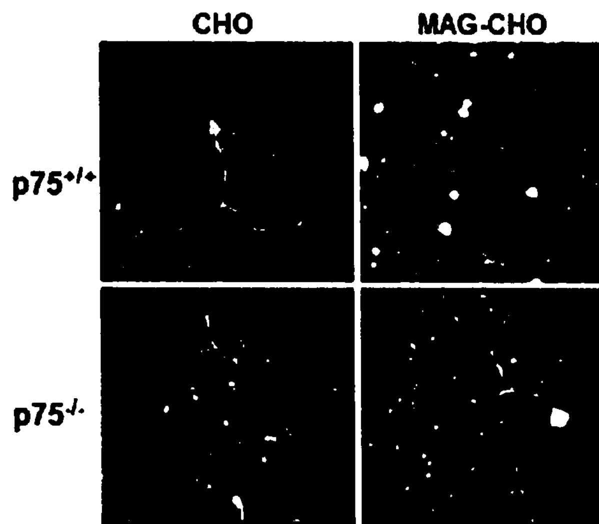


Figure 6.1 – MAG-Fc and Nogo66 co-precipitate p75NTR.
Lysates of PND13 cerebellar neurons were incubated with MAG-Fc, Nogo66-AP, NgR-AP or an anti-p75NTR antibody as indicated. Precipitated proteins were dissociated, separated in a 12% PAGE, transferred to PVDF membranes, and immunostained for p75NTR.



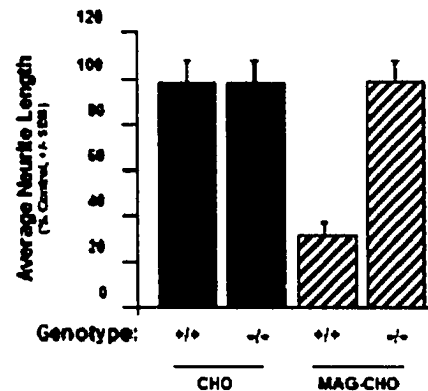


Figure 6.2- Inhibition of Neurite outgrowth by MAG requires p75NTR.
DRG neurons (P7) from either wild type ($p75^{+/+}$) or p75NTR null ($p75^{-/-}$) mice were plated on confluent monolayers of MAG-expressing (hatched bars) or control (black bars) CHO cells. After 24 hr., the neurons were fixed and immunostained with anti-GAP43 antibody to visualize neurite outgrowth.

onto a substrate of immobilized L1-Fc, and MAG-Fc (20 μg/ml) was added. After 4 hr incubation, cultures were fixed and stained for GAP43. Results show the mean length of the longest neurite per neuron (+/- sem) for 180–200 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from wild type neurons grown on control CHO cells.

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Inhibition by MAG, Nogo66 and Myelin Requires p75NTR

To confirm that p75NTR is a required component of the MAG inhibitory pathway, we measured neurite outgrowth from $p75NTR^{exonIII-/-}$ (Lee et al. 1992) and wild-type mice. The mutant mice express a truncated p75NTR that lacks its ligand-binding domain. When cultured on monolayers of MAG-expressing CHO cells, DRG neurons from the p75NTR mutant mice were insensitive to MAG inhibition of neurite outgrowth (Figure 6.2). As DRG neurons from wild-type mice were strongly inhibited, we concluded that p75NTR is indeed required for MAG inhibition of neurite outgrowth.

Dorsal root ganglia contain a heterogeneous population of neurons. Different DRG subpopulations are known to respond to distinct trophic signals due to differential neurotrophin receptor expression (McMahon et al. 1994). Isolectin B_4 (IB4), a lectin from the plant *Griffonia simplicifolia*, has been found to recognise α-D-galactose carbohydrate residues on the surface of a subset of DRG neurons (Silverman and Kruger 1988). There is evidence that the IB4-positive ($IB4^+$) DRG neurons from adult rat do not express p75NTR (Bennett et al. 1996) and that they sprout robustly *in vivo* after spinal nerve injury (Li and Zhou 2001). Thus, we decided to verify if we could identify discrete DRG subpopulations with distinct responses to MAG within a single neurite outgrowth experiment.

Immunocytochemistry of P8 DRG neurons cultured on a laminin substrate indicate that the $IB4^+$ neurons express NgR but they do not express

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p75NTR (Figure 6.3). The IB4⁻ population is shown to express both NgR and p75NTR (Figure 6.3). Further, when cultured on a monolayer of MAG-expressing cells, the IB4⁺ subpopulation is insensitive to MAG inhibition of axonal growth (Figure 6.4).

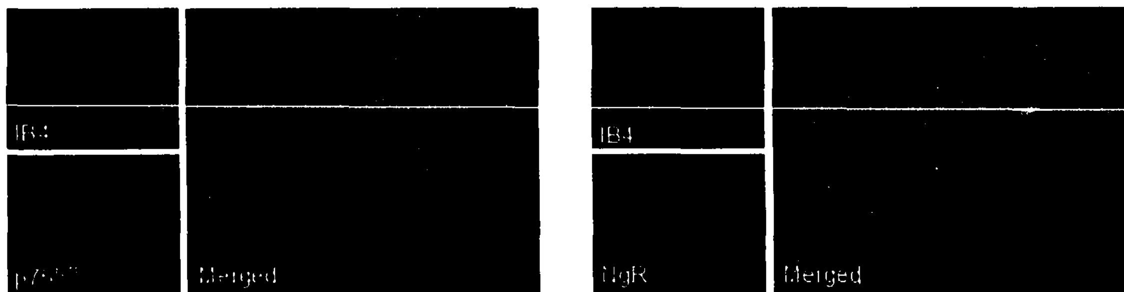

Figure 6.3 – IB4⁺ DRG neurons express NgR but not p75NTR.
P8 DRG neurons were plated onto laminin-coated 8-chamber slides and cultured overnight in SATO medium. Cultures were fixed with paraformaldehyde and double-stained, as indicated, with IB4-FITC and either anti-NgR or anti-p75^{NTR} antibodies. The arrowhead points to same neuron in both frames. Each larger image (merged) superimposes the two images (of the same field) to its left.

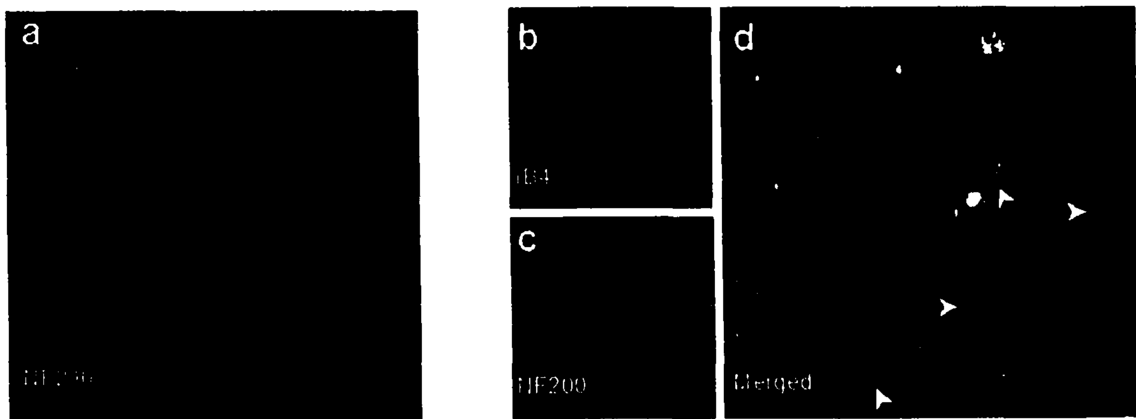

Figure 6.4 – IB4⁺ DRG neurons are insensitive to MAG.
P8 DRG neurons were plated on confluent monolayers of MAG-expressing (b-d) or control (a) CHO cells. After 24 hr., the neurons were fixed and double-stained with anti-NF200 antibody (a and c) and IB4-FITC (b). (b) and (c) are images of the same field and they are merged in (d). Arrowheads point to neurons extending long neurites.

As NgR mediates the inhibition of neurite outgrowth from neurons by MAG, Nogo-66 and OMgp, we decided to verify if p75NTR was required for

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inhibition by myelin in general. We transfected NG108 cells with a full-length p75NTR construct (HA-p75) or a dominant-negative p75NTR construct (Δ12-p75), which lacks the complete intracellular domain of p75NTR. After inducing cell differentiation, we cultured the transfected cells on myelin or PLL substrates and monitored their ability to extend processes. When MAG-Fc or Nogo66-AP proteins were added to the cultures, the full-length p75NTR transfected cells failed to extend neurites on the control substrate (Figure 6.5).

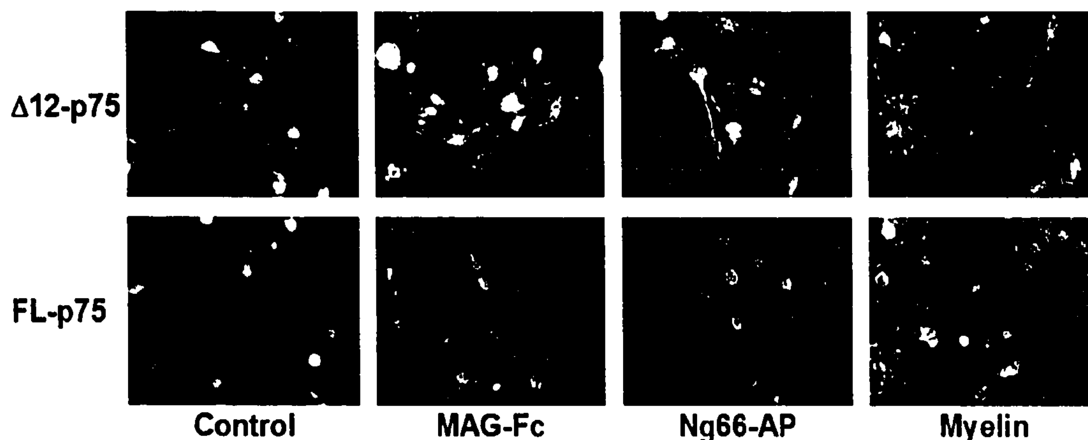

Figure 6.5 - A dominant-negative p75NTR blocks the inhibitory activity of MAG, Nogo-66 and myelin.

NG108 cells were transfected with cDNAs encoding either the full length $p75^{NTR}$ or $p75^{NTR}$ lacking the whole intracellular domain (Δ12- p75). Following transfection, cells were differentiated with dibutiryl-cAMP (1 mM) for 24h, prior to plating on substrates of poly-L-lysine (PLL) or purified myelin. MAG-Fc or Nogo66-AP (20nM) were added as indicated. The cultures were fixed after 24h and immunostained stained for GAP43.

The HA-p75 transfected cells also failed to extend processes on a myelin substrate. However, the Δ12-p75 transfectants produced robust outgrowth in the presence of MAG-Fc and Nogo66-AP as well as on the myelin substrate (Figure 6.5). Meanwhile, neither vector had an effect on axonal growth under control conditions. These results indicate that p75NTR is required for myelin inhibition of neurite outgrowth from primary neurons.

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We have demonstrated that p75NTR is required for MAG inhibition of neurite outgrowth from CNS neurons. Further, we expanded the p75NTR requirement to inhibition by myelin in general. The findings that p75NTR is the signal transducer for the NgR-p75NTR complex raises important questions about the mechanisms of myelin-induced inhibition of axonal growth.

The p75NTR was identified over a decade ago as the low-affinity neurotrophin receptor (Chao et al. 1986). Although initially relegated to play a secondary role to the Trk receptors, p75NTR is now known to have a key role in many aspects of cellular biology (Hempstead 2002). Among the complex set of p75NTR functions is axonal guidance during development of the nervous system. Examination of thalamic innervations of the cortex in p75NTR$^{-/-}$ mice revealed a role for p75NTR in supporting growth cone morphology and function in subplate neurons (McQuillen et al. 2002). Also, the p75NTR$^{-/-}$ mice phenotype includes pathfinding errors and mistargeting of sympathetic neurons (Lee et al. 1994).

There are similarities between the mechanisms underlying axon guidance and axonal growth inhibition. Axonal guidance can be viewed as the promotion or inhibition of growth in a determined direction. Endogenous cAMP levels can switch the turning behavior of spinal neurons in response to a MAG gradient (Song et al. 1998). High cAMP levels will induce a turn of the growth cone toward the MAG gradient (positive response) while low cAMP levels will induce a turn away from the gradient (negative response). Similarly, axonal

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growth from CNS neurons in the presence of MAG will be inhibited (negative response), when endogenous cAMP levels are low, or promoted (positive response), when endogenous cAMP levels are high (Cai et al. 2001; Qiu et al. 2002).

Growth cone motility is also influenced by intracellular calcium concentrations (Gomez and Spitzer 1999). High frequency transient elevations in Ca2+ levels result in stalling of the growth cone (negative response), while blocking the transients results in accelerated growth (positive response). Correspondingly, MAG induces a transient Ca2+ elevation during growth cone repulsion (negative response) (Wong et al. 2002). Not surprisingly, the MAG induced Ca2+ elevation is also mediated by the NgR-p75NTR complex (Wong et al. 2002).

With the discovery of the interaction between NgR and p75NTR, we now have a specific lead into the mechanism of how myelin inhibits neurite outgrowth. The identification of p75NTR, a known player in axon guidance (Lee et al. 1994; McQuillen et al. 2002), as the transducing unit of the inhibitory receptor complex may allow us to draw upon experimental knowledge from the guidance pathway and use it to further elucidate the inhibitory pathway. Growing axons depend on their ability to collect information about their environment. We believe that p75NTR operates within a growing axon as a sensor that acts on a molecular switch between positive and negative responses. We have demonstrated that inactivation of the NgR-p75NTR complex is an important tool to induce axonal regeneration in

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cultured neurons. Whether blocking NgR association with p75NTR or preventing p75NTR expression after injury can improve axonal regeneration *in vivo* remains a subject for further studies.

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CHAPTER 7 – CONCLUSIONS

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The work described herein indicates that NgR mediates the inhibition of axonal growth by MAG through activation of p75NTR and that the same receptor complex also mediates inhibition by Nogo and myelin. Indeed, work by others confirms that the same signal transduction pathway applies to growth inhibition by MAG, Nogo and OMgp (Wang et al. 2002a; Wong et al. 2002). This finding elucidates a potential target for developing therapies for spinal cord injury.

It should be noted that additional inhibitors of regeneration are also present in myelin, i.e. amino-Nogo and chondroitin-sulfate proteoglycans (CSPG). Though their activity is not dependent on the NgR/p75NTR complex, studies have shown that these additional inhibitors share a common pathway downstream of p75NTR: both amino-Nogo and CSPG depend on Rho-A to inhibit neurite outgrowth and altering the endogenous cAMP levels can block their activity (Schwab 2003; Silver 2003).

Immediately after injury and prior to the formation of the glial scar, the inhibitory molecules present in myelin and myelin debris represent the largest obstacle to axonal regeneration in the CNS. Progress has been made in keeping neurons alive through the administration of neurotrophic factors and anti-inflammatory drugs. Through a blockade of the NgR/p75NTR complex, we can now attempt to take advantage of this short window of opportunity and stimulate the initial phase of regeneration.

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CHAPTER 8 - MATERIALS AND METHODS

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Neurite outgrowth assays

The neurite outgrowth assay on MAG-expressing cells was carried out as described previously (Cai et al., 1999; Mukhopadhyay et al., 1994). In brief, 5×10^4 isolated cerebellar, hippocampal or DRG neurons from P4-8 rat pups were plated onto confluent monolayers of control and MAG-expressing CHO cells in 8-chamber tissue culture slides (Lab-Tek). Where indicated BDNF, pertussis toxin (PTX), pertussis toxin B-oligomer (PTB), NF023, polyclonal antibody to NgR (Santa Cruz) or NgR-AP, prepared as previously described, were included at the concentrations indicated or preincubated with the cells before the addition of neurons, After 18-24 hours of incubation, the cultures were fixed for 30 min with 4% paraformaldehyde, permeabilized with ice-cold methanol and immunostained with a rabbit polyclonal antibody against GAP43 (1:4000, from R. Curtis and G. Wilkins Imperial College, London). The slides were mounted in Permafluor (Immunon) and viewed under a fluorescence microscope. The length of the longest neurite for each GAP43-positive neuron for the first 180-200 neurons encountered when scanning the slide in a systematic manner was determined using a SimplePCI image analysis program.

For the assays with soluble MAG-Fc, prepared as described previously (Tang et al., 1997a) isolated neurons were plated onto an immobilized substrate of the growth-promoting molecule L1-Fc (20 μg/ml). Where indicated neurons were treated with compounds described above or with 2 U/ml of PI-PLC for 30 min at 37°C, before addition to the L1-Fc substrates. Incubation was for 4 h

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or 18 h before neurons were fixed and stained for GAP43 as described above. For the 4 h assays, 800-1000 neurons were scored for the presence of neurites and for the 18 h assays, the neurites from 180-200 neurons were measured as described above.

For the NG108 cells, the cDNA encoding the LRR and LRRCT domains of NgR was subcloned into pDisplay (Invitrogen) and the resultant construct was transfected into NG108 cells using Lipofetamine (Life technologies). Upon transfection, NG-108 cells were differentiated with dibutyl-cAMP (1 mM) for 24h, prior to plating on substrates of PDL or recombinant MAG (rMAG). After 24 hours, the cells were fixed with 4% paraformaldehyde, permeabilized and stained with an anti-HA antibody (Santa Cruz Biotechnology). Quantitation of neurite length and statistical analysis were performed as described previously.

For the NG108 cells, HA-tagged cDNAs encoding either the full length $p75^{NTR}$ or $p75^{NTR}$ lacking the intracellular domain (Δ12- p75) = $p75^{NTR}$ were transfected into NG108 cells using Lipofetamine (Life technologies). Upon transfection, NG-108 cells were differentiated with dibutiryl-cAMP (1 mM) for 24h, prior to plating on substrates of PLL or purified myelin. MAG-Fc or Nogo66-AP (20nM) was added where indicated. The cultures were fixed after 24h and stained as described above.

For the G-protein assays, encoding mutant G-protein subunits or wild type m2 muscarinic receptor were transfected into COS-7 cells, NG108 cells, cerebellar or DRG neurons using Lipofetamine (Life technologies). Cells were

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plated and used as described. Upon transfection, NG-108 cells were differentiated with dibutiryl-cAMP (1 mM) for 24h, prior to plating.

Binding assay and immunostaining for NgR

CHO cells expressing MAG or NgR (flag-tagged), or control cells were plated onto poly-l-lysine coated 8-chamber slides and grown to confluence. Cerebellar or DRG neurons (P4-7) were plated onto laminin-coated wells and cultured overnight. For binding of MAG-Fc, 20 μg/ml of MAG-Fc was added to the cells and allowed to bind for 2 hours before being washed, fixed and stained for MAG, using a MAG monoclonal antibody (Santa Cruz) and a phycoerthrin conjugated second antibody (Sigma). For binding of NgR-AP, 3.7 μg/μl of NgR-AP were added to each well and incubated for a further 2 h, before being washed, fixed and stained with a NgR polyclonal antibody (Santa Cruz), followed by FITC-conjugated second antibody (Santa Cruz). Where indicated neurons or NgR-expressing CHO cells were treated with PI-PLC or neuraminidase, 0.02 U/ml (Calbiochem) before addition of MAG-Fc or NgR antibody. Where indicated, Nogo-66-AP (3.5 and 7 mg) was included in the assays along with MAG-Fc. The recombinant Nogo-66 protein fused to AP was prepared as described before.

Precipitation assays

Cerebellar neurons, DRG neurons (P4-P7), CHO cells expressing NgR-flagged or control CHO cells were lysed in 50 mM Tris-HCl, Ph 7.5 RIPA

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buffer (Amersham). After pre-clearing with protein A sepharose, the lysates were incubated with either MAG-Fc (20 μg/ml), MAG (d1-3)-Fc (20 μg/ml), anti-p75NTR antibody (Moses Chao, 0.5 μg/ml) or an anti-flag antibody (1:1000, Santa Cruz) for 4 h at 4°C, after which 50 μl of 50% protein A sepharose beads were added and incubation continued for 60 min at 4°C. The samples were centrifuged and the pellet washed twice with lysis buffer. The proteins in the pellet were dissociated by the addition of SDS sample buffer and boiling for 10 min before being centrifuged and the proteins in the supernatant separated in a 10 % PAGE, before being transferred to PVDF membrane and stained for NgR or p75NTR.

Immunostaining

CN or DRG neurons were plated onto laminin-coated 8 wells slides and cultured overnight in SATO medium. Cultures were fixed for 30 min with 4% paraformaldehyde and immunostained with a rabbit polyclonal antibody against human NgR (Advance Diagnostics) or $p75^{NTR}$ extracellular domain (from Dr. Moses Chao), followed by biotinylated anti-rabbit and streptavidin-Texas Red.

Where indicated, IB4-FITC Sigma) was added to the Streptavidin-Texas Red.

Measurement of PLC activation

Approximately 24 hr after transfection, cells were transferred into six-well plates (±0.75 x 10^6 cells per well), and $_3$H-inositol (20 Ci/mmol, NEN) was

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added at 3 μCi/ml of inositol-free growth medium. After the labeling period, cell lysates were applied to an anion-exchange column and induced increases in intracellular inositol monophosphates measured by analysis of the fractions.

Expression of recombinant proteins

Vectors for expressing the various fusion proteins were generated as previously described. Proteins were expressed by transiently transfecting COS-7 cells with 40 g plasmid DNA/5 x10^6 cells/175 cm2 flask using a DEAE-dextran method. Purification of the Fc fusion proteins was done using a protein-A sepharose column. AP fusion proteins contain a poly-His tag for purification using a nickel column.

Prior to formation of multivalent complexes, concentrated tissue culture supernatants were biotinylated by incubation with recombinant BirA enzyme (Avidity, Denver, CO) in 10 mM Tris-HCl, pH 8, 7.5 mM MgCl2, 5mM

NaCl, 5mM ATP and 1 mM biotin, overnight. The proteins were then buffer-exchanged into PBS to remove free biotin prior to use.

Multivavalent complexes were formed by incubation of the biotinylated proteins with Streptavidin (Amersham) in a 6:1 ratio.

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