

Myelin-Associated Glycoprotein Interacts with the Nogo66 Receptor to Inhibit Neurite Outgrowth

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Summary

Myelin inhibitors of axonal regeneration, like Nogo and MAG, block regrowth after injury to the adult CNS. While a GPI-linked receptor for Nogo (NgR) has been identified, MAG's receptor is unknown. We show that MAG inhibits regeneration by interaction with NgR. Binding of and inhibition by MAG are lost if neuronal GPI-linked proteins are cleaved. Binding of MAG to NgR-expressing cells is GPI dependent and sialic acid independent. Conversely, NgR binds to MAG-expressing cells. MAG, but not a truncated MAG that binds neurons but does not inhibit regeneration, precipitates NgR from NgR-expressing cells, DRG, and cerebellar neurons. Importantly, NgR antibody, soluble NgR, or dominant-negative NgR each prevent inhibition of neurite outgrowth by MAG. Also, MAG and Nogo66 compete for binding to NgR. These results suggest redundancy in myelin inhibitors and indicate therapies for CNS injuries.

Introduction

Inhibitors of axonal regeneration that are present in myelin are one of a number of factors that prevent recovery after injury in the adult mammalian CNS (Qiu et al., 2000; Schwab and Bartholdi, 1996). Other factors include a lack of trophic factors and formation of the glial scar, which is accompanied by the upregulation of mainly proteoglycan inhibitors of regeneration (Fitch and Silver, 1999). However, immediately after injury and before the scar has had time to mature, the main obstacle to regeneration appears to be inhibitors, both secreted (Tang et al., 1997b, 2001) and membrane bound, in myelin. Previous work has shown that if mice are immunized with myelin before a spinal cord injury is inflicted, regeneration occurs (Huang et al., 1999). This implies that if inhibitors of regeneration in myelin are neutralized, at least immediately after injury, regeneration can occur

with the glial scar forming around these regenerated axons.

To date, three inhibitors of regeneration have been identified in myelin. These are NogoA (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), an antigen of the IN-1 antibody (Caroni and Schwab, 1988), myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), and oligodendrocyte-myelin glycoprotein (OMgp), which was recently shown to inhibit neurite outgrowth in vitro (Wang et al., 2002). All three of these inhibitors are found in a myelin membrane, immediately adjacent to the axon (Baumann and Pham-Dinh, 2001; Mikol et al., 1990; Trapp, 1988; Huber et al., 2002). Therefore, they are in an optimal location to mediate axon-glia interactions. Unlike the wealth of information on receptors and signaling cascades of inhibitory molecules involved in axonal guidance during development (Skaper et al., 2001; Tessier-Lavigne and Goodman, 1996), very little is known about how myelin inhibitors exert their effects. Currently, two events have been associated with signaling by myelin inhibitors. If the small GTPase, Rho, is inactivated (Lehmann et al., 1999) or if neuronal cAMP is elevated (Cai et al., 1999), inhibition by not only an individual myelin inhibitor, MAG, is blocked, but so also is inhibition by myelin in general. This suggests that there is either a common signaling pathway for all myelin inhibitors or different pathways converge at a point upstream from Rho and cAMP involvement.

Although two distinct inhibitory domains have been described for NogoA, the isoform of Nogo expressed by oligodendrocytes (Chen et al., 2000; Fournier et al., 2001), only a single receptor has been identified to date (Fournier et al., 2001). This Nogo receptor, NgR, binds with high affinity to a 66 amino acid, extracellular sequence, termed Nogo66, that is found in all three Nogo isoforms (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000) and is anchored to the membrane via a glycosylphosphatidylinositol (GPI) linkage (Fournier et al., 2001). Interestingly, OMgp has also been shown to interact with the NgR and to require this interaction for its inhibitory effects (Wang et al., 2002).

Here, we show that like Nogo and OMgp, MAG interacts with the NgR receptor to exert its inhibition. MAG binds specifically and competes with Nogo66 for binding to an NgR-expressing cell line. Conversely, NgR binds specifically to a MAG-expressing cell line. Also, MAG precipitates NgR specifically from NgR-expressing CHO cells, cerebellar neurons, and DRG neurons, while a truncated, noninhibitory form of MAG that binds neurons in a sialic acid-dependent manner does not. Importantly, a soluble form of NgR, an antibody to NgR, or a dominant-negative NgR each block inhibition of neurite outgrowth by MAG. These results indicate redundancy in the inhibitors of regeneration in myelin and point to NgR as an attractive target for development of possible therapies to encourage regeneration after CNS injury.

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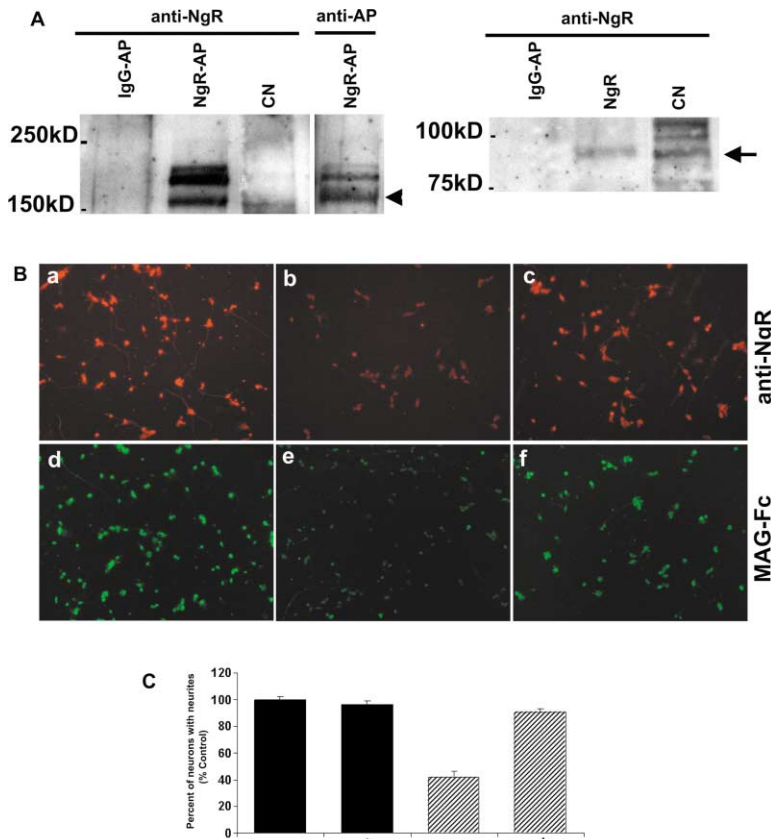


Figure 1. Binding of MAG-Fc and Inhibition of Neurite Outgrowth Requires GPI-Anchored Neuronal Proteins

(A) Proteins from lysates of NgR-expressing CHO cells and cerebellar neurons or 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. CN = cerebellar neuron lysate

(B) Cerebellar neurons (P4) were plated onto laminin-coated wells and cultured overnight before NgR antibody (1:1000) (a–c) or MAG-Fc (25 μ g/ml) (d–f) were added and incubated for 2 hr, after which 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-phycoerythrin-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.

(C) Cerebellar neurons (P4), either treated (+) or not (–) with PI-PLC, were plated onto a substrate of immobilized L1-Fc, and MAG-Fc (20 μ g/ml) was added (hatched bars) and cultured for 4 hr, after which the cultures were fixed and stained for GAP43. Results are the number of neurons with neurites, expressed as a percentage of the control, untreated neurons, in the absence of MAG-Fc (black bars). The results are the mean of at least four experiments, \pm SEM.

Results

Binding and Inhibition of Neurite Outgrowth by MAG Each Require a GPI-Linked Protein

Previously, we showed that a soluble, chimeric form of MAG, MAG-Fc (the extracellular domain of MAG fused to the Fc portion of human IgG), was able to specifically precipitate a number of neuronal surface proteins (De Bellard and Filbin, 1999). Of these proteins, one of about 80 kDa is approximately the same molecular weight as the recently cloned Nogo receptor, NgR. Because of this similarity in size, we wanted to determine if MAG was bringing about its inhibitory effect through an interaction with this same receptor. As the NgR receptor is a GPI-linked protein (Fournier et al., 2001), neurons were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove all GPI-linked proteins, and the ability of MAG to bind and inhibit neurite outgrowth was assessed. We first wanted to ensure that the commercially available NgR antibody did indeed recognize the NgR. Figure 1A shows that this antibody recognizes an 80 kDa protein expressed by NgR-expressing CHO cells and by cerebellar neurons. In addition, it binds to a recombinant, soluble form of NgR consisting of the entire extracellular domain fused to alkaline phosphatase (NgR-AP Mol.Wt. 150 kDa). This same band is recognized by an antibody to alkaline phosphatase.

The NgR antibody was then used to immunostain neurons. Strong binding of both this NgR antibody and MAG-Fc to cerebellar neurons is observed (Figure 1B).

In contrast, 1 hr after treatment of these neurons with PI-PLC, the binding of NgR antibody and of MAG-Fc are greatly reduced. However, 8 hr after PI-PLC treatment, the binding of NgR antibody and MAG-Fc to these neurons is restored, indicating the replacement of the GPI-linked proteins within this time (Figure 1B). Since the GPI-linked proteins are so rapidly replaced, we had to modify the neurite outgrowth assay we routinely use by reducing culture times from 18–24 hr to 2–6 hr, and rather than measuring neurite length, the number of neurons with neurites were counted. Removal of GPI-linked proteins from cerebellar neurons prevents the inhibition of neurite extension by MAG-Fc, while the same treatment has no effect on the ability of cerebellar neurons to extend neurites in the absence of MAG-Fc (Figure 1C). The reduced culture time required for this assay was not suitable for DRG neurons because under control conditions, they failed to extend detectable neurites by 6 hr. However, these results indicate that binding of MAG-Fc to neurons and its ability to inhibit neurite outgrowth are dependent on a neuronal GPI-linked protein.

MAG Binds to NgR in a Sialic Acid-Independent Manner

To determine if the PI-PLC-sensitive binding of MAG to neurons is attributable to the NgR, the ability of MAG-Fc to bind to NgR-expressing Chinese hamster ovary (CHO) cells and the ability of a soluble form of NgR, NgR-AP, to bind to MAG-expressing CHO cells was assessed. Figure 2Ab shows that MAG-Fc binds to NgR-express-

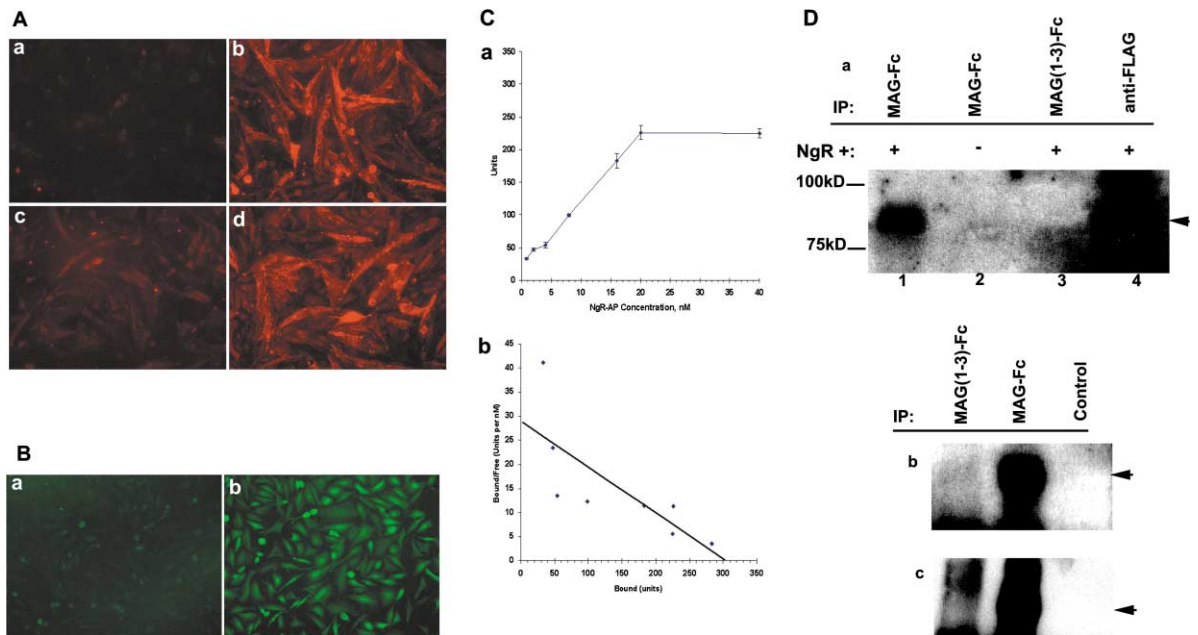


Figure 2. MAG Interacts with NgR

(A) Confluent monolayers of CHO cells expressing NgR (b-d) or control CHO cells (a) were incubated with MAG-Fc (20 μ g/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. (C) NgR-AP binding, at various concentrations, to immobilized MAG-Fc was measured (a). The K_d of NgR-MAG binding was calculated from bound NgR/free NgR plotted against bound NgR and calculated to be 8 nM (b). (D) Lysates of NgR-expressing or control CHO cells (a), cerebellar neurons (b), or DRG neurons (c) were precipitated with MAG-Fc, MAG (d1-3)-Fc, or an anti-flag antibody as indicated. Precipitated proteins were dissociated and separated in a 10% PAGE, transferred to PVDF membranes, and stained for NgR, using the ECL detection system. For (b) and (c), controls were without MAG-Fc. Arrows indicate NgR.

ing CHO cells. After PI-PLC treatment, this binding is lost (Figure 2Ac), and there is no binding of MAG-Fc to control CHO cells that do not express NgR (Figure 2Aa). In addition, although MAG is a sialic acid binding protein (Kelm et al., 1994), the binding of MAG-Fc to NgR CHO cells is sialic acid independent as treatment of these cells with sialidase has no effect on MAG-Fc binding (Figure 2Ad). In the converse experiment, NgR-AP bound specifically to MAG-expressing CHO cells and not to control CHO cells (Figure 2B). To determine the affinity of MAG's interaction with NgR, the binding of NgR-AP, over a range of concentrations, to MAG-expressing CHO cells (not shown) or to immobilized MAG-Fc (Tang et al., 1997a) was measured. A K_d of 8 nM was calculated (Figure 2C) using both approaches. This value is very similar to the affinities of both Nogo66 (7 nM) and OMgp (5 nM) in binding to NgR. Importantly, consistent with a direct interaction of these two molecules, MAG-Fc was able to precipitate NgR from lysates of NgR-expressing CHO cells, DRG neurons, or cerebellar neurons (Figure 2D). NgR was not precipitated from control CHO cells by MAG-Fc, nor from neurons by MAG (d1-3)-Fc, a truncated form of MAG that binds neurons in a sialic acid-dependent manner but has no effect on neurite outgrowth (Tang et al., 1997a) (Figure 2D).

Blocking NgR Prevents Inhibition of Neurite Outgrowth by MAG

If indeed MAG interacts with the NgR to bring about inhibition of neurite outgrowth, then blocking that inter-

action should block inhibition of axonal extension by MAG. To block NgR on neurons, a polyclonal NgR antibody was included in the neurite outgrowth assays with MAG-expressing CHO cells (Figure 3B) or with MAG-Fc (Figure 3C). The NgR antibody blocked inhibition of neurite outgrowth by MAG for both DRG and cerebellar neurons (Figure 3B). The antibodies had no effect on axonal growth on control CHO cells and a control antibody had no effect on inhibition by MAG (not shown). The NgR antibody also blocked the inhibition of neurite outgrowth from DRG neurons by MAG-Fc (Figure 3C). An alternative approach to blocking the interaction between MAG and its neuronal receptor is to include a soluble form of the receptor in the cultures (Flanagan and Cheng, 2000). In this way, the soluble receptor will compete for MAG binding with the neuronal receptor and so prevent interaction and, in turn, prevent the signal for inhibition from being transduced to the neuron. To test this possibility, soluble NgR-AP was included in the cultures. As shown in Figure 3A, NgR-AP blocked the ability of MAG-expressing CHO cells to inhibit neurite outgrowth in a dose-dependent manner. The NgR-AP protein had no effect on neurite outgrowth on the control CHO cells. Inhibition of neurite outgrowth by MAG-Fc can also be blocked by NgR-AP (Figure 3C).

In addition to soluble NgR-AP, inhibition of neurite outgrowth by MAG was blocked using a dominant-negative form of the NgR. The amino-terminal region of NgR covering both the leucine rich repeats (LRR) and C-terminal LRR (LRRCT) domains has been shown to be nec-

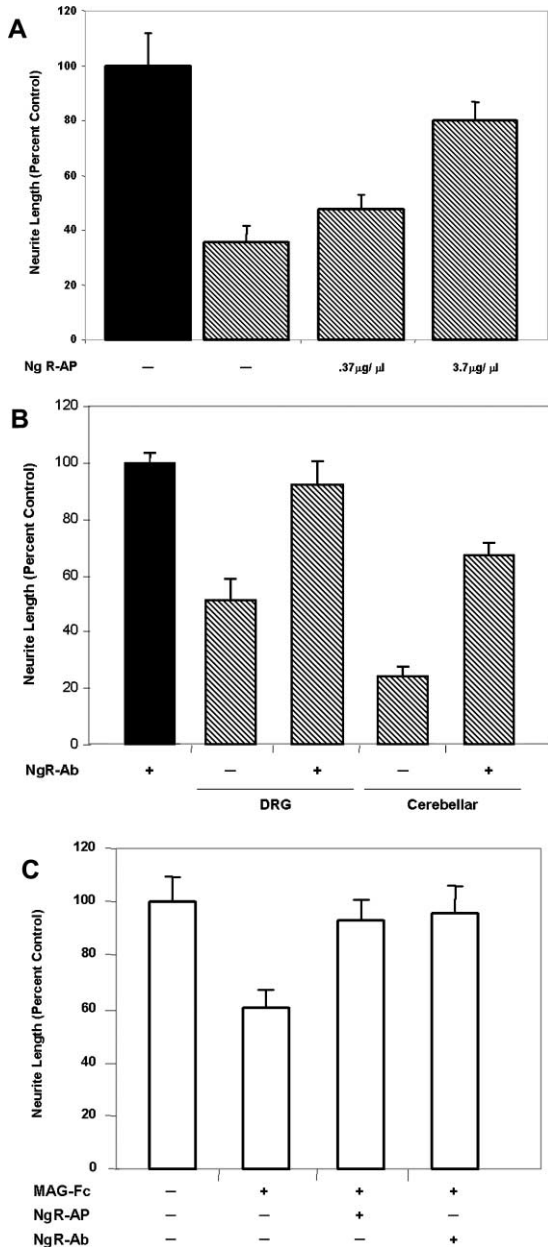


Figure 3. Soluble NgR and NgR Antibody Each Block MAG's Inhibition of Neurite Outgrowth

DRG neurons (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 $\mu\text{g}/\text{ml}$) was added (C). Where indicated, the monolayers were incubated with soluble NgR-AP at either 0.37 or 3.7 $\mu\text{g}/\mu\text{l}$ or NgR antibody (1:100) for 30 min prior to addition of neurons. For MAG-Fc, NgR-AP (3.7 $\mu\text{g}/\mu\text{l}$) 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, \pm 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; white bars, neurons with soluble MAG-Fc as indicated.

essential for interaction with both OMgp and Nogo66 (Wang et al., 2002). Therefore, we reasoned that the unique extreme C-terminal region of NgR, which is not required for ligand binding, might interact with a signaling coreceptor since the GPI-anchored NgR alone would not be expected to be able to transduce the inhibitory signal into the interior of responding neurons. Thus, it is conceivable that overexpression of a truncated NgR lacking only the extreme C-terminal region (N-NgR), but still containing the ligand binding LRR and LRRCT domains, would inhibit the function of wild-type NgR in a dominant-negative manner. As primary neurons proved to be refractory to transfection, we tested this hypothesis by introducing full-length (FL-NgR) or truncated forms of NgR, N-NgR, and, as a control, NgR missing the ligand binding domains (C-NgR) into NG108 cells, a neuronal cell line that is responsive to both MAG and myelin (Lehmann et al., 1999; McKerracher et al., 1994). As shown in Figure 4, immobilized recombinant MAG inhibited neurite outgrowth from differentiated NG108 cells. In contrast to full-length NgR or C-NgR, N-NgR overexpression resulted in robust neurite outgrowth of NG108 cells (Figures 4B and 4C). Expression of this mutant NgR had no effect on the ability of NG108 cells to extend processes in the absence of MAG. Together, these results suggest that the neutralizing effects of the dominant-negative NgR are specific for MAG.

MAG and Nogo66 Compete for Binding to NgR

If MAG and NogoA interact with the same receptor, the question becomes whether the interactions are competitive or noncompetitive. To address this issue, the ability of Nogo66, the extracellular sequence of Nogo that interacts with NgR, to block binding of MAG-Fc to neurons was assessed. When Nogo66-AP and MAG-Fc were included at the same concentration, there was little effect on MAG-Fc binding to NgR-expressing CHO cells (Figure 5A). However, when Nogo66-AP was added in a 2-fold molar excess, MAG-Fc binding was greatly reduced, indicating direct competition for the same binding site (Figure 5B). To assess more precisely the competition of MAG and Nogo66 for NgR, the IC_{50} (concentration of Nogo66 at which binding of MAG is reduced by 50%) was determined. By measuring MAG-Fc binding to cerebellar neurons in the presence of increasing concentrations of Nogo66, the IC_{50} was calculated to be approximately 120 nM (Figure 5C). An IC_{50} in the nanomolar range indicates that Nogo66 effectively displaces MAG binding and therefore that the two ligands compete directly for the same receptor.

Discussion

The results presented here demonstrate that the Nogo receptor, NgR, which specifically binds the extracellular sequence of 66 amino acids common to all Nogo isoforms, also binds MAG and is essential for MAG-mediated inhibition of neurite outgrowth from a variety of neurons. Furthermore, although MAG and Nogo66 have no obvious sequence similarities, they compete with each other for binding to NgR. In addition to Nogo and MAG, another myelin-specific protein, OMgp, which has recently been shown to inhibit neurite outgrowth, also

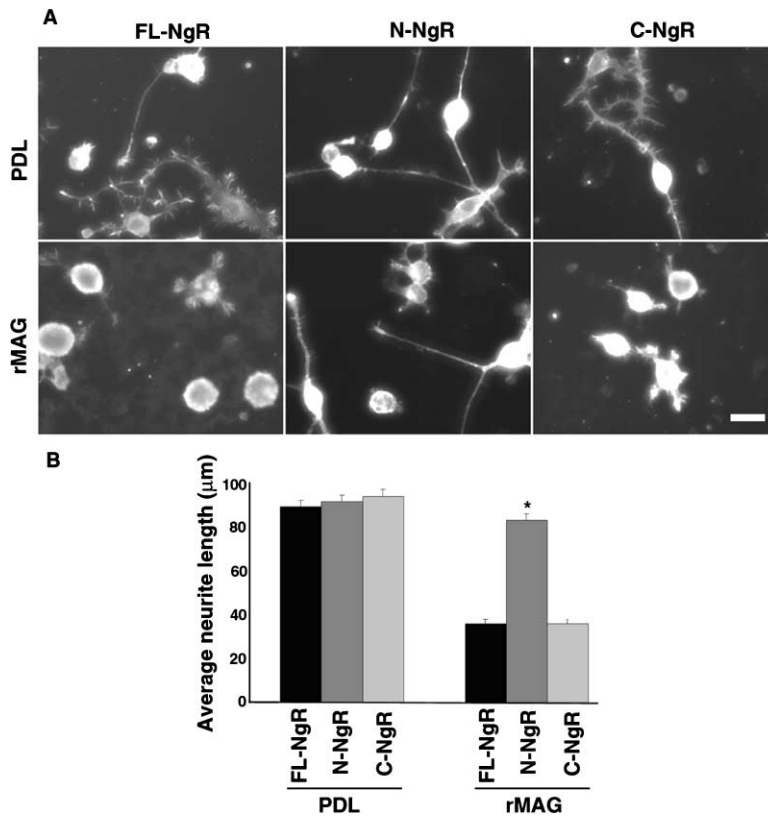


Figure 4. A Dominant-Negative NgR Blocks the Inhibitory Activity of MAG

(A) Differentiated NG108 cells expressing full-length (FL-NgR), N-terminal (dominant-negative) (N-NgR), or C-terminal (C-NgR) NgR were plated on either PDL or immobilized MAG substrates, incubated overnight, and then stained for GFP. Scale bar: 12 µm. (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.

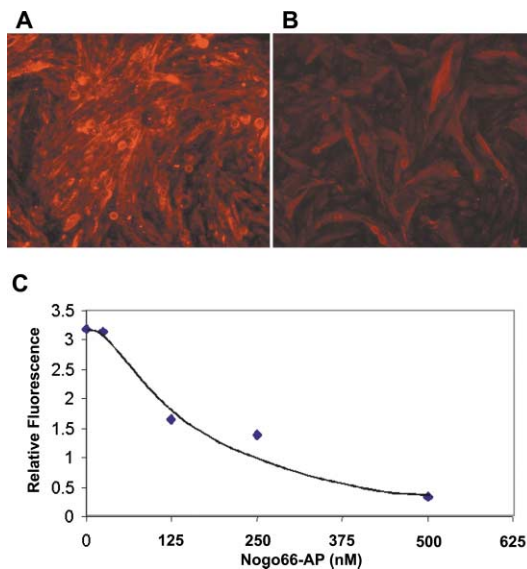


Figure 5. Nogo66 Competes with MAG for Binding to NgR
Confluent monolayers of CHO cells expressing NgR were incubated with MAG-Fc (20 µg/ml) along with Nogo66-AP at 3.5 µg/ml (A) or 7 µg/ml (B) for 2 hr before being fixed and immunostained for MAG. The IC_{50} for Nogo66-MAG competition for the NgR (C) was determined by measuring binding of fluorescently 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. 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.

interacts with the NgR and competes with Nogo66 for this receptor in exerting its inhibitory effect (Wang et al., 2002). Although we have not assessed whether MAG and OMgp also compete with each other for binding, given that Nogo66 competes with both MAG and OMgp for binding to NgR, it seems that OMgp is also likely to compete with MAG for binding to this receptor. This competition for binding to the same receptor may explain the apparent redundancy observed when a single inhibitory component is blocked in vivo. For example, after injury in the MAG knockout mouse, there is only very little, or no, improvement in axonal regeneration in vivo (Li et al., 1996; Montag et al., 1994). Likewise, in vivo application of a monoclonal antibody to Nogo, the IN-1 antibody, after injury, results in only a small percentage of regenerating axons (Schnell and Schwab, 1990, 1993). The effects on regeneration of blocking OMgp in vivo have not yet been reported. However, extrapolating from the results with MAG and Nogo, the prediction is that there would be little effect on regeneration in vivo if only OMgp is blocked. These results are important because the effects of these three potent inhibitors of regeneration can be blocked completely, at least in culture, by blocking a single receptor, NgR. Indeed, blocking the NgR with an antagonist of Nogo66 binding has been shown to induce more regeneration after injury (GrandPre et al., 2002) than when Nogo is blocked with IN-1 (Schnell and Schwab, 1990, 1993) or in MAG knockout mice. It remains to be determined if an effective antagonist can be developed that allows complete functional recovery. Conceivably, such an antagonist could be a sequence derived from any one of these three inhibitors.

From the time it was realized that there were multiple inhibitors of regeneration in myelin (McKerracher et al., 1994; Mukhopadhyay et al., 1994), the debate has been which, if any, of the inhibitors makes the largest contribution to the inhibitory effect of myelin. Because all the inhibitors identified to date seem to share the same receptor, and alternative receptors have yet to be described, the relative contribution which each inhibitor makes to the block of regeneration would then depend on (1) the affinity of each inhibitor for the receptor, (2) the relative concentration of each inhibitor in myelin, and (3) the likelihood of a regenerating axon encountering that particular inhibitor. From binding studies, Nogo66 (7 nM), OMgp (5 nM), and MAG (8 nM) all appear to bind NgR with the same affinity. Furthermore, it has been estimated that MAG represents 1% of total CNS myelin proteins (Quarles, 1997) and that OMgp is a relatively minor component (Baumann and Pham-Dinh, 2001). The amount of NogoA in myelin, relative to other myelin proteins, has not been reported. Hence, a direct comparison of relative abundance cannot be made at this time. Although all three of these inhibitors have been reported to be present in a myelin membrane in direct contact with the axon, the majority of NogoA is intracellular (Chen et al., 2000; GrandPre et al., 2000; Huber et al., 2002). It is likely that after injury, and consequential disruption of cellular integrity, this intracellular NogoA would be exposed to the axons attempting regeneration. This is important not only because it could expose more of the 66 residue luminal domains to NgR, but also because the N terminus of the molecule would also be exposed and a second inhibitory domain has been mapped to the N terminus of NogoA. The N-terminal sequence of Nogo, when immobilized, has been shown to inhibit neurite outgrowth (Chen et al., 2000; Fournier et al., 2001; Prinjha et al., 2000). Although the identity of the receptor for the N-terminal region of NogoA is still elusive, the fact that the three inhibitory components require a single NgR receptor and that blocking this receptor completely overcomes inhibition by myelin in general (GrandPre et al., 2002) suggests, first, that signaling through this receptor is the major route whereby inhibition is transduced to the neuron. Second, these results point to considerable redundancy of these inhibitory ligands (Filbin, 1996). Consistent with the observations from earlier *in vivo* studies (Li et al., 1996; Montag et al., 1994; Schnell and Schwab, 1990, 1993), it seems unlikely, then, that an individual inhibitor will be the major effector of myelin inhibition.

It is not known if there are other functional receptors for OMgp and MAG. A number of neuronal and extracellular matrix binding partners have been reported for MAG (Fahrig et al., 1987; Franzen et al., 2001; Yamashita et al., 2002; Yang et al., 1996). Of these, two, gangliosides and the neurotrophin receptor p75 (Vinson et al., 2001; Yamashita et al., 2002; Vyas et al., 2002), have been suggested as functional MAG receptors involved in inhibition. As MAG is a member of the Siglec family of sialic acid binding proteins (Crocker et al., 1998; Kelm et al., 1994), it can bind specific gangliosides (as well as sialic acid-bearing glycoproteins), most notably GT1b and GD1A (Collins et al., 1997). However, results presented here are consistent with our previous conclusion (Tang et al., 1997a) that sialic acid-dependent binding

of MAG to neurons is neither necessary nor sufficient for its ability to inhibit axonal regeneration. First, we show that binding of MAG to NgR-expressing CHO cells is unaffected by removal of sialic acid residues; interaction of MAG and NgR does not require sialic acid (Figure 2A). Second, MAG (d1-3)-Fc, a truncated form of MAG missing Ig-like domains 4 and 5, which binds to neurons in a sialic acid-dependent manner but has no effect on neurite outgrowth, does not precipitate NgR (Figure 2D). As clustering of the ganglioside GT1b with antibodies in the absence of MAG appears to be sufficient to inhibit neurite outgrowth (Vinson et al., 2001; Vyas et al., 2002), it is possible that this inhibition is completely independent of MAG. Another study reports that GT1b interaction with p75 transduces the signal for inhibition by MAG (Yamashita et al., 2002). It is possible that MAG's ability to bind gangliosides potentiates its inhibitory effects by clustering signaling molecules, but there is no evidence to suggest that gangliosides are necessary for MAG's inhibition.

Previous reports that either inactivation of Rho (Lehmann et al., 1999) or elevation of cAMP (Cai et al., 1999; Song et al., 1998) can overcome inhibition by myelin in general and encourage regeneration *in vivo* (Qiu et al., 2002; Neumann et al., 2002) indicate that there could be a convergence in the signaling pathways of all the inhibitors in myelin. Alternatively, Rho and/or cAMP may be dominant contributors to individual signaling pathways. Although the proteins Nogo66, MAG, and OMgp share no apparent sequence similarities, their ability to compete for receptor binding suggests that they may share structural similarities. This point cannot be addressed until the crystal structure of all three proteins has been resolved. On the other hand, because the NgR is a GPI-linked protein without a transmembrane domain, it alone cannot transduce the signal across the membrane but requires a signaling partner(s). It seems possible, but we would argue unlikely given the apparent convergence in signaling, that the transducing partner in the NgR receptor complex is different for each of these three inhibitory ligands.

It is well established that MAG is a bifunctional molecule in that it promotes axonal growth from young neurons but, with development, the response of these same neurons switches to inhibition by MAG (DeBellard et al., 1996; Johnson et al., 1989; Mukhopadhyay et al., 1994; Song et al., 1998). A similar switch occurs in response to myelin in general. We have reported that a spontaneous decrease in the endogenous levels of neuronal cAMP, and not a change in the MAG/myelin receptor(s), can account for this switch in response (Cai et al., 2001). Consistent with this suggestion, P1 DRG neurons, which are promoted by MAG and not inhibited by myelin, express the NgR (not shown). Although it has not yet been demonstrated directly that NgR is indeed responsible for the effects of MAG on young neurons, it is possible that depending on the intrinsic state of the neuron—high or low cAMP—NgR can transduce either an inhibitory signal or a signal that promotes growth.

The results presented here, together with the findings of others (Fournier et al., 2001; Wang et al., 2002), show that three distinct inhibitors in myelin each require an interaction with the NgR to exert their inhibitory effects.

This finding poses the NgR as a very attractive target for developing therapies for spinal cord and CNS injury.

Experimental Procedures

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). Briefly, 5×10^4 isolated cerebellar or DRG neurons from P4–P8 rat pups were plated onto confluent monolayers of control and MAG-expressing CHO cells in 8-chamber tissue culture slides (Lab-Tek). Where indicated, a polyclonal antibody to NgR (Santa Cruz) was included or NgR-AP, prepared as previously described, was preincubated with the cells before the addition of neurons, at the concentrations indicated. After 16–18 hr 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, United Kingdom). 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 the Simple PCI 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 μ g/ml). Where indicated, neurons were treated with 2 U/ml of PI-PLC for 30 min at 37°C, before addition to the L1-Fc substrates. Incubation was for 4 hr or 18 hr before neurons were fixed and stained for GAP43 as described above. For the 4 hr assays, 800–1000 neurons were scored for the presence of neurites and for the 18 hr assays, the neurites from 180–200 neurons were measured as described above. Where indicated, the NgR antibody or the NgR-AP were included in the cultures.

For the NG108 cells, the cDNA encoding the entire extracellular domain (amino acids 27–445; FL-NgR), the N-terminal (27–309; N-NgR), or the C-terminal (301–445; C-NgR) of human 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 24 hr, prior to plating on substrates of PDL or recombinant MAG (rMAG) (Huang et al., 1999; Lehmann et al., 1999). After 24 hr, 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 (Cohen-Cory and Fraser, 1995).

Binding Assays and Immunostaining for NgR

CHO cells expressing MAG or NgR (flag-tagged), or control cells, were plated onto poly-lysine-coated 8-chamber slides and grown to confluency. Cerebellar or DRG neurons (PND4–7) were plated onto laminin-coated wells and cultured overnight. For binding of MAG-Fc, 20 μ g/ml of MAG-Fc was added to the cells and allowed to bind for 2 hr before being washed, fixed, and stained for MAG using a MAG monoclonal antibody (Santa Cruz) and a phycoerythrin-conjugated second antibody (Sigma). For binding of NgR-AP, 3.7 μ g/ μ l of NgR-AP was added to each well and incubated for a further 2 hr, before being washed, fixed, and stained with a NgR polyclonal antibody (Santa Cruz), followed by FITC-conjugated second antibody (Santa Cruz). To calculate the K_d of the MAG:NgR interaction, binding over a range of concentrations of NgR-AP (1–40 nM) to either MAG-expressing CHO cells or to immobilized MAG-Fc (Tang et al., 1997a) was measured and the bound NgR/free NgR plotted against bound NgR. Where indicated, neurons or NgR-expressing CHO cells were treated with PI-PLC or α -2,3, 6,8-neuraminidase (0.02 U/ml) (Calbiochem) before addition of MAG-Fc or NgR antibody. Where indicated, Nogo66-AP (3.5 and 7 μ g) was included in the assays along with MAG-Fc and immunostained for MAG-Fc. To calculate the IC_{50} for Nogo66-MAG, the binding of vitally labeled, fluorescent (CalceinAM) neurons to immobilized MAG-Fc in the presence of various concentrations of Nogo66-AP (5–500 nM) was measured as described previously (Tang et al., 1997a). The recombi-

nant Nogo66 protein fused to AP was prepared as described before (Fournier et al., 2001; Wang et al., 2002).

Precipitation Assays

Cerebellar neurons, DRG neurons (PND4–7), CHO cells expressing NgR-FLAG[®], or control CHO cells were lysed in 50 mM Tris-HCl (pH 7.5) RIPA buffer (Amersham). After pre-clearing with protein A sepharose, the lysates were incubated with either MAG-Fc (20 μ g/ml), MAG (d1-3)-Fc (20 μ g/ml), or an anti-flag antibody (1:1000, Santa Cruz) for 4 hr at 4°C, after which 50 μ l of 50% protein A sepharose beads was 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, prior to being transferred to PVDF membrane and stained for NgR.

Acknowledgments

We thank Maria Handler and Vaciliki Gioka for their outstanding technical support and Dr. Wilfredo Mellado for all his help. This work was supported by grants from the New York State Spinal Cord fund, the NIH NS 37060, the National Multiple Sclerosis Society, and core facility grants from the Research Centers for Minorities Institute-NIH and Specialized Neuroscience Research Programs-NIH (NS41073). Z.H. was supported by grants from the Whitehall Foundation, the International Spinal Research Trust, and the NIH. K.C.W. is a recipient of a Howard Hughes Predoctoral Fellowship.

Received: June 13, 2002

Revised: June 27, 2002

Published online: June 28, 2002

References

- Baumann, N., and Pham-Dinh, D. (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol. Rev.* 81, 871–927.
- Cai, D., Shen, Y., De Bellard, M., Tang, S., and Filbin, M.T. (1999). Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* 22, 89–101.
- Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., and Filbin, M.T. (2001). Neuronal cyclic amp controls the developmental loss in ability of axons to regenerate. *J. Neurosci.* 21, 4731–4739.
- Caroni, P., and Schwab, M.E. (1988). Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron* 1, 85–96.
- Chen, M.S., Huber, A.B., van der Haar, M.E., Frank, M., Schnell, L., Spillmann, A.A., Christ, F., and Schwab, M.E. (2000). Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 403, 434–439.
- Cohen-Cory, S., and Fraser, S.E. (1995). Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature* 378, 192–196.
- Collins, B.E., Yang, L.J., Mukhopadhyay, G., Filbin, M.T., Kiso, M., Hasegawa, A., and Schnaar, R.L. (1997). Sialic acid specificity of myelin-associated glycoprotein binding. *J. Biol. Chem.* 272, 1248–1255.
- Crocker, P.R., Clark, E.A., Filbin, M., Gordon, S., Jones, Y., Kehrl, J.H., Kelm, S., Le Douarin, N., Powell, L., Roder, J., et al. (1998). Siglecs: a family of sialic-acid binding lectins [letter]. *Glycobiology* 8, v.
- De Bellard, M., and Filbin, M.T. (1999). Myelin-associated glycoprotein, MAG, selectively binds several neuronal proteins. *J. Neurosci. Res.* 56, 213–218.
- DeBellard, M.E., Tang, S., Mukhopadhyay, G., Shen, Y.J., and Filbin, M.T. (1996). Myelin-associated glycoprotein inhibits axonal regeneration from a variety of neurons via interaction with a sialoglycoprotein. *Mol. Cell. Neurosci.* 7, 89–101.

- Fahrig, T., Landa, C., Pesheva, P., Kuhn, K., and Schachner, M. (1987). Characterization of binding properties of the myelin-associated glycoprotein to extracellular matrix constituents. *EMBO J.* 6, 2875–2883.
- Filbin, M.T. (1996). The Muddle with MAG. *Mol. Cell. Neurosci.* 8, 84–92.
- Fitch, M.T., and Silver, J. (1999). Beyond the glial scar. In *CNS Regeneration*, M.H. Tuszynski and J.H. Kordower, eds. (San Diego, CA: Academic Press), pp. 55–88.
- Flanagan, J.G., and Cheng, H.J. (2000). Alkaline phosphatase fusion proteins for molecular characterization and cloning of receptors and their ligands. *Methods Enzymol.* 327, 198–210.
- Fournier, A.E., GrandPre, T., and Strittmatter, S.M. (2001). Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* 409, 341–346.
- Franzen, R., Tanner, S.L., Dashiell, S.M., Rottkamp, C.A., Hammer, J.A., and Quarles, R.H. (2001). Microtubule-associated protein 1B: a neuronal binding partner for myelin-associated glycoprotein. *J. Cell Biol.* 155, 893–898.
- GrandPre, T., Nakamura, F., Vartanian, T., and Strittmatter, S.M. (2000). Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* 403, 439–444.
- GrandPre, T., Li, S., and Strittmatter, S.M. (2002). Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature* 417, 547–551.
- Huang, D.W., McKerracher, L., Braun, P.E., and David, S. (1999). A therapeutic vaccine approach to stimulate axon regeneration in the adult mammalian spinal cord. *Neuron* 24, 639–647.
- Huber, A.B., Weinmann, O., Brosamle, C., Oertle, T., and Schwab, M.E. (2002). Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. *J. Neurosci.* 22, 3553–3567.
- Johnson, P.W., Abramow-Newerly, W., Seilheimer, B., Sadoul, R., Tropak, M.B., Argum, M., Dunn, R.J., Schachner, M., and Roder, J.C. (1989). Recombinant myelin-associated glycoprotein confers neural adhesion and neurite outgrowth function. *Neuron* 3, 377–385.
- Kelm, S., Pelz, A., Schauer, R., Filbin, M.T., Tang, S., de Bellard, M.E., Schnaar, R.L., Mahoney, J.A., Hartnell, A., Bradfield, P., et al. (1994). Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. *Curr. Biol.* 4, 965–972.
- Lehmann, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebok, A., Leclerc, N., Tigyi, G., and McKerracher, L. (1999). Inactivation of the signaling pathway promotes CNS axon regeneration. *J. Neurosci.* 19, 7537–7547.
- Li, M., Shibata, A., Li, C., Braun, P.E., McKerracher, L., Roder, J., Kater, S.B., and David, S. (1996). Myelin-associated glycoprotein inhibits neurite/axon growth and causes growth cone collapse. *J. Neurosci. Res.* 46, 404–414.
- McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., and Braun, P.E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* 13, 805–811.
- Mikol, D.D., Gulcher, J.R., and Stefansson, K. (1990). The oligodendrocyte-myelin glycoprotein belongs to a distinct family of proteins and contains the HNK-1 carbohydrate. *J. Cell Biol.* 110, 471–479.
- Montag, D., Giese, K.P., Bartsch, U., Martini, R., Lang, Y., Bluthmann, H., Karthigasan, J., Kirschner, D.A., Wintergerst, E.S., Nave, K.A., et al. (1994). Mice deficient for the myelin-associated glycoprotein show subtle abnormalities in myelin. *Neuron* 13, 229–246.
- Mukhopadhyay, G., Doherty, P., Walsh, F.S., Crocker, P.R., and Filbin, M.T. (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* 13, 757–767.
- Neumann, S., Bradke, F., Tessier-Lavigne, M., and Basbaum, A.I. (2002). Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. *Neuron* 34, 885–893.
- Prinjha, R., Moore, S.E., Vinson, M., Blake, S., Morrow, R., Christie, G., Michalovich, D., Simmons, D.L., and Walsh, F.S. (2000). Inhibitor of neurite outgrowth in humans. *Nature* 403, 383–384.
- Qiu, J., Cai, D., and Filbin, M.T. (2000). Glial inhibition of nerve regeneration in the mature mammalian CNS. *Glia* 29, 166–174.
- Qiu, J., Cai, D., Dai, H., McAtee, M., Hoffman, P.N., Bregman, B.S., and Filbin, M.T. (2002). Spinal axon regeneration induced by elevation of cAMP. *Neuron* 34, 895–903.
- Quarles, R.H. (1997). Glycoproteins of myelin sheaths. *J. Mol. Neurosci.* 8, 1–12.
- Schnell, L., and Schwab, M.E. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature* 343, 269–272.
- Schnell, L., and Schwab, M.E. (1993). Sprouting and regeneration of lesioned corticospinal tract fibres in the adult rat spinal cord. *Eur. J. Neurosci.* 5, 1156–1171.
- Schwab, M.E., and Bartholdi, D. (1996). Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol. Rev.* 76, 319–370.
- Skaper, S.D., Moore, S.E., and Walsh, F.S. (2001). Cell signalling cascades regulating neuronal growth-promoting and inhibitory cues. *Prog. Neurobiol.* 65, 593–608.
- Song, H., Ming, G., He, Z., Lehmann, M., McKerracher, L., Tessier-Lavigne, M., and Poo, M. (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* 281, 1515–1518.
- Tang, S., Shen, Y.J., DeBellard, M.E., Mukhopadhyay, G., Salzer, J.L., Crocker, P.R., and Filbin, M.T. (1997a). Myelin-associated glycoprotein interacts with neurons via a sialic acid binding site at ARG118 and a distinct neurite inhibition site. *J. Cell Biol.* 138, 1355–1366.
- Tang, S., Woodhall, R.W., Shen, Y.J., deBellard, M.E., Saffell, J.L., Doherty, P., Walsh, F.S., and Filbin, M.T. (1997b). Soluble myelin-associated glycoprotein (MAG) found in vivo inhibits axonal regeneration. *Mol. Cell. Neurosci.* 9, 333–346.
- Tang, S., Qiu, J., Nikulina, E., and Filbin, M.T. (2001). Soluble myelin-associated glycoprotein released from damaged white matter inhibits axonal regeneration. *Mol. Cell. Neurosci.* 18, 259–269.
- Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. *Science* 274, 1123–1133.
- Trapp, B.D. (1988). Distribution of the myelin-associated glycoprotein and P0 protein during myelin compaction in quaking mouse peripheral nerve. *J. Cell Biol.* 107, 675–685.
- Vinson, M., Strijbos, P.J., Rowles, A., Facci, L., Moore, S.E., Simmons, D.L., and Walsh, F.S. (2001). Myelin-associated glycoprotein interacts with ganglioside GT1b. A mechanism for neurite outgrowth inhibition. *J. Biol. Chem.* 276, 20280–20285.
- Vyas, A.A., Patel, H.V., Fromholt, S.E., Heffer-Laue, M., Vyas, K.A., Dang, J., Schachner, M., and Schnaar, R.L. (2002). From the cover: Gangliosides are functional nerve cell ligands for myelin-associated glycoprotein (MAG), an inhibitor of nerve regeneration. *Proc. Natl. Acad. Sci. USA* 99, 8412–8417.
- Wang, K.C., Koprivica, V., Kim, J.A., Sivasankaran, R., Guo, Y., Neve, R.L., and He, Z. (2002). Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* 417, 941–944. Published online June 16, 2002.
- Yamashita, T., Higuchi, H., and Tohyama, M. (2002). The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. *J. Cell Biol.* 157, 565–570.
- Yang, L.J., Zeller, C.B., Shaper, N.L., Kiso, M., Hasegawa, A., Shapiro, R.E., and Schnaar, R.L. (1996). Gangliosides are neuronal ligands for myelin-associated glycoprotein. *Proc. Natl. Acad. Sci. USA* 93, 814–818.