

# New roles for old proteins in adult CNS axonal regeneration

Timothy Spencer, Marco Domeniconi, Zixuan Cao and Marie T Filbin\*

The past year has yielded many insights and a few surprises in the field of axonal regeneration. The identification of oligodendrocyte-myelin glycoprotein as an inhibitor of axonal growth, and the discovery that the three major myelin-associated inhibitors of CNS regeneration share the same functional receptor, has launched a new wave of studies that aim to identify the signaling components of these inhibitory pathways. These findings also offer new avenues of research directed toward blocking possible therapeutic targets that inhibit regeneration and toward encouraging axonal regeneration in the CNS after injury.

## Addresses

Department of Biological Sciences, Hunter College, The City University of New York, 695 Park Avenue, New York, NY 10021, USA  
\*e-mail: filbin@genectr.hunter.cuny.edu

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## Abbreviations

<b>CSPG</b>	chondroitin sulfate proteoglycan
<b>DRG</b>	dorsal root ganglion
<b>GPI</b>	glycosylphosphatidylinositol
<b>MAG</b>	myelin-associated glycoprotein
<b>NgR</b>	Nogo-66 receptor
<b>OMgp</b>	oligodendrocyte-myelin glycoprotein
<b>PtdIns-PLC</b>	phosphatidylinositol-specific phospholipase C

## Introduction

The local environment of the adult central nervous system (CNS) could be the ‘deciding factor’ in determining whether spontaneous axonal regeneration occurs. This is implied by the fact that, unlike peripheral neurons and certain embryonic CNS neurons, axons in the adult mammalian CNS do not regenerate after injury. Indeed, it appears that the failure of adult CNS axons to regenerate is not due to an intrinsic and irreversible lack of regenerative ability; axonal extension is possible if a permissive environment such as a peripheral nervous system (PNS) tissue graft is provided [1]. Several factors may account for the normal regenerative failure seen in the adult CNS, including a post-natal decline in available neurotrophic factors and intracellular cyclic nucleotides, the formation of a glial scar, the presence of myelin-associated inhibitors of axonal extension, and possibly

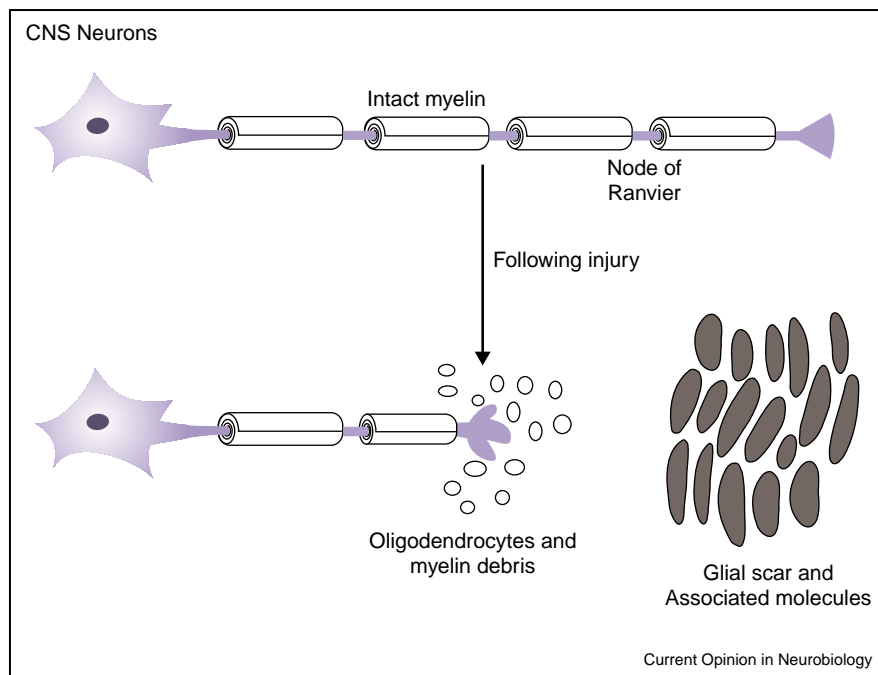
the presence of developmental repulsive guidance cues. During the past three years, several new and potent inhibitors of regeneration have been identified in myelin, as have many surprises regarding their proposed neuronal receptor(s) and mode of signaling. In this review, we focus on the latest advances in identifying the inhibitors of regeneration present in myelin, the receptor(s) that mediate their effects, and some of the proposed signaling mechanisms that may prove to be therapeutic targets for encouraging regeneration in the adult CNS following injury.

## Myelin-associated inhibitors of axonal regeneration in the adult CNS

After injury to the adult CNS, axons attempting to regenerate have two major obstacles to overcome (Figure 1): first, the inhibitors in myelin that are exposed by the damage, and second a glial scar. The glial scar acts as a physical barrier to regeneration and contains inhibitory molecules that are associated with the extracellular matrix such as chondroitin sulphate proteoglycans (CSPGs) [2,3]. It has recently been reported [4\*\*] that removal of glycosaminoglycan (GAG) chains from CSPGs *in vivo* resulted in improved regeneration and functional recovery. This demonstrates that CSPG molecules do indeed play a role in blocking axonal regeneration after injury [4\*\*]. However, because the glial scar takes a considerable time to become fully mature, the major impediments to regeneration immediately after injury are most likely to be inhibitors in myelin.

Although myelin had been proposed to inhibit regeneration before [5], it was the pioneering work of Martin Schwab and co-workers [6,7] that showed that an antibody to an inhibitory fraction of myelin, termed IN-1, could permit limited axonal regeneration and some functional recovery in rats. This work firmly established myelin as a potent inhibitor of axonal regeneration [8]. However, specific myelin inhibitors have been described only recently. Despite the body of work utilizing the IN-1 antibody, the first such inhibitory molecule to be identified was not the IN-1 antigen but was myelin-associated glycoprotein (MAG) [9,10], a protein initially described in 1973 [11]. As a member of the immunoglobulin (Ig)-superfamily and a sialic acid-binding glycoprotein, MAG is a Siglec family protein (Siglec 4). In the CNS, MAG is found in the periaxonal myelin membrane, and in the PNS, it is also found in the outermost membrane of the myelin sheath [12,13]. Several years after the identification of MAG, an antigen of IN-1, Nogo, was cloned independently by three separate groups [14–16]. Nogo is expressed as the distinct isoforms A, B and C, and

Figure 1



Following injury, a lesioned axon faces several inhibitors that prevent regeneration in the CNS environment. The glial scar contains several inhibitory molecules and acts as a physical barrier to axonal growth. In addition, a regenerating axon will encounter many myelin-associated molecules — expressed on the membrane face or released after damage — that can inhibit axonal extension. These are believed to provide the primary block to regeneration before glial scar formation.

mediates its inhibitory activity via two spatially separated domains: an amino-terminal domain specific to Nogo-A (Amino-Nogo) [14,16] and an extracellular 66 amino acid sequence, which is found in all three isoforms (Nogo-66) [15,17]. Nogo-A is the predominant CNS isoform of Nogo. The carboxy-terminal region of all three isoforms exhibits a considerable degree of homology (~70%) to the reticulon family of proteins [15]. Like the other reticulon family members, Nogo appears to be predominantly associated with the endoplasmic reticulum (ER). Following injury and the attendant damage to myelin and oligodendrocytes, ER-associated Nogo would become exposed to the extracellular environment along with the inhibitory amino terminus of surface-expressed Nogo-A. An alternative topology for Nogo-A has been proposed in which the inhibitory amino terminus is expressed on the extracellular membrane surface before injury [18]. Regardless of the native topology, both Nogo-66 and the amino-terminus of Nogo-A have been shown to be potent inhibitors of axonal extension and to induce growth cone collapse *in vitro*.

The latest myelin-associated inhibitor of regeneration to be described is the glycosylphosphatidylinositol (GPI)-linked oligodendrocyte-myelin glycoprotein (OMgp). Identified 15 years ago [19], OMgp has only recently been revealed as the inhibitory component of the fraction

of bovine brain myelin initially termed arretin. Like MAG and Nogo, OMgp inhibits axonal extension and induces growth cone collapse *in vitro* [20,21].

It seems evident, therefore, that improving the recovery of function and sensation following injury to the CNS will require the simultaneous blocking of all of the major myelin-associated inhibitors to permit the regrowth of axons before formation of the glial scar. Recent advances in identifying the receptors that mediate the neuronal activity of these inhibitors have yielded encouraging surprises that may eventually facilitate this block of inhibition.

### Receptors and binding partners

How do these inhibitors interact with regenerating axons to exert their effects? The neuronal receptor for Nogo-66 (NgR) was identified by screening a cDNA expression library. NgR is a GPI-linked protein that consists of eight consecutive leucine-rich-repeat (LRR) domains followed by a carboxy-terminal LRR. When expressed after transfection *in vitro*, NgR binds to Nogo-66 directly to mediate growth cone collapse in dorsal root ganglion (DRG) neurons and to confer sensitivity to normally unresponsive cells [22]. In addition, studies of the expression patterns of Nogo and NgR suggest that these proteins are both present and juxtaposed at the myelin–axon interface, which is consistent with NgR acting as the

functional Nogo receptor [23<sup>•</sup>]. The NgR receptor does not, however, appear to interact or mediate the effects of amino-Nogo. Therefore, it has been proposed that a separate, as yet unidentified, receptor or complex must exist but its contribution to inhibition may be relatively minor. With the receptor and ligand for one myelin inhibitor identified, it is now possible to design potential therapeutic agents to disrupt their interaction and so attempt to encourage regeneration. Strittmatter and co-workers [17<sup>•</sup>] found that the amino-terminal residues of the Nogo-66 sequence are essential for binding to the receptor but do not contribute to the inhibitory activity of the protein. They therefore reasoned that a small peptide consisting of the first 40 residues of the Nogo-66 sequence (NEP1-40) should compete with native Nogo for binding but not itself induce inhibition [17<sup>•</sup>]. This peptide competitor significantly blocks the inhibitory effects of Nogo-66 both *in vitro* and via intrathecal delivery *in vivo*, demonstrating its potential for therapeutic use.

In the study illustrating the inhibitory role of OMgp, He's group also demonstrated, surprisingly, that the functional receptor for this molecule is none other than NgR. Using alkaline phosphatase-fused OMgp as a probe, they identified NgR as a binding partner from cDNA expression library pools. In addition, the axonal inhibition induced by OMgp is both phosphatidylinositol-specific phospholipase C (PtdIns-PLC)-sensitive and induced in non-responsive neurons via ectopic expression of NgR [21<sup>••</sup>]. These investigators also show that OMgp and Nogo-66 bind to NgR with a similar affinity (EC<sub>50</sub> values for OMgp and Nogo-66 are 1.5 nM and 1.0 nM, respectively) and their effects are not synergistic, they are redundant. Taken together, these findings suggest that NgR acts as the neuronal receptor for OMgp, and hence OMgp is likely to induce intracellular signaling that is identical to that of Nogo-66 to effect inhibition.

Since MAG's identification as an inhibitor of axon outgrowth in 1994, many researchers have sought to identify its neuronal receptor but until recently, it has remained elusive. As MAG is a sialic-acid-binding protein, it can bind both sialoglycoproteins and sialolipids, in particular the gangliosides GD1a and GT1b. Recently, these gangliosides were proposed as function-mediating binding partners of MAG. Indeed, the clustering of GT1b with a multivalent IgM antibody mimics the inhibitory effects of MAG via signaling to the intracellular mediator Rho [24,25]. However, it had been shown previously that sialic acid binding was unnecessary for MAG to exert inhibition [26]. If the sialic-acid-binding site on MAG, Arg118, is mutated, the sialic-acid-dependent binding is lost. Nevertheless, when expressed by cells, this mutant MAG still inhibits axonal growth as effectively as wild-type MAG [26]. That MAG's ability to inhibit axonal growth is sialic-acid-independent but PtdIns-PLC-sensitive [27<sup>••</sup>,28<sup>••</sup>] suggests that gangliosides are not

required to mediate the effects of MAG. This observation set the stage for some highly unexpected findings. Independently, two groups identified MAG as yet another ligand of the NgR through screening of a cDNA expression library [27<sup>••</sup>], and immunoprecipitation, and analysis of cell-surface binding [27<sup>••</sup>,28<sup>••</sup>]. These studies show that the interaction of MAG with NgR is specific, sialic-acid-independent, and has an affinity comparable to that of Nogo-66 and OMgp. In addition, application of soluble or dominant-negative forms of NgR can effectively block the inhibitory growth effects of MAG *in vitro*. One issue that has yet to be resolved, however, is that of the location of the respective binding sites. One group [28<sup>••</sup>] has reported that excess soluble Nogo-66 can effectively block the binding of soluble MAG to NgR-expressing cells with an IC<sub>50</sub> (the concentration of competitive peptide necessary to achieve 50% maximal binding of target protein) of 120 nM, suggesting identical or at least overlapping binding regions for MAG and Nogo-66. In contrast, the report from Strittmatter's group [27<sup>••</sup>] indicates that neither excess Nogo-66 nor the Nogo-66-inhibiting peptide termed NEP1-40 significantly reduce MAG-NgR binding. The resolution of this issue is imperative for the design of an NgR inhibitor that effectively blocks the effects of all the myelin-associated inhibitors.

As NgR is a GPI-linked protein, it lacks transmembrane and cytosolic domains. Hence, to effect inhibition, a transducing partner molecule is required. A likely candidate for this transducer is the neurotrophin receptor, p75 (p75<sup>NTR</sup>). An initial report implicated p75<sup>NTR</sup> as the signal transducer for MAG-mediated inhibition [29<sup>•</sup>]. Although neurons from the p75<sup>-/-</sup> mouse were not inhibited by MAG, a direct interaction between MAG and p75<sup>NTR</sup> was not demonstrated. It was suggested that MAG's ability to bind GT1b bridged the gap in the MAG-p75<sup>NTR</sup> interaction. More recently, p75<sup>NTR</sup> (along with NgR but without gangliosides) was firmly established as the signaling partner in the receptor complex that effects inhibition by MAG, Nogo-66, and OMgp. Experiments in our laboratory [M Domeniconi, M Filbin, unpublished data] and from the He group [30<sup>••</sup>] show that NgR, MAG and Nogo-66 can immunoprecipitate p75<sup>NTR</sup>, and as previously shown neurons from p75<sup>NTR</sup><sup>-/-</sup> mice are unresponsive to the myelin-associated inhibitors. In addition, soluble or virally introduced dominant-negative forms of p75<sup>NTR</sup> can abrogate the effects of individual inhibitors as well as of purified myelin. The ability of these molecules to associate with the p75<sup>NTR</sup> receptor complex is not affected by other p75<sup>NTR</sup> neurotrophic binding partners.

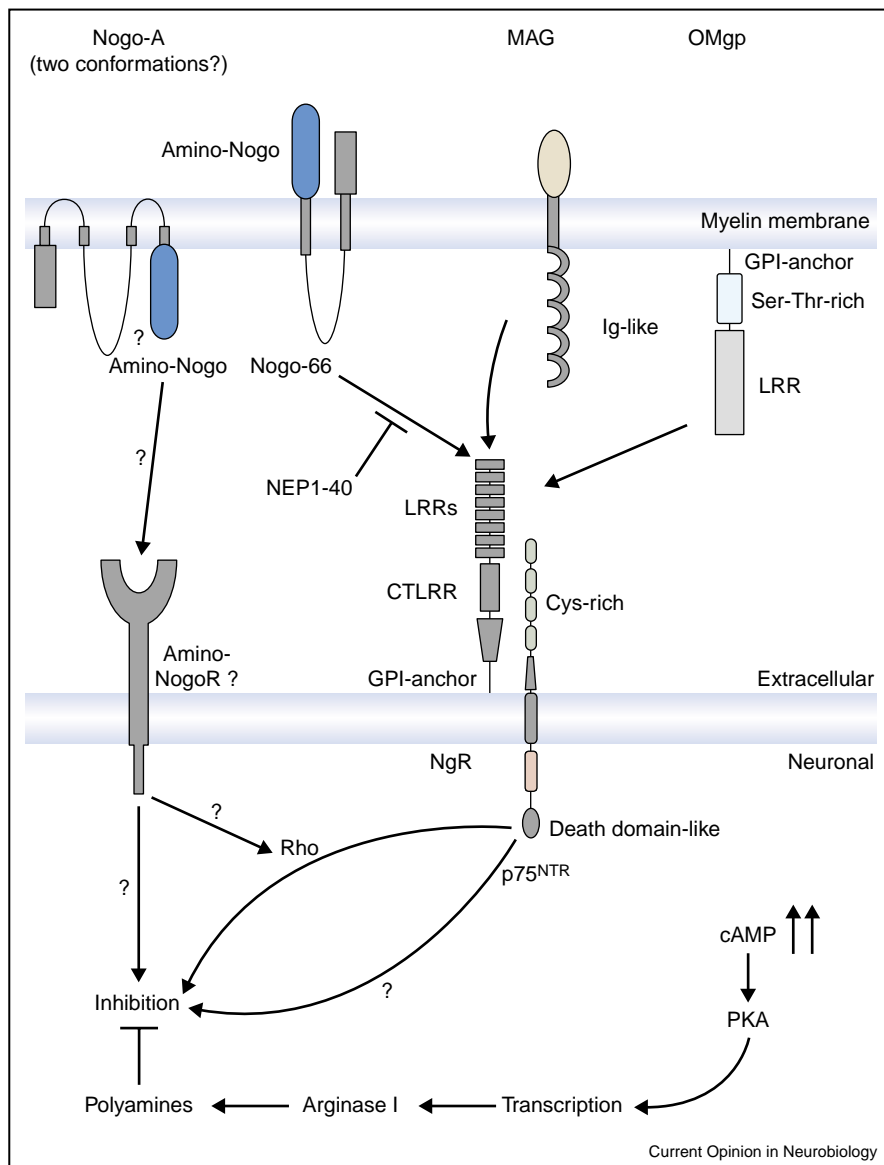
The question that remains is what role, if any, do the gangliosides play in this model? Gangliosides appear to be neither necessary nor sufficient to achieve inhibition by MAG but could augment inhibition by facilitating the clustering of signaling molecules.

## Implications for regeneration

The recent explosion in our understanding of the nature of the myelin-associated inhibitors and how they interact with neurons has vastly increased the number of potential therapeutic targets. The finding that a single neuronal receptor binds with comparable affinities and mediates the effects of all three of the major myelin-associated inhibitors was not only a surprise but also suggested redundancy of activity amongst these inhibitors. In other

words, the effects of these myelin-associated inhibitors are not cumulative but are independent of one another, and the relative contribution of each to the block of axonal extension will depend on their relative level of expression at the periaxonal surface. This suggests that it may be possible to block a single target and thereby abrogate the inhibition of axonal regrowth by myelin and hence, induce regeneration after injury. However, if an agent to block the binding of inhibitors to the NgR is to be

Figure 2



Schematic representation of the major myelin-associated inhibitors and the proposed receptor complex that mediates their signaling. All three inhibitory molecules (Nogo-66, MAG and OMgp) bind to NgR. This in turn associates with p75<sup>NTR</sup> to transduce a signal that results in the activation of the small GTPase Rho as well as other, as yet unidentified, signaling cascades leading to the inhibition of axonal growth following injury. The intracellular upregulation of the small second messenger molecule cAMP leads to the activation of protein kinase A. This signaling cascade initiates the induction of gene transcription, including the synthesis of Arginase I, the rate limiting enzyme in the polyamine synthesis pathway. Upregulation of polyamines in turn initiates a block of the growth inhibition induced by the myelin-associated inhibitors. The small peptide NEP1-40 can bind to NgR and inhibit binding of the myelin inhibitor Nogo-66 but not of MAG.

designed, extensive knowledge of the relevant binding site(s) must be gained.

The redundancy of all three inhibitors is consistent with findings that the effects of all myelin-associated inhibitors can be overcome simultaneously by altering certain integral, intracellular signaling molecules. One such molecule is the small GTPase, Rho. Blocking Rho signaling can promote axonal regeneration both in the presence of MAG and myelin *in vitro* and following CNS injury *in vivo* [31]. It has been suggested that it is the interaction of the inhibitory signaling complex with p75<sup>NTR</sup> that modulates Rho's activity [29\*,30\*\*]. Hence, therapeutic approaches that target the Rho signaling pathway may be one method by which a simultaneous block of all the major myelin-associated inhibitors could be achieved. Two recent studies by McKerracher's group [32\*\*,33] indicate that this may indeed be the case. Inactivation of Rho or its downstream effector Rho-associated kinase (ROK) can induce improved axonal growth of primary neurons on inhibitory substrates *in vitro* [32\*\*,33], and following CNS injury, can permit increased regeneration and functional recovery *in vivo* [32\*\*].

Another potential target for improving axonal regeneration is the intracellular second messenger, cAMP. It has previously been shown that increasing the levels of cAMP can mediate a reversal of the effects of MAG on both axonal extension [34] and growth cone turning [35] *in vitro*. In addition, it has long been recognized that inflicting a pre-conditioning peripheral lesion on DRG neurons results in the regeneration of the CNS branch of the same neuron when it is subsequently lesioned [36–38]. Recently, it has been suggested that CNS regeneration as a consequence of peripheral lesioning is also mediated by an increase in intracellular cAMP levels [39\*\*,40\*\*]. In support of this hypothesis, studies show that microinjection of a cAMP analogue in the absence of a conditioning lesion can mimic the regenerative effects of such a lesion. Elevation of cAMP *in vivo* can also improve subsequent axonal growth of neurons when they are cultured on inhibitory substrates *in vitro*. Furthermore, we have shown that one of the downstream components of this signaling pathway is a synthesis of polyamines that results from an upregulation of Arginase I, which is a key enzyme in their synthesis [41\*]. Overexpression of Arginase I or exogenous application of polyamines can mediate improved axonal regeneration on myelin substrates, suggesting a mechanism by which myelin-associated inhibitors can be overcome. These findings can be explained by a model in which the binding of a single receptor by multiple ligands (i.e. the myelin inhibitors) initiates the activity of the same intracellular signaling pathway(s); thus, mechanisms that overcome the action of one inhibitor may be able to overcome the inhibitory actions of all three of the major myelin associated inhibitors. These experiments reaffirm the idea that actions mediated by

NgR are the major inhibitory components of myelin-associated inhibition.

## Conclusions

There has been a recent explosion in the identification of specific myelin-associated inhibitors, and the receptor that mediates their actions has also been unveiled. This represents a major leap forward in our understanding of the contribution of the damaged myelin sheath to the block of axonal regeneration, which occurs following injury to the adult CNS. In addition, these findings provide a starting point for new avenues of research, which will result in the elucidation of the downstream signaling components of this inhibitory complex.

Furthermore, recent evidence suggests that blockage of the inhibitory signaling can be achieved via an elevation of the intracellular second messenger, cAMP. This elevation of cAMP has been shown to induce transcriptional activation and a subsequent increase in the synthesis of polyamines, which may play a role in this block of inhibition.

Taken together, the body of work presented in the past year reveals a single theme: blocking a common receptor prevents a common signaling pathway (Figure 2). This in turn, allows for the abrogation of the effects of all of the myelin-associated inhibitors. If applied before formation of the glial scar, blocking the receptor could result in improved regeneration and functional recovery after CNS injury.

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The authors describe a downstream consequence of elevated cAMP, which allows axons to grow on a myelin substrate. The enzyme Arginase I (ArgI), which is key in the synthesis of polyamines, is up-regulated by cAMP. Polyamines are also elevated in response to treatment with db-cAMP. Either overexpression of ArgI or addition of polyamines is sufficient to overcome inhibition by myelin. These findings illustrate yet another downstream, therapeutic target for encouraging axonal regeneration after injury.