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Myelin-Associated Inhibitors in Axonal Growth After CNS Injury

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Abstract

There are multiple barriers to axonal growth after CNS injury. Myelin-associated inhibitors represent one group of barriers extrinsic to the injured neurons. Nogo, MAG and OMgp are three prototypical myelin inhibitors that signal through multiple neuronal receptors to exert growth inhibition. Targeting myelin inhibition alone modulates the compensatory sprouting of uninjured axons but the effect on the regeneration of injured axons is limited. Meanwhile, modulating sprouting, a naturally occurring repair mechanism, may be a more attainable therapeutic goal for promoting functional repair after CNS injury in the near term.

1. Introduction

It is notorious that after injury of the adult mammalian central nervous system (CNS), damaged axons cannot regenerate to a significant extent, leading to major functional impairments in patients of spinal cord injury (SCI). Because the peripheral nervous system (PNS) has a remarkable ability to regenerate axons, extensive efforts have been focusing on understanding the differences between the PNS and the CNS. The key observation that CNS axons can regenerate in a PNS environment [1] prompted the notion that the environment in the PNS, but not the CNS, is conducive to axon regeneration. One major distinction between the CNS and the PNS is the origin of the myelin and its composition. This led to the hypothesis that CNS myelin is inhibitory to axon regeneration. The production of the IN-1 antibody against an inhibitory activity from CNS myelin [2], the identification of Nogo [3], other myelin-associated inhibitors (MAIs) and their receptors, and the many in vitro and in vivo studies since have contributed much to our understanding of the molecular regulation of axonal growth after CNS injury. It is now widely recognized that both neuron-intrinsic and extrinsic mechanisms contribute to the lack of CNS axon regeneration. Here we discuss the role of the prototypical myelin inhibitors in the context of recent development in the field of axon growth and repair after CNS injury.

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2. Definition of regeneration and sprouting

The literature on MAIs in axonal repair is abundant, mostly aimed at addressing the key question: can the manipulation of the MAIs and their receptors promote axon regeneration in vivo? The short answer is: yes and no. Indeed, the answer depends on the definition of regeneration. There are many different terms used to describe axon growth after injury: regeneration, sprouting, regenerative sprouting, or even axonal plasticity. Use of inaccurate or ambiguous terminology has been a major issue in the field, leading to confusion and disagreement. This is partly due to the continuous evolution of scientific concepts and partly to the limitations of the experimental tools available at any given time.

To allow for a meaningful discussion, here we provide one way to define regeneration and sprouting. In this definition, whether any axonal growth after injury is regeneration or sprouting depends solely on whether or not a neuron has been injured in the first place. Regeneration is axonal growth from injured neurons, while sprouting is axonal growth from uninjured neurons (Fig. 1). Under this definition, there are three typical scenarios for regeneration. First, regeneration can originate from the cut end (or tip) of injured axons (Fig. 1.3), which is the most classic type of regeneration. In the literature regenerating axons often have to grow beyond (either through or around) the injury site and towards their original targets to be considered significant or relevant. However, this may not be necessary if neurons proximal to the injury can relay information from regenerated axons [4]. Second, regeneration can originate from the shaft of injured axons, forming new branches de novo (Fig. 1.5). In this scenario, regeneration can initiate close to the injury site or at a distance, and the growth can cover a short or long distance (Fig. 1.5). Third, regeneration can be extension from pre-existing, non-injured axonal branches of injured neurons (Fig. 1.6). In contrast, as axonal growth from uninjured neurons, sprouting generally occurs as a compensatory response to injury of other neurons. Just as regeneration, sprouting may also initiate at different locations (proximal or distal, close or distant) relative to the injury site, and the growth can also be for short or long distances (Fig. 1.4).

It should be noted that even though regeneration and sprouting can be strictly defined conceptually, it is not always technically straightforward to distinguish the different types of axonal growth depicted in Fig. 1. For instance, axonal growth represented in Fig. 1.5 (a, a') and 1.6 (a) are often collectively referred to as "regenerative sprouting" in the literature. Note that in all these three cases, growth is from injured neurons, thus the term "regenerative sprouting" contradicts with the definition of sprouting as growth from uninjured neurons and could be confusing. It is therefore always advisable to describe in great detail the axon growth phenotype one observes in spinal cord injury models.

Distinguishing regeneration from sprouting based on the injury status of the neurons will be useful in investigating the molecular mechanisms because injured and uninjured neurons are likely to be differentially regulated in their axon growth abilities [5]. Using more defined terms to describe axonal growth also has important bearing on clinical applications. A treatment that promotes sprouting but not regeneration can be efficacious for anatomically incomplete but not complete injuries. Targeting the appropriate cohort of patients would be critical for the success of clinical trials.

3. Multiple ligands and multiple receptors involved in axon growth inhibition

There are three prototypical MAIs: Nogo, MAG and OMgp, all of which have potent inhibitory activity on neurite growth in vitro. These MAIs signal through multiple neuronal receptors and co-receptors to effect cytoskeleton rearrangement and neurite inhibition through a signaling pathway involving Rho and Rho-associated kinase (ROCK) (Fig. 2). There are other potential MAIs expressed by myelin and oligodendrocytes. Here we focus on the prototypical myelin inhibitors and their receptors.

3.1. Multiple Ligands

MAG was the first MAI characterized molecularly [6]. It is a transmembrane glycoprotein (Fig. 2) produced by myelinating glial cells: oligodendrocytes in the CNS and Schwann cells in the PNS, with a higher expression level in the CNS. MAG functions in the maintenance of myelinated axons [7]. Its effects on axon growth are bi-modal: MAG promotes axon growth from young neurons and inhibits growth from older neurons, a switch that is age and neuron type dependent [6]. MAG has been widely used as an inhibitory substrate for neurite growth assays using postnatal and adult neurons. However, relatively few studies addressed its function in axonal growth after injury in vivo. In genetic studies, targeting MAG alone did not improve axon regeneration [8,9••,10••]. Interestingly, both genetic deletion and intrathecal delivery of sialidase to interfere MAG binding to sialoglycans (gangliosides GD1a and GT1b) enhanced serotonergic (5-HT) axon sprouting [10••,11]. Surprisingly, genetically deleting MAG *reduced* corticospinal tract (CST) axon sprouting [10••]. The CST is a functionally important tract that controls voluntary movements in humans, and has been extensively studied in rodent models of spinal cord injury [12]. Thus, MAG may have opposing roles – growth inhibitory on some neurons but promoting on other neurons - even in the adult CNS. Moreover, MAG may mediate axon stability and integrity, and protect axons under pathological conditions [13,14•]. Genetically deleting MAG led to accelerated axonal loss in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis [15•]. Together, these studies indicate that MAG has divergent roles in axonal response to injury and disease: in addition to its well-publicized role in growth inhibition, MAG may *promote* axonal growth and/or protect axons from further degeneration in the adult CNS.

OMgp is a glycosylphosphatidylinositol (GPI)-linked protein (Fig. 2). Originally found in CNS myelin extract, it is expressed not only by oligodendrocytes but also by neurons, including adult CNS neurons [16,17]. Its role in developmental axon sprouting is still poorly understood [18]. Interestingly, OMgp is involved in the regulation of synaptic plasticity and activity-dependent synaptic strength [19], and it may influence axonal target specification during the development of thalamocortical projections [20]. In three independent studies, genetically deleting OMgp did not promote CST axon regeneration [9••,10••,21]. While deleting OMgp similarly did not lead to 5-HT axon regeneration [9••,10••], it enhanced 5-HT axon sprouting [10••,21]. Thus, OMgp inhibits axon sprouting after CNS injury in vivo.

Nogo (or Rtn4) is the most extensively studied MAI, with Nogo-A being the isoform most abundantly expressed by oligodendrocytes [22]. Alternative splicing and alternative promoter usage generate two other isoforms: Nogo-B and Nogo-C. Nogo-A is a transmembrane protein expressed in the endoplasmic reticulum but may reach cell surface. An extracellular 66 amino acid loop named Nogo-66 in the C-terminus (shared among the 3 isoforms) induces growth cone collapse and inhibits neurite growth [3] (Fig. 2). A Nogo-A specific region is also inhibitory to neurite growth independently of Nogo-66 [23]. Interestingly, Nogo deficient mice display a delayed closure of the critical period for ocular dominance plasticity, implicating a role for Nogo in regulating experience dependent plasticity [24].

Multiple different Nogo knockout mice have been analyzed for axon growth and repair after injury [25-28]. The different Nogo knockout lines all lack Nogo-A but have different effects on Nogo-B and C [29••]. Consistent among the different studies, CNS myelin preparations made from various Nogo mutant mice all exhibited a reduced inhibitory effect on neurite growth in vitro, indicating that Nogo has a substantial contribution to myelin-associated growth inhibition [25-27]. In vivo data were rather different, ranging from extensive [25], suggestive (i.e. with a non-significant trend)[26] to no enhanced CST regeneration [27] in various Nogo mutant mice. Different factors have been proposed to explain these discrepancies, such as the definition of regeneration/sprouting, the type and severity of the lesion, the age of the mice at the time of the lesion, the genetic background or the configuration of the gene disruption [29••]. It was found later that the evidence for the most extensive CST regeneration in a Nogo-A,B gene trap mutant appeared to have risen from an inadvertent axon labeling artifacts, providing a cautionary tale for detailed anatomical analyses with spinal injury models [30]. When the Nogo-A,B gene trap mutant was re-assessed by a different group under conditions to minimize axon labeling artifacts, CST regeneration was no longer observed [28]. Together, these studies indicate that the effect of deleting Nogo on CST regeneration after experimental spinal cord injury is limited at best.

In contrast, most studies in the literature agree on a role for Nogo in CST axon sprouting. In this regard, it should be noted that any partial injury that spare even a minor population of CST axons may allow for CST sprouting that gives the appearance of regeneration [31]. Indeed, the earlier studies using the IN-1 antibodies [32], and later more specific Nogo-A antibodies in rodent models [33] and in primate models [34] could be reconciled with genetic studies if sprouting had been the emphasis. In genetic studies, an increase of CST axon sprouting was consistently reported in Nogo mutant mice by different labs, independent of the mutation analyzed and the strain background as long as the CST was not completely severed [9••,10••,26,35]. It is interesting to note that chondroitin sulfate proteoglycans (CSPGs), the astroglia-derived axon growth inhibitors, also appear to exert their effect on CNS repair primarily through modulating axon sprouting rather than regeneration [36•,37••].

Distinguishing sprouting from regeneration is not only important in investigating the underlying mechanisms, it is also important in translational effort: a therapy that is designed to improve functional recovery primarily based on enhanced sprouting is unlikely to have any chance of success for anatomically complete injuries. Sprouting occurs spontaneously

without any treatment after injury. It is the body's natural repair mechanism for the CNS that can be modulated by targeting glia-derived growth inhibitors. Sprouting axons do not have to travel far to reach appropriate targets while regenerating axons may have to travel long distance in order to make functional connections. For these reasons, modulating sprouting, rather than regeneration, might be a more attainable therapeutic goal in the near term to promote functional recovery. The mechanisms by which sprouting leads to functional recovery remain to be extensively investigated.

3.2. Multiple Receptors

NgRs (NgR1, NgR2 and NgR3) are a family of three leucine-rich repeat GPI-linked proteins that have been shown to bind axon growth inhibitors. The first MAI receptor discovered was Nogo receptor 1 (NgR1 or Nogo-66 receptor) because of its binding to Nogo-66 [38]. Later on it was found that NgR1 binds to MAG [39] and OMgp [40] as well, despite the three MAIs not sharing structural similarities (Fig. 2). NgR2 also binds to MAG, with even a higher affinity than NgR1 [41]. Unexpectedly, NgR1 along with NgR3 bind to CSPGs [42••], highlighting potential functional redundancy and crosstalk between the two different classes of inhibitors (Fig. 2).

NgR1 forms a complex with co-receptors, LINGO-1 and $P75^{NTR}$ or TROY, to initiate intracellular signaling [43-46]. One working model is that the formation of ligand/receptor/co-receptors complex promotes proteolysis of $p75^{NTR}$ (via α- and γ-secretase), which activates protein kinase C and the small GTPase RhoA/ROCK/Cofilin pathway, thereby promoting actin depolymerization in the growth cones and blocking neurite extension [47]. However, whereas NgR1 is required to promote growth cone collapse from Nogo-66, it is not for required for its longer-term effect on neurite extension [48-50]. Physiologically, NgR1 has a role in activity-dependent synaptic strength and plasticity [24,51] and is involved, as are NgR2/3, in restricting synapse formation during development [52•]. It will be interesting to find out whether (and if so, how) these physiological functions relate to their function in CNS repair.

Among NgRs, NgR1 is the most extensively studied for its function in vivo after injury. A peptide blocking Nogo66-NgR1 interaction had mixed results in CST regeneration and functional recovery [53-55,56••]. Genetic studies by two independent labs found no enhancement in CST regeneration in two different NgR1 knockout mouse lines [48,49]. In line with this, deleting $p75^{NTR}$, a co-receptor for NgR1, also did not enhance CST axon regeneration [49,57]. In NgR1 knockout mice, even 5-HT axons did not exhibit enhanced regeneration after a complete spinal transection [58]. However, deleting NgR1 enhanced CST axon sprouting across the midline following a unilateral lesion [35], consistent with a role for myelin-mediated inhibition in axon sprouting after injury. Further studies are required to substantiate the robustness of such enhanced sprouting and associated functional benefit.

PirB. Paired immunoglobulin-like receptor B (PirB, LILRB2 in human) is another major MAI receptor that binds to Nogo, MAG and OMgp (Fig. 2) [59]. MAI binding triggers PirB's interaction with $p75^{NTR}$ [60], leading to the recruitment of phosphatases (SHP-1 and SHP-2), which then modulate tropomyosin-receptor kinase phosphorylation and associated

signal transduction pathways [61••]. Interestingly, PirB is associated with ocular-dominance plasticity [62], just as Nogo, NgR1 and CSPGs [24,63]. However, deleting PirB did not enhance axon regeneration after optic nerve crush [61••], traumatic brain lesion [64] or spinal cord injury [56••]. Furthermore, even blocking NgR1 with NEP1-40 in PirB knockout mice did not enhance CST regeneration [56••]. Interestingly, however, SHP-1/2 knockdown promoted optic nerve regeneration [61••].

Other receptors. Beside NgRs and PirB, other receptors or signaling mediators have been proposed for MAIs. For example, both MAG and amino-Nogo can signal through an integrin-based mechanism [65,66] (Fig. 2). However, a direct interacting partner for amino-Nogo remains to be identified.

4. Combined effects of targeting multiple growth inhibitors

The presence of multiple myelin inhibitors along with multiple receptors prompted the question of functional redundancy among the different myelin inhibitors. Two independent groups generated and characterized Nogo/MAG/OMgp triple knockout mice [9••,10••]. A detailed discussion of the different mutations and genetic background used can be found in a previous review [29••]. The results are summarized here. Using in vitro neurite growth assays, both studies found a substantial contribution from Nogo to the inhibitory activity of CNS myelin [9••,10••]. In vivo results were more divergent. In one study, deleting Nogo-, MAG, OMgp or all three inhibitors together did not promote CST regeneration after dorsal hemisection; deleting all three together did not promote 5-HT regeneration after complete transection [10••]; deleting Nogo promoted CST sprouting after pyramidotomy while deleting MAG or OMgp promoted 5-HT sprouting after lateral hemisection. In no case was any additive or synergistic effect seen on axon sprouting, implicating a potential ceiling effect of manipulating myelin inhibitors. What was most surprising was that deleting MAG *reduced* CST sprouting, as discussed above [10••]. Again, this study emphasizes the importance of understanding the in vivo role of myelin-associated inhibitors – or, perhaps more correctly, myelin-associated axon growth modulators – before targeting these molecules in therapeutic development. No functional recovery was reported in this study.

In the other study, the investigators confirmed their previous finding that deleting Nogo alone promoted CST regeneration [9••]. Deleting MAG or OMgp alone did not lead to CST regeneration but in combination with Nogo deletion led to more CST regeneration than Nogo deletion alone [9••]. Targeting Nogo or all three inhibitors also promoted 5-HT sprouting with a partial lesion model [9••]. Taken together, these two studies reinforce the notion that manipulating MAIs has more consistent and reproducible effect on axon sprouting than regeneration. The complexity involving molecule and tract specific effects on axonal growth after injury remains to be fully elucidated. Again, it is extremely important to understand the in vivo roles of individual MAIs under physiological and pathophysiological conditions before targeting them in therapies. Moving therapies forward without a clear understanding of the in vivo function of the intended molecular targets will lead to unnecessary failure in translational effort.

Functional redundancy may also exist between the myelin inhibitors and CSPGs. Using NEP1-40 and chondroitinase treatment, two different groups did not see a synergistic effect on axon growth in a slice culture and organotypic co-culture system respectively [67,68]. In contrast, acute treatment with Nogo-A antibody and delayed Chondroitinase treatment have been reported to promote CST growth additively and combining the two treatments was more effective in promoting functional recovery when applied together with a rehabilitation scheme [69••]. The authors made an interesting observation that the diameters of CST axons affected by the two treatments are different, with targeting Nogo promoting growth of larger diameter axons while targeting CSPGs promoting the growth of finer processes with varicosities. The molecular mechanism underlying this phenomenon warrants further investigation.

5. Concluding remarks

Myelin-Associated Inhibitors (MAIs) are molecules present in the CNS myelin that modulate axon growth. Most evidence in the literature is consistent with a role for MAIs in axon sprouting, reproducible with a variety of injury models, axonal tracts and across different labs. Axon regeneration, however, remains limited by targeting these molecules alone. Both regeneration and sprouting can contribute to functional recovery. Distinguishing these two forms of injury-induced axonal growth is important not only to the understanding of the underlying molecular regulation but also to the development of effective therapeutic strategies to treat CNS injuries and other neurological conditions. Indeed, promoting sprouting could be as functionally important, if not more, as regeneration. Indeed, it may be more realistic to target sprouting than frank regeneration in the near term. The roles of MAIs in axon sprouting are complex. Once the neuron-intrinsic growth state is elevated, extrinsic axon growth modulators including MAIs are more likely to stand out as the next barrier for regeneration. Regardless of sprouting or regeneration, the anatomical substrate provided by enhanced growth is only likely to be useful for functional gains with additional, activity-dependent mechanisms such as that provided by rehabilitation or training.

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Highlights

- Regeneration and sprouting are two forms of injury-induced axonal growth
- Myelin inhibitors modulate axonal sprouting after CNS injury
- Regeneration elicited intrinsically can be further modulated by myelin inhibitors
- Promoting sprouting to restore function may be a more attainable near-term goal

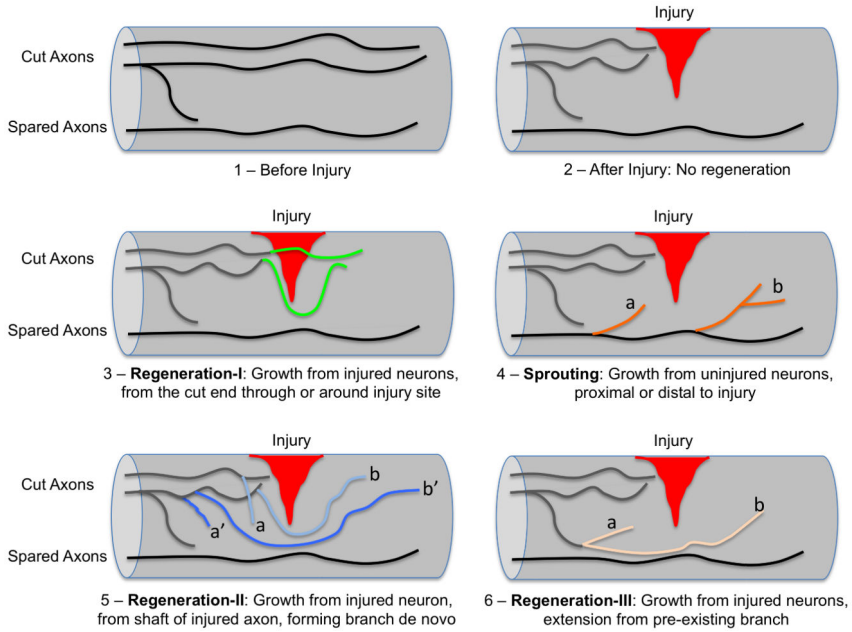

Figure 1. Axon regeneration versus axon sprouting after injury in the spinal cord

1) Axons in the non-injured spinal cord. **2)** After a partial injury, injured axons normally do not regenerate. **3)** Regeneration scenario I: injured axons grow from the cut end (i.e. injured axonal tip), through or around the injury site. This is the typical definition of regeneration. **4)** Sprouting is any new axonal growth from uninjured neurons. This occurs in response to injury of other neurons. It can occur proximal (a) or distal (b) to the injury site. **5)** Regeneration scenario II: axonal growth from the shaft of injured axons, forming new branches de novo. The growth can originate close to the injury site (a, b) or at a distance (a', b'); it can be for a short (a, a') or long (b, b') distance. **6)** Regeneration scenario III: axonal extension from pre-existing branches of injured neurons. It can be for a short (a) or long (b) distance. The common theme for all scenarios of regeneration here is that axonal growth is from injured neurons.

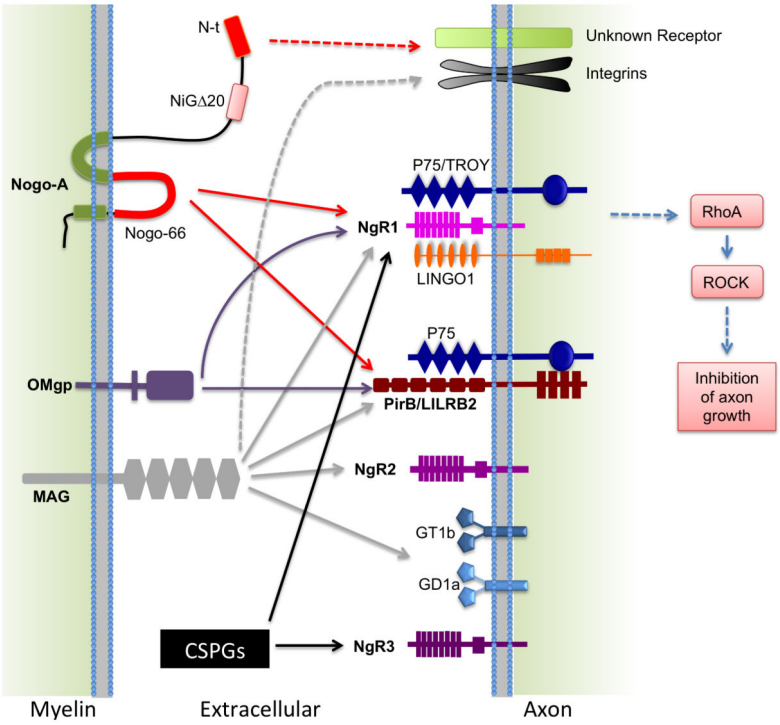

Figure 2. Interaction of the prototypical myelin-associated inhibitors with their receptors

Nogo, MAG and OMgp all bind to NgR1 and PirB receptors. NgR1 forms a complex with LINGO-1 and $p75^{NTR}$ (or TROY) to signal growth inhibition in the axons. PirB can also bind to $p75^{NTR}$. In addition, MAG can bind NgR2 and gangliosides GD1a and GT1b. N-terminal (N-t) Nogo and MAG may also signal through an integrin-based mechanism. Other unknown receptors may exist. Chondroitin sulfate proteoglycans (CSPGs), the glial scar-derived inhibitors, can bind to NgR1 and NgR3. Thus, myelin inhibitors and CSPGs share some receptors.