

# Overcoming inhibitors in myelin to promote axonal regeneration

Marco Domeniconi, Marie T. Filbin\*

*Hunter College of the City University of New York, Department of Biological Sciences, 695 Park Avenue Room 807N, New York, NY 10021, USA*

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## Abstract

The lack of axonal growth after injury in the adult central nervous system (CNS) is due to several factors including the formation of a glial scar, the absence of neurotrophic factors, the presence of growth-inhibitory molecules associated with myelin and the intrinsic growth-state of the neurons. To date, three inhibitors have been identified in myelin: Myelin-Associated Glycoprotein (MAG), Nogo-A, and Oligodendrocyte-Myelin glycoprotein (OMgp). In previous studies we reported that MAG inhibits axonal regeneration by high affinity interaction ( $K_D$  8 nM) with the Nogo66 receptor (NgR) and activation of a p75 neurotrophin receptor (p75NTR)-mediated signaling pathway.

Similar to other axon guidance molecules, MAG is bifunctional. When cultured on MAG-expressing cells, dorsal root ganglia neurons (DRG) older than post-natal day 4 (PND4) extend neurites 50% shorter on average than when cultured on control cells. In contrast, MAG promotes neurite outgrowth from DRG neurons from animals younger than PND4. The response switch, which is also seen in retinal ganglia (RGC) and Raphe nucleus neurons, is concomitant with a developmental decrease in the endogenous neuronal cAMP levels. We report that artificially increasing cAMP levels in older neurons can alter their growth-state and induce axonal growth in the presence of myelin-associated inhibitors.

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## 1. Discussion

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 [1–3]. There is no a priori reason for this failure, since lower vertebrates can regenerate a severed spinal cord [4]. 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 [5] and the immature CNS, as the one of neonatal rats, easily regenerates after injury [6]. Aguayo et al. used peripheral nerve segments to make bridges between the medulla and spinal cord to demonstrate that central neurons could grow in a peripheral environment [7]. 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 [7,8]. Others have shown that isolated CNS neurons can extend long processes in vitro [9,10]. 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 environment [2,11]. Thus, any successful strategy for the regeneration of injured CNS axons will likely include multiple steps: keeping neurons alive and in a positive growth-state, prevent the formation of a glial scar, overcome inhibitory molecules present in the myelin debris, and give direction to the growing axons. We have focused our research on myelin-associated inhibitors and ways to block their influences on injured axons.

To date, three major inhibitors of growth have been identified in myelin: myelin-associated glycoprotein (MAG) [12,13], oligodendrocyte-myelin glycoprotein (OMgp) [14,15], and Nogo-A [16], an antigen of the IN-1 antibody

\* Corresponding author.

*E-mail address:* Filbin@genectr.hunter.cuny.edu (M.T. Filbin).

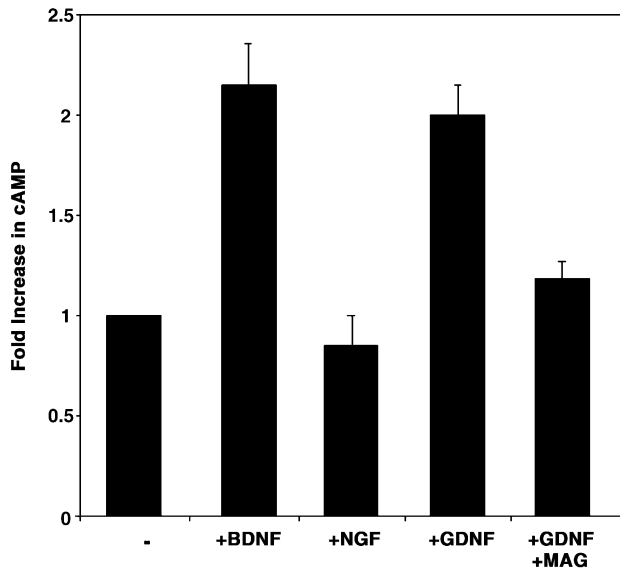


Fig. 1. Dissociated cerebellar neurons were plated in a 96-well dish. The neurons were cultured for at least 6 h after which time BDNF, GDNF, or NGF, each at 200 ng/ml, was added as indicated and incubated for a further 30 min. Where indicated, neurotrophin was added with MAG-Fc (+MAG) at a concentration of 20  $\mu$ g/ml. Following incubation, the cAMP levels were measured and compared to a standard. The results are the mean of between four and seven experiments, each done in quadruplicate. Results represent the fold-increase relative to neurons incubated for the same length of time but without the addition of neurotrophin (reproduced from Cai et al. [26], with permission).

[17] containing two inhibitory domains, termed Nogo-66 [16] and amino-Nogo [18]. Although these three inhibitors have no structure similarity, they all appear to be located in the periaxonal surface of the myelin membrane placing them in an optimal location to mediate axon-glia interactions. In addition, the three proteins have been shown to bind the same neuronal receptor. The Nogo-66 receptor (NgR) was first identified by screening a cDNA expression library for binding partners of Nogo-66 [19], a 66 amino acids peptide containing inhibitory activity, and later shown to bind all three inhibitors with similar affinities [15,20,21]. Importantly, introduction of a dominant-negative form of NgR can dramatically reduce the effects of Nogo, MAG, OMgp and myelin in general [15,20]. Since NgR is a glycosylphosphatidylinositol (GPI)-linked protein, a second molecule is required for the transduction of the inhibitory signal. This co-receptor was soon identified as the p75 common neurotrophin receptor (p75<sup>NTR</sup>) [22]. Although the details of the signaling pathway are still being investigated, it has been demonstrated that MAG, Nogo-66 and OMgp activate the small GTPase Rho-A [23] and they do so in a p75<sup>NTR</sup>-dependent manner [22,24].

The discovery of a receptor complex common to the three major myelin inhibitors of regeneration has presented an important target for therapeutic intervention following injury to the CNS. However, targeting a large receptor complex may prove a difficult endeavor and changing the inherent growth state of the injured neurons could prove a

better approach. Since recent work by the Bregman group had suggested that exposure to neurotrophic factors can facilitate axonal growth into grafted tissue [25] we decided to investigate if such exposure could indeed change the neuronal responses to inhibitory cues. Postnatal CNS neurons fail to extend neurites when grown on a substrate of MAG-expressing cells or purified myelin. Although the simple addition of neurotrophic factors to the neuronal cultures did not result in axonal growth, we found that neurons that had been exposed to specific factors for some time prior to their encounter with the inhibitors were no longer sensitive to the inhibitory signals [26]. It seemed that a signaling pathway activated by neurotrophins could “prime” the neurons and overcome the effects of MAG and myelin.

We proceeded to test an array of compounds involved in transduction pathways and identified one that could mimic the “priming” effect seen with neurotrophins. Dibutylryl-cAMP (db-cAMP) could not only abrogate the inhibitory activity of MAG and myelin, but it did so without requiring a “priming” period. This was a most interesting find since it suggested that db-cAMP was an integral component of the neurotrophin signaling pathway and that it could be used to modulate axonal responses. Indeed, competitive immunoassay measurements show a two-fold increase in endogenous cAMP levels in response to treatment with neurotrophic factors (Fig. 1). Furthermore, the neurotrophin “priming” effect requires activation of the cAMP-dependant protein kinase (PKA), as pharmacological inhibition of the enzyme abrogates the improved regenerative ability seen after both neurotrophin priming and db-cAMP treatment [26].

One interesting feature of MAG is its bi-functionality. Early during embryonic development, although both MAG and its receptor complement are already present, MAG does

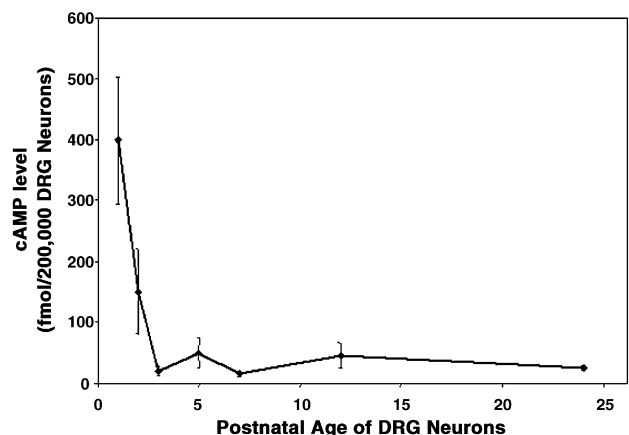


Fig. 2. Dissociated DRG neurons from P1–P22 animals, as indicated, were cultured overnight in a 96-well dish. cAMP levels were measured using a competitive immunoassay. Results are the mean ( $\pm$ SE) of at least three experiments, each performed in sextuplicate (reproduced from Cai et al. [28], with permission).

not hinder axonal growth. It is only at a later stage that neurons become sensitive to its activity. While the switch in response mainly occurs embryonically, in the specific case of dorsal root ganglia neurons (DRG) it occurs sharply at postnatal day 3 (PND3) [27]. Notably, the regenerative ability of neurons also switches during development: young or embryonic neurons have been known to spontaneously regenerate while the older ones do not [6]. We questioned what could be the cause of this change in response. Quantification of cAMP levels in pre- and postnatal neurons by both immunostaining and immunoassay demonstrate that the endogenous cAMP levels are quite elevated in younger

neurons and drop sharply in the older ones. As to DRG, the levels decrease dramatically at PND3 (Fig. 2). The time course of the cAMP decline in DRG coincides with their loss of regenerative ability in the presence of MAG and myelin. Further, we found that blocking the cAMP signaling pathway in young DRG completely abrogates their ability to grown on inhibitory substrates. How does this apply to an in vivo model? We proceeded to examine the role of cAMP in the spontaneous regeneration of neonatal spinal cord. In the young animal, dorsal column hemisection is normally followed by regeneration of the axotomized axons. Notably, if an inhibitor of PKA is applied simultaneously to injury at

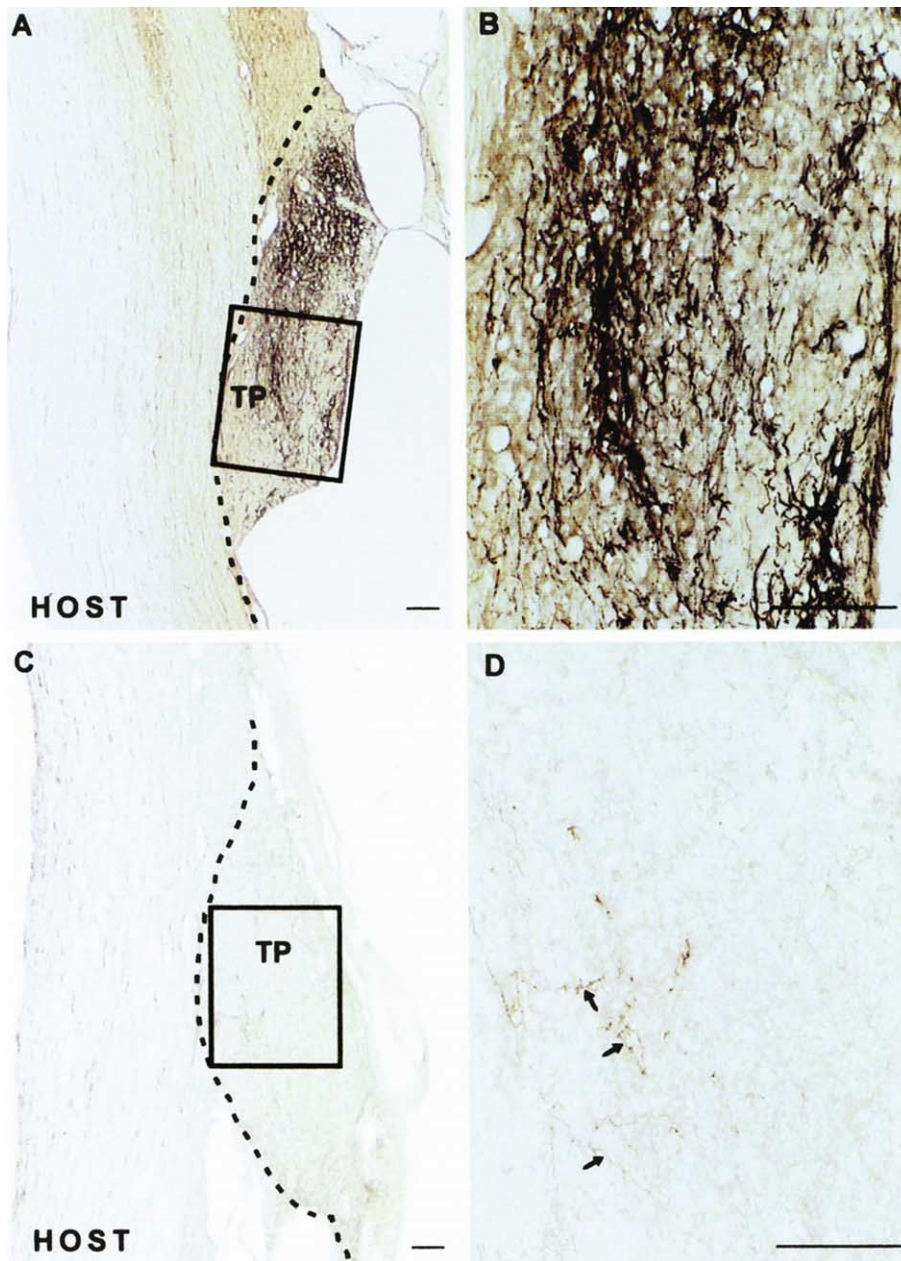


Fig. 3. Regeneration of neonatal corticospinal axons after overhemisection lesion. BDA anterograde labeling of axons shows extensive growth in the untreated animals (A and B) but not in the animals treated with the PKA inhibitor H89 (C and D). Boxed areas in A and C are shown at higher magnification in B and D, respectively. Scale bars, 100  $\mu$ m (reproduced from Cai et al. [28], with permission).

the lesion site, the spontaneous regeneration is completely blocked (Fig. 3) [28].

There is an interesting paradigm in which adult CNS neurons have been shown to regenerate *in vivo*: the conditioning lesion model. Dorsal root ganglia neurons are unique in that they 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 [29]. The mechanism leading to the conditioning lesion effect is unknown, but our recent findings raise an intriguing possibility. Does the PNS branch lesion induce an elevation in the neuronal cAMP levels and, thus, facilitate the CNS branch regeneration? To test this hypothesis we measured cAMP levels in DRG at 1 day and 1 week after a sciatic nerve lesion. Once again, we found a substantial increase in the endogenous cAMP levels of the axotomized DRG neurons. The cAMP levels rose sharply after 1 day and returned to baseline

levels after a week (Fig. 4) [30]. When the DRG neurons were isolated and cultured on MAG-expressing cells or purified myelin, they extended long neurites in a PKA-dependant manner and without the additions of neurotrophins or db-cAMP.

Although it has long been held that CNS neurons do not regenerate after injury, it is now clear that their failure to do so is largely due to the presence of inhibitors in myelin and that they will grow despite the presence of growth inhibitory molecules in several different situations. Indeed, embryonic and young neurons, DRG neurons after a conditioning lesion and adult neurons primed with neurotrophins will regenerate following axotomy. It is remarkable that all these situations involve elevation of the cAMP levels as the determining factor. Furthermore, it is remarkable that a single injection of db-cAMP applied 1 week prior to dorsal column lesion is sufficient to facilitate extensive axonal regeneration [30,31].

Following our work identifying the neuronal receptors mediating inhibition by MAG and myelin, we have now

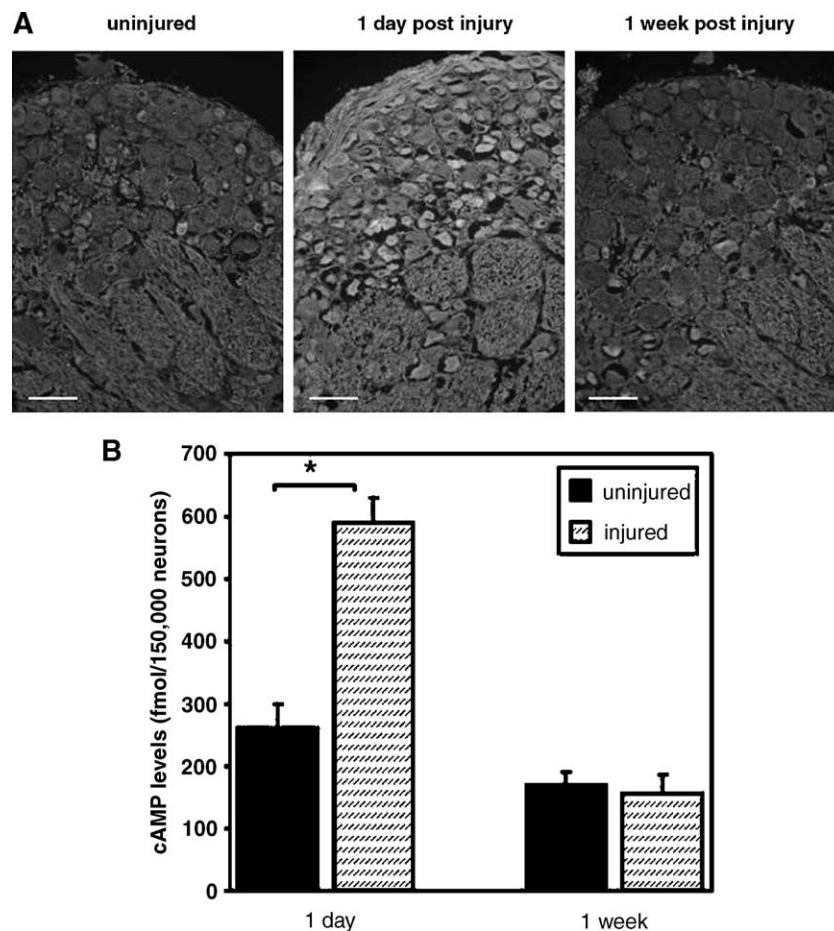


Fig. 4. Changes in cAMP levels in DRG neurons in response to a peripheral nerve lesion. Sciatic nerves were unilaterally lesioned either 1 day or 1 week prior to when L4 and L5 DRG neurons were removed. (A) DRGs were fixed in acrolin immediately upon removal, sectioned, and immunostained for cAMP. Scale bar, 20  $\mu$ m. (B) cAMP levels were measured using a competitive immunoassay after plating 200,000 dissociated DRG neurons per well. For each condition, in each experiment the DRGs from 8 animals were combined. Results are the mean ( $\pm$ SEM) of at least six experiments, each carried out in quadruplet. Black bar, uninjured nerve; stippled bar, lesioned nerve. Asterisk (\*) indicates results are significantly different,  $p < 0.05$  (reproduced from Qiu et al. [30], with permission).

shown that elevation of cAMP by either administration of cAMP analogues or priming with neurotrophic factors can promote axonal regeneration further expanding the range of possible therapeutic strategies for the treatment of spinal cord injury.

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