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Prior Exposure to Neurotrophins Blocks Inhibition of Axonal Regeneration by MAG and Myelin via a cAMP-Dependent Mechanism

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Summary

MAG is a potent inhibitor of axonal regeneration. Here, inhibition by MAG, and myelin in general, is blocked if neurons are exposed to neurotrophins before encountering the inhibitor; priming cerebellar neurons with BDNF or GDNF, but not NGF, or priming DRG neurons with any of these neurotrophins blocks inhibition by MAG/myelin. Dibutyryl cAMP also overcomes inhibition by MAG/myelin, and cAMP is elevated by neurotrophins. A PKA inhibitor present during priming abrogates the block of inhibition. Finally, if neurons are exposed to MAG/myelin and neurotrophins simultaneously, but with the G_i protein inhibitor, inhibition is blocked. We suggest that priming neurons with particular neurotrophins elevates cAMP and activates PKA, which blocks subsequent inhibition of regeneration and that priming is required because MAG/myelin activates a G_i protein, which blocks increases in cAMP. This is important for encouraging axons to regrow in vivo.

Introduction

The immature mammalian CNS will readily regrow in vivo after injury (Bates and Stelzner, 1993; Hasan et al., 1993). In contrast, mature axons of the mammalian CNS will not regenerate if damaged (see Johnson, 1993; Schwab and Bartholdi, 1996). Although the precise mechanisms regulating this developmental switch are not known, it is widely accepted that both environmental cues and neuronal response change with maturity and contribute to the lack of regeneration (Keirstead et al., 1992; Li and Raisman, 1993; Li et al., 1995; see Schwab and Bartholdi, 1996). One of the most notable differences between the mature and the immature nervous systems is the presence of myelin. Indeed, from both in vivo (Schnell and Schwab, 1990; Keirstead et al., 1992; Cheng et al., 1996) and in vitro (Crutcher, 1989; see Schwab et al., 1993) studies, strong evidence implicates CNS myelin as a potent inhibitor of axonal regeneration in the damaged, adult CNS. While myelin probably has many inhibitory components, very few have been identified and characterized (see Johnson, 1993; Schwab et al., 1993; Filbin, 1995, 1996; Keynes and Cook, 1995). It is generally accepted that the antigen(s) recognized by the IN-1 antibody contributes substantially to overall inhibition by myelin since the presence of antibody allows extensive regrowth of neurons on myelin in vitro or after injury in vivo (Caroni and Schwab, 1988; Schnell and Schwab, 1990). In addition, we (Mukhopadhyay et al., 1994) and others (McKerracher et al., 1994) have shown myelin-associated glycoprotein (MAG), a well characterized myelin component, potently inhibits axonal regeneration in culture. Interestingly, MAG is a bifunctional molecule inhibiting regeneration from a wide variety of postnatal neurons (McKerracher et al., 1994; Mukhopadhyay et al., 1994; De Bellard et al., 1996) yet promoting axonal growth from dorsal root ganglion (DRG) neurons up to postnatal day 4, after which DRG regrowth is also inhibited by MAG (Johnson et al., 1989; Mukhopadhyay et al., 1994). Although it is demonstrably a strong inhibitor of axonal regrowth in vitro, MAG's contribution to inhibition by myelin in vivo is less clear. Using the MAG knockout mice, one group reported not only an improvement in the distance damaged axons regenerated but also an improvement in the number of axons regrowing (Li et al., 1996). Their results contrast, however, with the report by Bartsch et al. (1995), who observed no difference in regeneration between $MAG^{-/-}$ and wild-type mice.

In apparent contradiction to the dogma that CNS myelin is inhibitory, two recent studies report extensive axonal growth into white matter distal to the site of a lesion (Berry et al., 1996; Bregman, 1998). Bregman and her colleagues showed that when embryonic tissue was implanted into a lesion created in rat spinal cord, there was extensive axonal growth into the embryonic tissue but not out into the host white matter beyond the lesion. Significantly, they noted that when pumping either of two neurotrophins—brain-derived neurotrophic factor (BDNF) or neurotrophic factor 3 (NT-3)—into the implanted experimental tissue, axons not only grew into the embryonic tissue, but they also extended long processes into the white matter beyond. Berry and coworkers independently (Berry et al., 1996) showed that if an explant of peripheral nerve tissue was placed in the retina after a lesion was created in the optic nerve, optic nerve axons grew extensively into the white matter, distal to the lesion site. It should be noted that in this experiment, unlike previous studies of Aguayo and his colleagues (David and Aguayo, 1981; Benfey and Aguayo, 1982), the regenerating axons never contacted the peripheral nerve explant. The unexpected results from both these studies suggest that the neurotrophins used for the spinal cord study and some component secreted from the peripheral nerve explants affects how the growing axon responds to myelin, even to the extent of neutralizing its ability to inhibit axonal regrowth.

In addition to supporting neuronal survival and differentiation (see Barde, 1990; Thoenen, 1991; Klein, 1994; Snider, 1994; Lindsay, 1996), neurotrophic factors can also behave as neurotropins. Axons will turn and grow toward a high concentration of nerve growth factor (NGF) (Letourneau, 1978), and *Xenopus* spinal neurons in culture turn toward a gradient of BDNF (Ming et al., 1997a; Song et al., 1997). Interestingly, this chemoattractant effect of BDNF can be switched to a repulsive

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one if a competitive analog of cAMP or an inhibitor of protein kinase A is added to the culture (Song et al., 1997). Furthermore, a recently identified axonal guidance molecule, netrin-1, also attracts *Xenopus* spinal neurons (Ming et al., 1997b), and like MAG, netrin-1 is bifunctional (Colamarino and Tessier-Lavigne, 1995). As with BDNF, netrin-1 repels axons that have been cultured with a cAMP competitive analog or with an inhibitor of protein kinase A (Ming et al., 1997b). Curiously, under these same conditions, a soluble recombinant form of MAG repels these *Xenopus* growth cones, but this repulsion is switched to attraction by addition of a cAMP agonist to the culture media (Song et al., 1998). These studies imply that the neuronal cytosolic cAMP levels may dictate whether a particular molecular guidance cue is repulsive/inhibitory or attractive/promoting.

In this study, we ask if the inhibitory effects of MAG and of CNS myelin can be modulated by neurotrophins and if this modulation involves cAMP. We show that growth inhibition by MAG and myelin is indeed blocked in cerebellar neurons that are first cultured overnight (primed) with BDNF or with glial-derived neurotrophic factor (GDNF), but not with NGF. For DRG neurons, inhibition by MAG and myelin is blocked when the neurons are primed with BDNF, GDNF, or NGF. The priming effect is both time dependent and dose dependent. Adding neurotrophins at the same time as exposure to MAG or myelin has no effect on inhibition. In addition, we show that artificial elevation of cAMP levels with dibutyryl cAMP also blocks inhibition by both MAG and myelin. Following from this, we show that neurotrophins increase neuronal cAMP levels, and if neurons are primed with neurotrophins in the presence of a protein kinase A (PKA) inhibitor, the block of MAG and myelin inhibition is completely abrogated. Finally, if neurons are not primed but are instead exposed simultaneously to neurotrophins and MAG or myelin along with the G protein inhibitor, pertussis toxin, inhibition is also blocked. Together, these results indicate that increased cAMP levels and activation of PKA can block the inhibitory effects of MAG and myelin, but only when they are elevated/activated prior to exposure to MAG or myelin. The requirement for priming indicates that binding MAG or myelin to neurons activates an inhibitory G protein that blocks any increase in cAMP, a conclusion supported by finding that MAG blocks neurotrophin-induced elevation of cAMP and pertussis toxin neutralizes the block by MAG. Along with recent studies in vivo (Berry et al., 1996; Bregman, 1998), our results point to a model of enhanced regeneration and functional recovery in vivo by elevating cAMP and activation of PKA in neurons or growth cones very soon after injury, before growth cones encounter myelin inhibitors.

Results

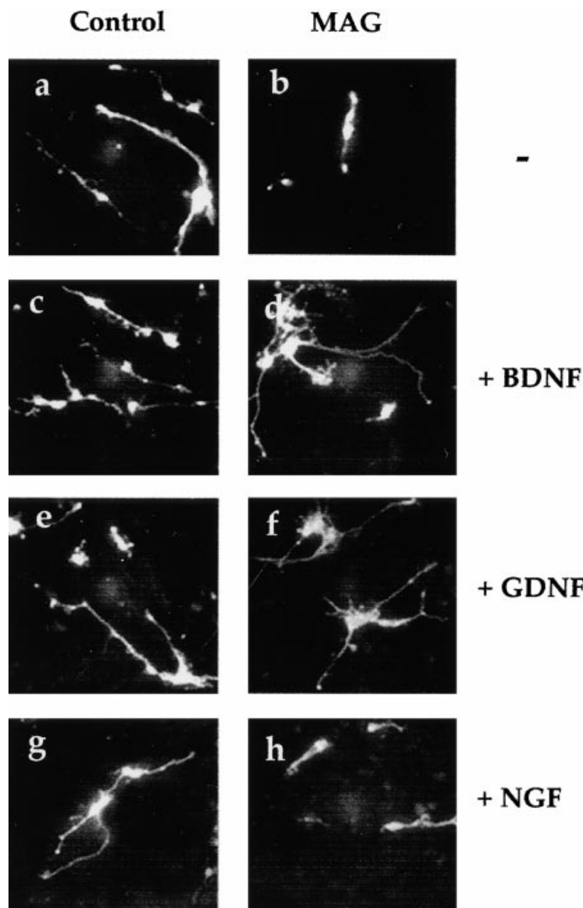
Priming Neurons with Neurotrophins prior to Exposure to MAG

To determine if neurotrophins can reverse the inhibitory effects of MAG on neurite outgrowth, BDNF, NGF, or GDNF were added separately at a concentration of 200 ng/ml to either cerebellar (usually P7–P9) or older DRG neurons grown on monolayers of MAG-expressing or control cells. (Older DRGs always refers to older than postnatal day 5, selected because MAG is bifunctional and promotes neurite outgrowth from DRG neurons up to P3–P4, after which it inhibits regeneration [Mukhopadhyay et al., 1994; De Bellard et al., 1996].) As we reported previously (Mukhopadhyay et al., 1994), neurite outgrowth from neonatal cerebellar neurons and from older DRG neurons is inhibited by about 70% and 50%, respectively, when grown on MAG-expessing cells compared to control cells, regardless of whether neurotrophins were present in the media. Therefore, of the neurotrophins tested, none had an effect on MAG's inhibition of neurite outgrowth when added at the same time as cultured neurons are exposed to MAG.

However, we noted from Bregman (1998) that spinal neurons in vivo that extended long axons into white matter distal to a lesion site had been exposed to neurotrophins prior to encountering myelin. Therefore, we asked if this prior exposure to neurotrophins affects the ability of MAG to inhibit outgrowth. Cerebellar and older DRG neurons were isolated as usual, but instead of plating them directly onto the MAG-expressing and control cells, they were first grown overnight on poly-l-lysine in the presence or absence of one of the neurotrophins, each at a concentration of 200 ng/ml. After overnight priming, the neurons were then replated onto the MAG-expressing or the control cells. For cerebellar neurons primed with BDNF or GDNF, but not NGF, before being exposed to MAG, the inhibition by MAG was completely blocked. Figure 1 shows that priming with BDNF or GDNF resulted in the same neurite growth in the presence of MAG as on the control cells (Figures 1c–1f). In contrast, priming with NGF or without neurotrophin has no effect on the ability of MAG to inhibit axonal regeneration (Figures 1a, 1b, 1g, and 1h): inhibition is about 70%, as we found previously for unprimed neurons (Mukhopadhyay et al., 1994). These results demonstrate that if cerebellar neurons are exposed to specific neurotrophins (BDNF or GDNF but not NGF) before being exposed to MAG, inhibition is completely blocked.

Similar experiments with older DRG neurons, which are also inhibited by MAG (Mukhopadhyay et al., 1994; De Bellard et al., 1996), showed that priming the neurons with BDNF or GDNF completely blocked the inhibitory effects of MAG (Figure 2). However, unlike cerebellar neurons, older DRG neurons primed with NGF also resisted the inhibitory effects of MAG (Figures 2g and 2h). Overnight culture in the absence of growth factor had no effect on the ability of MAG to subsequently inhibit axonal growth, nor did priming with any of the neurotrophins have any effect on neurite outgrowth on the control cells (Figures 1 and 2). Therefore, depending on the type of neuron, priming with specific neurotrophins can completely and specifically block the inhibitory effects of MAG. It is important to note that exactly the same inhibition, or block of inhibition, was recorded whether total process outgrowth or length of the longest neurite was measured. Similarly, the same inhibition by MAG or its block by neurotrophin priming was seen when cerebellar or DRG neurons from adult rats (>P60) were used (data not shown); although, in the majority of these experiments, neurons from P7–P9 rats were used to facilitate neuronal preparation.

To further establish the specificity of neurotrophin



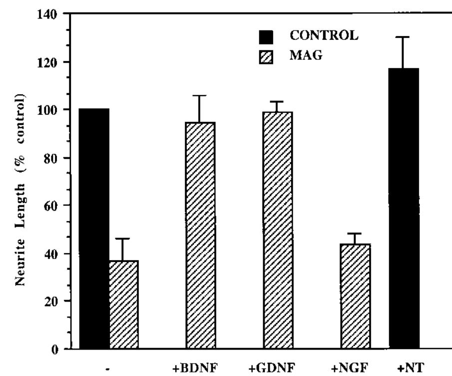


Figure 1. The Effect of Priming Cerebellar Neurons with Neurotrophins on Subsequent Inhibition of Neurite Outgrowth by MAG

Dissociated cerebellar neurons were first primed overnight on poly-l-lysine without neurotrophin (a and b) or with BDNF (c and d), GDNF (e and f), or NGF (g and h), each at 200 ng/ml, before being trypsinized and transferred to a monolayer of either MAG-expressing CHO cells (b, d, f, and h) or control CHO cells (a, c, e, and g) for further overnight culture before being fixed and immunostained for GAP43. Results in graph show the mean length of the longest neurite per neuron (+/− sem) for 180–200 individual neurons grown on either MAG-expressing CHO cells (stippled bars) or control cells (solid bars). Results are standardized to percentage of control, taken as neurite length from neurons primed without neurotrophin, and then grown on control CHO cells. NT refers to neurons primed with either BDNF, GDNF, or NGF before being subsequently cultured on control cells.

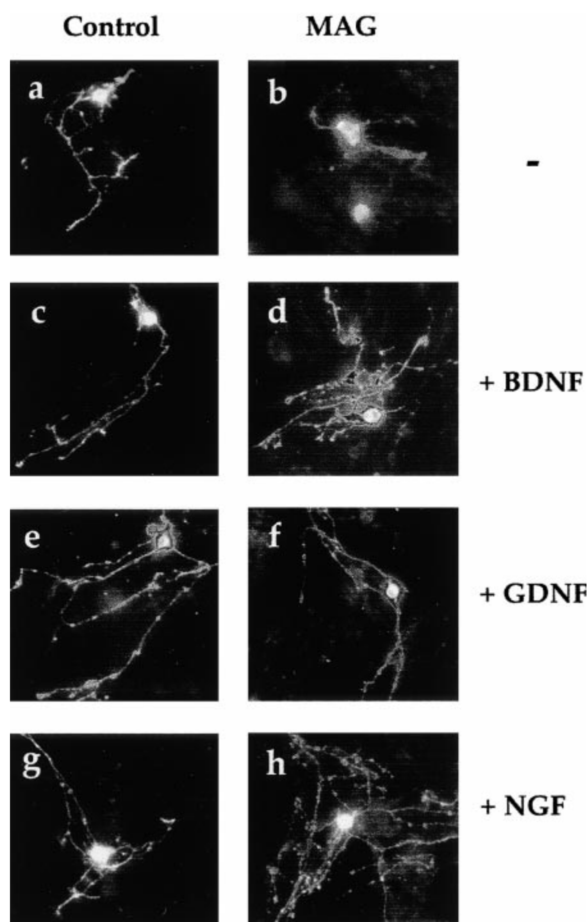
priming on blocking the inhibition by MAG, we investigated the dose dependence and time dependence of this effect. Cerebellar neurons were primed for different times in the presence of 200 ng/ml BDNF, GDNF, or NGF and then transferred to either MAG-expressing or control CHO cells. Figure 3 shows that for BDNF and GDNF, but not for NGF, longer priming times increase the block of outgrowth inhibition by MAG. BDNF and GDNF blocks MAG inhibition quite rapidly; 6 hr of priming prevents the majority of inhibition by MAG. Overnight priming blocks inhibition by MAG completely. As before, priming with NGF for any length of time did not affect MAG's ability to inhibit neurite outgrowth from cerebellar neurons. To determine if the priming effect is dose dependent, cerebellar neurons were primed overnight with different concentrations of either BDNF, GDNF, or NGF. As the concentration of BDNF and GDNF increases, the block of MAG inhibition also increases, essentially neutralizing inhibition by MAG at 200 ng/ml of either growth factor (Figure 4). Throughout this concentration range, NGF has little effect on MAG's inhibition of axonal growth. Similar effects of time dependence and dose dependence of priming were shown for older DRG neurons primed with any one of these three growth factors (data not shown).

Binding of MAG to Neurons Primed with Neurotrophins

One possibility is that priming neurons with neurotrophins downregulates the putative MAG receptor. If so, then primed neurons should no longer bind MAG. In our previously established assay to measure neuronal binding of MAG (De Bellard et al., 1996; Tang et al., 1997a), MAG-Fc—extracellular domain of MAG fused to Fc—is immobilized, and neurons, vitally labeled with a fluorescent dye, are allowed to bind. After washing, the number of bound neurons is assessed in a FluorImager and compared to binding to a control chimera, MUC18-Fc, also a 5 Ig domain–containing protein (Kelm et al., 1994). Priming either cerebellar or DRG neurons with any of the neurotrophins has no effect on their binding to MAG-Fc, and there is only background binding to MUC18-Fc (data not shown). These results demonstrate that priming neurons with BDNF, GDNF, or NGF has no effect on neuronal binding to MAG and suggests that surface expression of the MAG receptor is unaffected by priming.

Priming Neurons with Neurotrophins prior to Exposure to Myelin

MAG is a component of myelin, and CNS myelin has long been known to be inhibitory for axonal regeneration (see Schwab et al., 1993). We asked if the inhibitory properties of myelin itself could be blocked by priming neurons with growth factors. To do this, cerebellar or older DRG neurons were primed as described above, but instead of plating on CHO cells, they were transferred to a substrate of purified CNS myelin and cultured overnight. Under these conditions, BDNF and GDNF, but not NGF, extended neurites that were about twice as long



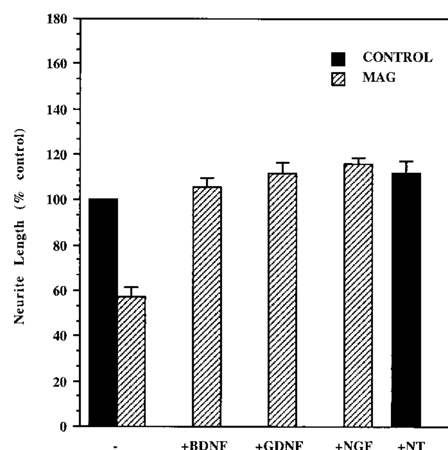


Figure 2. The Effect of Priming Older DRG Neurons with Neurotrophins on Subsequent Inhibition of Neurite Outgrowth by MAG

Dissociated older DRG neurons were first primed overnight on poly-l-lysine without neurotrophin (a and b) or with BDNF (c and d), GDNF (e and f), or NGF (g and h), each at 200 ng/ml, before being trypsinized and transferred to a monolayer of either MAG-expressing CHO cells (b, d, f, and h) or control CHO cells (a, c, e, and g) for further overnight culture before being fixed and immunostained for GAP43. Results in graph show the mean length of the longest neurite per neuron (+/− sem) for 180–200 individual neurons grown on either MAG-expressing CHO cells (stippled bars) or control cells (solid bars). Results are standardized to percentage of control, taken as neurite length from neurons primed without neurotrophin, and then grown on control CHO cells. NT refers to neurons primed with any one of the neurotrophins used, before being subsequently cultured on control cells.

as for control neurons primed without growth factor or not primed at all (Figure 5a). Similarly, if older DRG neurons were primed with BDNF or GDNF and also with NGF, they extended neurites on CNS myelin that were also about twice as long as controls (Figure 5b). These results suggest that the inhibitory effects of myelin, like those of MAG, can be reversed by exposing neurons to specific neurotrophins prior to when they encounter inhibitors in myelin.

Involvement of cAMP in Inhibition by MAG and Myelin

Since binding of MAG to neurons is unaffected by neurotrophin priming, the block of inhibition is unlikely to result from downregulation of the putative MAG receptor. Instead, the difference between the neurotrophin-primed and unprimed neurons probably occurs as a signal transduction step downstream from receptor–ligand interaction. To investigate this idea, we tested a battery of reagents known to block or activate various components of signaling mechanisms. Of all the reagents tested (data not shown), only dibutyryl cAMP (db cAMP), an analog of cAMP readily taken up by cells, reversed inhibition completely when added to cerebellar neurons growing on MAG-expressing cells without affecting neurite outgrowth on the control cells (Figure 6a). Furthermore, the effect of db cAMP was dose dependent (data not shown). Inhibition by MAG of neurite outgrowth from older DRG neurons is also completely blocked by 1 mM db cAMP (data not shown). Importantly, when 1 mM db cAMP is added to either cerebellar (Figure 6b) or DRG neurons (data not shown) growing on purified myelin, neurites were about twice as long as those grown in the absence of db cAMP.

Since artificially elevating the cAMP levels in neurons with db cAMP or priming neurons with specific neurotrophins can each block the inhibitory effects of MAG and myelin, we asked if particular neurotrophins increase the intracellular levels of neuronal cAMP. Measured by competitive immunoassay, the cAMP levels in cerebellar and older DRG neurons, after priming for 24 hr with BDNF, GDNF, or NGF, initially showed no consistent increase. However, a consistent increase in cAMP was observed after neuronal priming for 30 min. Figure 7 shows that exposure of cerebellar neurons to either BDNF or GDNF results in a 2-fold increase in cAMP levels. Under the same conditions, NGF had no effect (Figure 7a). In contrast, exposure of older DRG neurons to BDNF, GDNF, or NGF each resulted in a 2-fold increase in neuronal cAMP (Figure 7b). The 2-fold increase is relative to basal levels of cAMP, which range from 20–50 fmol/10^5 cerebellar neurons and 150–280 fmol/10^4 DRG neurons. Within any one experiment, the increase in cAMP was always near 2-fold, except, as noted above, for cerebellar neurons treated with NGF. Therefore, depending on the neuron and the particular neurotrophin, exposure to neurotrophins can elevate the endogenous neuronal levels of cAMP. This suggests that

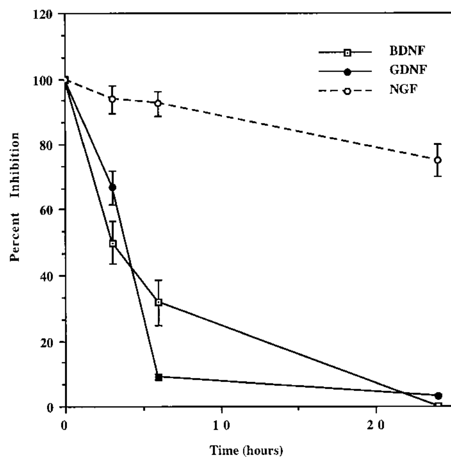


Figure 3. The Effect of Priming Neurons for Different Times with Neurotrophins on Subsequent Inhibition of Neurite Outgrowth by MAG

Cerebellar neurons were primed for different times with BDNF (open squares), GDNF (closed circles), or NGF (dotted line), each at 200 ng/ml, before transfer to either MAG-expressing or control CHO cells and cultured for a further 18 hr. The neurons were then fixed and immunostained for GAP43, and the longest neurite from 180–200 neurons was measured. Results are expressed as percentage inhibition (+/− sem), where 100% inhibition is taken as the neurite outgrowth of neurons primed without neurotrophin and subsequently grown on MAG-expressing CHO cells.

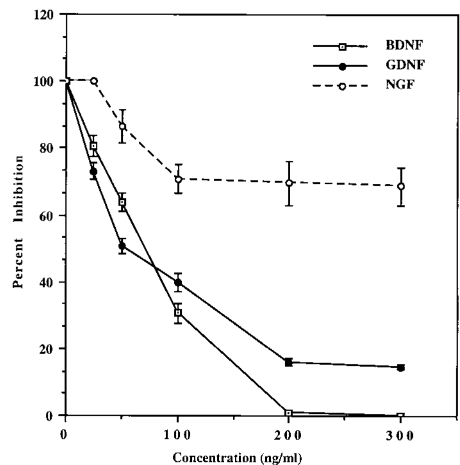


Figure 4. The Effect of Priming Neurons with Different Concentrations of Neurotrophins on Subsequent Inhibition of Neurite Outgrowth by MAG

Cerebellar neurons were primed overnight with various concentrations (25–300 ng/ml) of BDNF (open squares), GDNF (closed circles), or NGF (dotted line) before transfer to either MAG-expressing or control CHO cells and cultured for a further 18 hr. The neurons were then fixed and immunostained for GAP43, and the longest neurite from 180–200 neurons was measured. Results are expressed as percentage inhibition (+/− sem), where 100% inhibition is taken as the neurite outgrowth of neurons primed without neurotrophin and subsequently grown on MAG-expressing CHO cells.

priming with neurotrophins may block inhibition by MAG and myelin in part by elevating endogenous cAMP levels. However, although we measure an increase in cAMP after 30 min, 6 hr of priming are required to block significantly inhibition of neurite outgrowth. This suggests that downstream events required for block of inhibition, subsequent to elevation of cAMP, require longer to become activated. This appears to be the case because if cerebellar neurons are primed for 1 hr with BDNF or GDNF, inhibition is only partially blocked (Figure 3). On the other hand, if cerebellar neurons are primed for 1 hr with BDNF or GDNF and the neurotrophin is then removed but the neurons are not exposed to MAG for a further 5 hr, inhibition is almost completely blocked (data not shown).

Priming Neurons with Neurotrophins in the Presence of a Protein Kinase A Inhibitor

Elevation of cytosolic cAMP activates protein kinase A (PKA). To determine if activation of PKA is necessary for the block of MAG and myelin's inhibition by priming with particular neurotrophins, the PKA inhibitor KT5720 (200 nM) was included during neurotrophin priming. Figure 8 shows that addition of KT5720 during priming with either BDNF or GDNF for cerebellar neurons and BDNF, GDNF, or NGF for DRG neurons completely abrogates the block of MAG's inhibition of axonal growth; in the presence of KT5720, MAG again inhibits regeneration from those neurons primed with growth factors. Importantly, the presence of KT5720 during priming of either cerebellar or DRG neurons also prevents the neurotrophin block of inhibition by myelin; myelin is as effective an inhibitor of neurons that were primed in the presence of KT5720 as neurons that were never exposed to neurotrophin (Figure 9). Similar results were obtained if the cAMP antagonist, Rp cAMP, was used at 100 nM (data not shown). These results indicate that before exposure to the inhibitor, elevation of cAMP, leading to activation of PKA, can overcome inhibition by MAG and myelin.

Involvement of G_i in Inhibition by MAG and Myelin

Pertussis toxin binds to and inactivates the heterotrimeric GTP-binding proteins, G_i and G_o (see Post and Brown, 1996). To assess if either G protein must be activated for inhibition by MAG, we first cultured the neurons for 1–2 hr in the presence of toxin before exposing them to MAG. This procedure had no effect on the inhibition of neurite outgrowth by MAG (data not shown). However, if cerebeller neurons were cultured first with pertussis toxin and then exposed simultaneously to neurotrophins and MAG or myelin (i.e., without priming), BDNF and GDNF blocked the inhibitory effects of MAG and myelin (Figure 10) while BDNF, GDNF, or NGF blocked inhibition of older DRG neurons by MAG and myelin (data not shown). Pertussis toxin, therefore, eliminates the need to prime with neurotrophins to block inhibition by MAG and myelin. This suggests that priming neurons is required because MAG/myelin activates a pertussis toxin–sensitive G protein that prevents neurotrophin-induced elevation of cAMP. This idea is supported by the fact that soluble MAG-Fc, which effectively inhibits axonal regeneration (Tang et al., 1997b), can prevent the neurotrophin-induced increase in cAMP, and pertussis toxin neutralizes the MAG-Fc block of increasing cAMP, allowing cAMP to increase (Figure 7). Figure 7 shows effects of MAG-Fc and pertussis toxin on GDNF-induced cAMP increases in cerebellar neurons

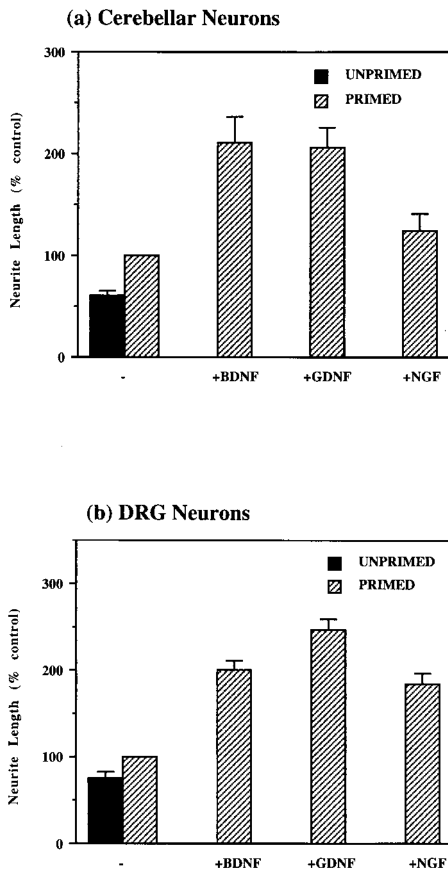


Figure 5. The Effect of Priming Neurons with Neurotrophins on Subsequent Inhibition by Myelin

Dissociated cerebellar neurons (a) or older DRG neurons (b) were first primed overnight on poly-l-lysine with no neurotrophin or with BDNF, GDNF, or NGF (each at 200 ng/ml as indicated) before being transferred to a substrate of isolated myelin (1 μg/ml) for further overnight culture before being fixed and immunostained for GAP43. Results show the mean length of the longest neurite per neuron (+/− sem) for 180–200 individual unprimed neurons (solid bar) or neurons primed (stippled bar) without neurotrophin or with the various neurotrophins as indicated. Results are percent of control, with 100% control taken as growth of neurons primed in the absence of neurotrophin before subsequent culture on myelin.

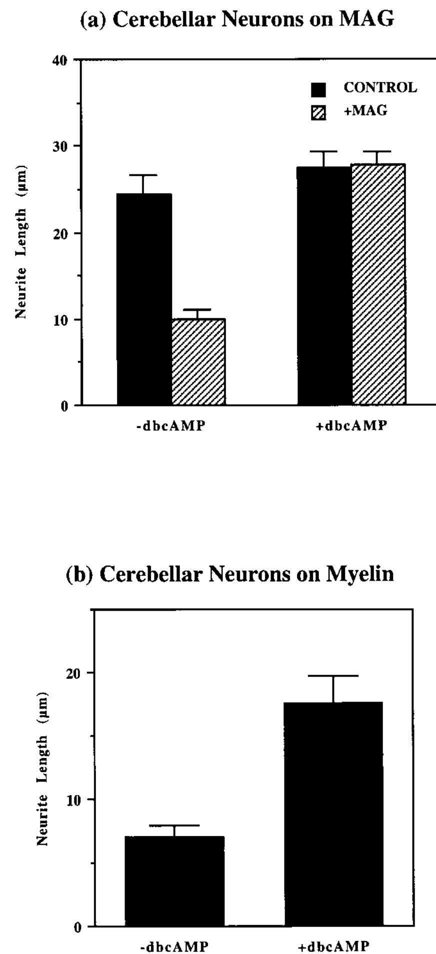


Figure 6. The Effect of Dibutyryl cAMP on Inhibition by MAG and Myelin of Neurite Outgrowth from Cerebellar Neurons

Cerebellar neurons were plated onto monolayers of either (a) MAG-expressing CHO cells (solid bars) or control cells (stippled bars) or (b) immobilized myelin membranes and cultured overnight in the presence or absence of dibutyryl cAMP at a concentration of 1 mM. The cultures were then fixed and immunostained for GAP43, and the neurites were measured. Results show the mean length of the longest neuron (+/− sem) for 180–200 neurons.

and NGF-induced increases in DRG neurons. Identical effects were observed for BDNF-induced elevation in cerebellar and for BDNF- and GDNF-induced cAMP elevation in DRG neurons (data not shown).

Discussion

For a number of years, the dogma has been that damaged axons of the mammalian CNS will not regrow into areas of white matter, which is essentially myelin (see Johnson, 1993; Schwab and Bartholdi, 1996). Recent studies by two groups of workers appear to contradict this long-held view by showing extensive axonal regrowth into white matter, distal to a site of injury, without any obvious inhibition by myelin (Berry et al., 1996; Bregman, 1998). We now offer an explanation for these surprising results by providing evidence that when neurons are first exposed to particular neurotrophins before encountering MAG or myelin, their axonal growth is no longer inhibited. Furthermore, since priming with neurotrophins is so effective in blocking inhibition by myelin, both in culture and in vivo, it strongly implies that either (1) the action of all the major myelin-specific inhibitors is blocked by priming with neurotrophins or (2) that MAG is, itself, the major contributor to the overall inhibition by myelin. For the neurotrophins studied here, the effect of priming is clearly mediated through activation of a cAMP-dependent pathway. If priming with neurotrophin is carried out in the presence of an inhibitor of PKA, priming has no effect—myelin and MAG still inhibit axonal regeneration. In contrast to the effect of priming, simultaneous addition of neurotrophin and exposure to MAG or myelin has no effect on inhibition. This distinction deserves emphasis: in order to grow in the presence of myelin's inhibitory components, neuronal cAMP must

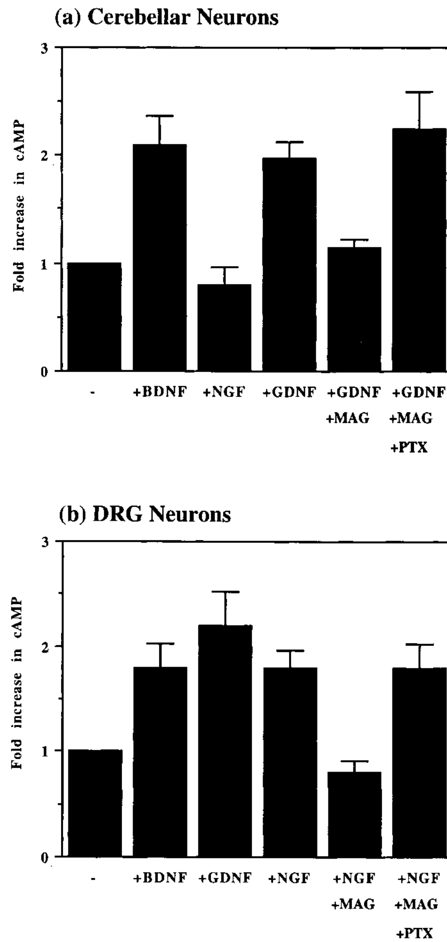


Figure 7. Measurement of cAMP in Neurons after Exposure to Neurotrophins

Dissociated cerebellar (2×10^5) or DRG (5×10^4) neurons were placed in individual wells of a 96-well dish. The neurons were cultured for at least 6 hr 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 μg/ml or neurons were incubated with 2 ng/ml pertussis toxin (+PTX) prior to addition of neurotrophin and MAG-Fc. 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.

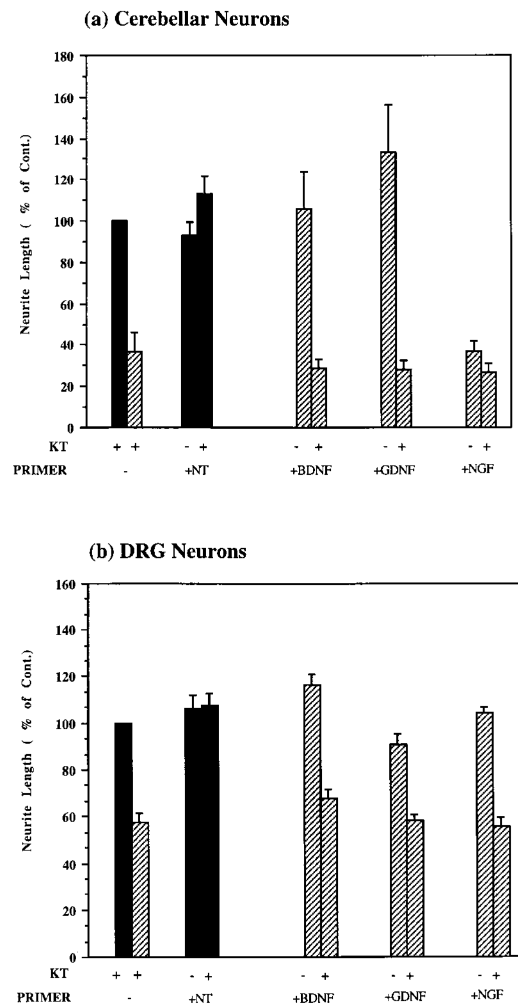


Figure 8. The Effect of Priming Neurons with Neurotrophins in the Presence of an Inhibitor of Protein Kinase A on Subsequent Inhibition of Neurite Outgrowth by MAG

Dissociated cerebellar neurons (a), or older DRG neurons (b), were first primed overnight on poly-l-lysine with no neurotrophin or in the presence of BDNF, GDNF, or NGF each at 200 ng/ml, with and without the PKA inhibitor KT5720 at 200 nM as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (stippled bars) or control CHO cells (solid bars) for further overnight culture before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/− sem) for 180–200 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells. NT refers to neurons primed with either BDNF, GDNF, or NGF before being subsequently cultured on control CHO cells.

be elevated and PKA activated before the neuron or axon encounters the inhibitor. We interpret the requirement for priming to indicate that MAG or myelin activates an inhibitory, pertussis toxin–sensitive, heterotrimeric G protein—most likely G_i—which in turn prevents any elevation of cAMP that would otherwise be stimulated by neurotrophin binding.

Based on these observations, we propose the model in Figure 11 to explain how priming with particular neurotrophins overcomes the inhibitory effects of MAG/myelin. In our model, neurotrophin binding results in a rise in intracellular cAMP in the absence of MAG/myelin. In turn, elevated cAMP activates PKA and other, as yet unidentified downstream proteins. If the neuron in this state is now exposed to MAG/myelin, the activated PKA or some downstream protein blocks or neutralizes the inhibitory signaling steps launched by MAG/myelin binding. It is important to note that PKA can inactivate the G protein RhoA by phosphorylation (Lang et al., 1996), and activated RhoA has been implicated in growth cone collapse and neurite retraction (Katoh et al., 1996; Tigyi et al., 1996), a possible step in the inhibition of axonal growth. On the other hand, if the neuron is exposed

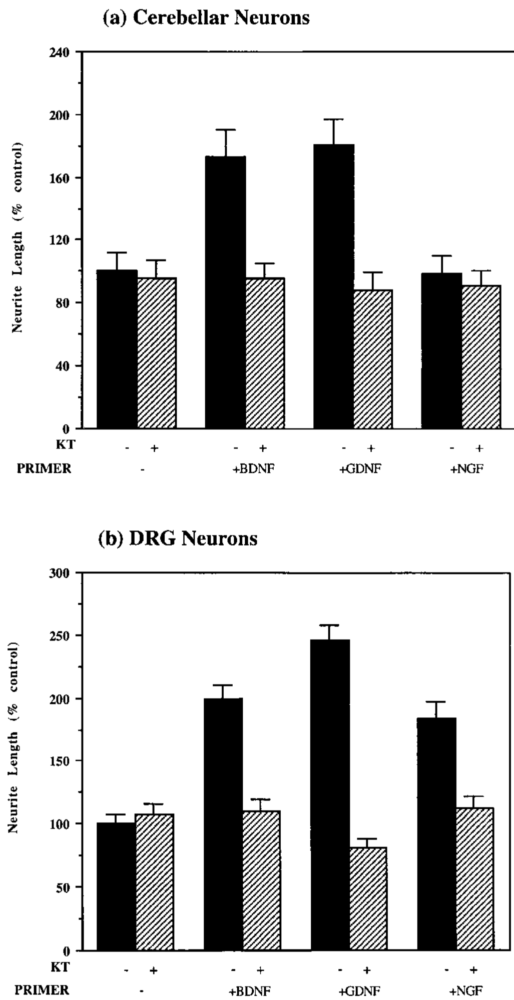


Figure 9. The Effect of Priming Neurons with Neurotrophins in the Presence of an Inhibitor of Protein Kinase A on Subsequent Inhibition of Neurite Outgrowth by Myelin

Dissociated cerebellar (a) or older DRG neurons (b) were first primed overnight on poly-l-lysine with no neurotrophin or in the presence of BDNF, GDNF, or NGF each at a concentration of 200 ng/ml, with (stippled bars) and without (solid bars) the PKA inhibitor KT5720 at 200 nM, as indicated, before being transferred to a substrate of immobilized myelin for further overnight culture before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/− sem) for 180–200 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on myelin.

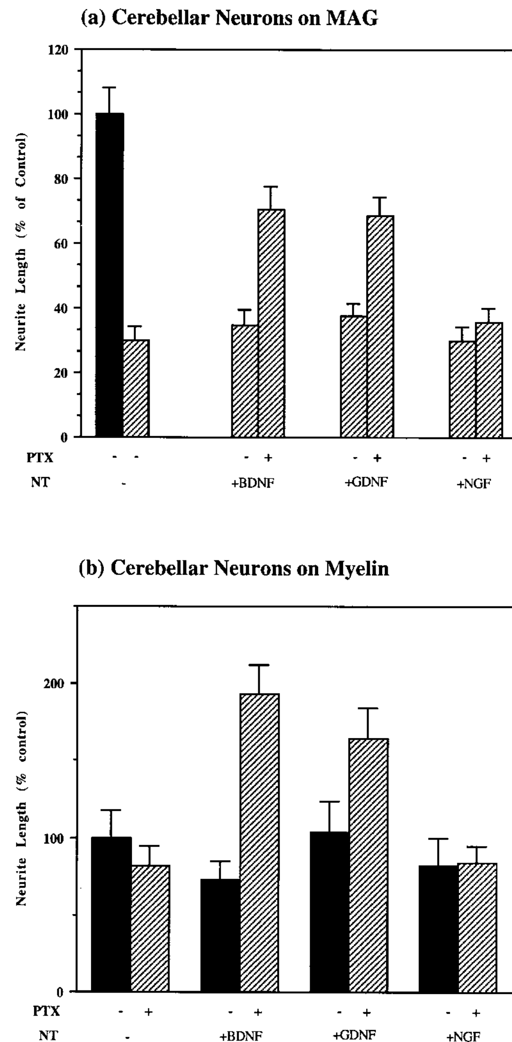


Figure 10. The Effect of Neurotrophins, without Priming, in the Presence of Pertussis Toxin on Inhibition of Neurite Outgrowth by MAG and Myelin

Cerebellar neurons were cultured for 1–2 hr in the presence of 2 ng/ml pertussis toxin (PTX) before being cultured overnight on (a) a monolayer of either MAG-expressing (stippled bars) or control CHO cells (solid bars) or (b) on a substrate of immobilized myelin and BDNF, GDNF, or NGF each at 200 ng/ml, as indicated, before being fixed and immunostained for GAP43. Results show the mean length of the longest neurite per neuron (+/− sem) for 180–200 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells (a) or on myelin in the absence of pertussis toxin (b).

to MAG/myelin and the neurotrophin simultaneously, endogenous cAMP levels are kept low by MAG/myelin-activated G_i, and inhibition by MAG/myelin persists. Activation of G_i by MAG/myelin, however, is not directly responsible for inhibition of axonal regeneration because pertussis toxin alone, in the absence of growth factor, has no effect on inhibition by MAG/myelin (Figure 10). One prediction from this model is that interfering with these signaling steps should interrupt the MAG/myelin inhibitory events and permit axonal regeneration through white matter in vivo.

These are important results for several reasons. First, they suggest a general strategy to overcome myelin-specific inhibitors of regeneration in vivo: the elevation of neuronal cAMP levels and activation of PKA before the axon is challenged to regrow into white matter. Second, they provide direct evidence of specific signal transduction pathways involved in inhibition by MAG and myelin. Third, they describe a novel function for neurotrophins in modulating an axon's response to guidance molecules. Finally, MAG, and perhaps all myelin-specific inhibitors, can now be added to the growing

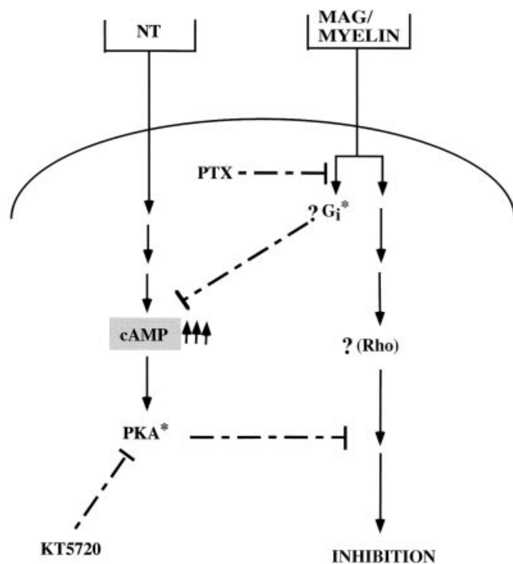


Figure 11. Model to Explain How Priming with Neurotrophins Blocks Inhibition by MAG or Myelin

During priming, neurotrophins (NT) interact with a surface neuronal receptor inducing an increase in neuronal cAMP, which in turn activates KT5720-sensitive protein kinase A (PKA*). Activation of PKA or some as yet unidentified downstream signal then blocks subsequent inhibition by MAG or myelin, perhaps by inactivating the small G protein, Rho. If, however, neurotrophin is added to the neuron at the same time as exposure to MAG or myelin, cAMP is prevented from increasing by MAG/myelin activation of a pertussis toxin (PTX)–sensitive G protein (G_i*) and so inhibition of axonal regeneration is not blocked. G protein activation by MAG or myelin has no direct effect on inhibition of axonal regeneration by MAG or myelin.

number of molecules whose effect as either inhibitors/repellents or promoters/attractants of axonal growth can be determined by the endogenous levels of neuronal cAMP. This underscores the suggestion by Poo and his coworkers (Song et al., 1997, 1998) that the response of an axon to a particular guidance molecule may readily switch between inhibition/repulsion and promotion/attraction by changes in the neuron's endogenous levels of cAMP. It should be noted, however, that cAMP-dependent signals are not the only mechanism whereby inhibition by myelin/MAG can be blocked. We found that priming either cerebellar or older DRG neurons with the neurotrophin NT-3 also blocked subsequent inhibition by MAG/myelin, but its block of inhibition was unaffected by inhibiting PKA (data not shown). It is possible the step directly responsible for blocking inhibition by neurotrophins is downstream from cAMP-dependent activation of PKA and that NT-3 activates the same downstream event via a cAMP-indepedent pathway. Indeed, NT-3, by itself, has been shown to exert neurotropic effects on axons via a cGMP- rather than cAMP-dependent pathway (Song et al., 1998; see discussion below).

The neurotrophin effect cannot be due to a simple competitive inhibition through neurotrophin binding at the MAG/myelin receptor since inhibition of PKA activation during priming permits subsequent inhibition by MAG/myelin, which is possible only if the MAG/myelin receptor is not occupied by neurotrophin. Along the same lines, direct competition by MAG for diverse neurotrophin receptors (Trk family members and GDNF family receptors) is not expected. Instead, as we show here, the neurotrophins BDNF, NGF, and GDNF activate PKA via elevation of cAMP, and we suggest that this is the mechanism by which MAG/myelin inhibition is neutralized. Both BDNF and NGF have been previously shown to increase cAMP levels in the PC12 neuronal line and intact isolated nerve endings from rat brain (Knipper et al., 1993a, 1993b). In those systems, the increase occurred within seconds and was believed to be via non-Trk receptors. Importantly, no functional consequence was attributed to the increase. We do not yet know what receptors mediate the effect we report here.

These results enlarge the view that levels of cAMP in neurons, with the subsequent activation of PKA, affect guidance responses by neurons (Ming et al., 1997a, 1997b; Song et al., 1997, 1998; Wang and Zheng, 1998). *Xenopus* spinal axons have been shown to grow toward a source of either BDNF (Song et al., 1997) or netrin-1 (Ming et al., 1997b), the axonal guidance molecule secreted from the floor plate (Kennedy et al., 1994). In the presence of a PKA inhibitor, however, these axons grow away from the source of BDNF or netrin-1. Blocking PKA activation does not merely neutralize the chemoattractant response to BDNF or netrin-1; it changes to a repellent response (Ming et al., 1997b; Song et al., 1997). In contrast, *Xenopus* spinal axons were repelled by soluble, recombinant MAG (Song et al., 1998). Consistent with our observations here, repulsion of *Xenopus* axons by MAG was switched to attraction if a cAMP agonist was added to the culture media (Song et al., 1998). Under defined conditions, then, BDNF, netrin-1, and MAG are all bifunctional. For MAG, we have shown that it promotes axonal growth from neonatal DRG neurons up to postnatal day 4, after which it inhibits growth (Mukhopadhyay et al., 1994; De Bellard et al., 1996). Netrin-1 acts as a chemoattractant for axons that typically grow toward the floorplate and as a chemorepellent for those axons that typically grow away (Colamarino and Tessier-Lavigne, 1995). In freshly isolated *Xenopus* spinal neurons, BDNF was recently shown to induce growth cone collapse (Wang and Zheng, 1998), but when the neurons are first cultured for longer periods, exposure to BDNF leads to growth toward the BDNF source (Song et al., 1997). The bifunctional behavior of MAG and BDNF correlates with the changes in endogenous cAMP levels. In DRG neurons from newborns, promotion by MAG can also be prevented by blocking PKA activation (D. Cai et al., submitted) while, as we now report, inhibition can be blocked by elevating cAMP. Similarly, when *Xenopus* spinal neurons are first isolated and cultured (within 6 hr), BDNF induces growth cone collapse, which can be overcome by artificially elevating endogenous cAMP levels (Wang and Zheng, 1998). After longer periods in culture, when these neurons grow toward BDNF, blocking PKA activation blocks chemoattraction (Song et al., 1997). In these studies, it is assumed that for *Xenopus* spinal neurons, the endogenous cAMP levels are initially low and rise either spontaneously when cultured or as a consequence of the culture media, and so PKA is activated. It appears that elevated cAMP levels and activation of PKA are key components of a general

mechanism to override the inhibition of axonal growth caused by a variety of molecules: MAG, BDNF, and netrin-1. In contrast, attraction of *Xenopus* growth cones to NT-3 and repulsion by the guidance molecule semaphorin have been shown to be brought about by a cGMP-dependent mechanism (Song et al., 1998). Curiously, NT-3 can overcome inhibition by MAG/myelin in a cAMP-independent manner (data not shown). For inhibition to occur, cAMP levels may have to be low and PKA may have to be inactive. On the other hand, while elevating cAMP blocks inhibition of axonal regeneration, other mechanisms, bypassing the cAMP step, may also block inhibition.

One key difference between growth inhibition by MAG/myelin and inhibition by BDNF or netrin-1 may be that MAG/myelin prevents endogenous cAMP from rising in addition to inhibiting axonal regeneration. From our studies here, we know that BDNF certainly does not prevent cAMP accumulation; in fact, we use BDNF to induce a cAMP increase. Although we find that pertussis toxin has no direct effect on inhibition of axonal regeneration by MAG/myelin, others have found that pertussis toxin can partially block growth cone collapse by myelin (Igarashi et al., 1993). The more dynamic, transient phenomenon of growth cone collapse could well be more sensitive to G protein activation than the long-term regulation of net axon growth. We did not examine the dynamics of the growth cone in these studies.

Our present results allow us to expand the repertoire of neurotrophins beyond their well studied effects on neuronal survival and differentiation (see Barde, 1990; Thoenen, 1991; Klein, 1994; Snider, 1994; Lindsay, 1996). We can also now characterize them as modulators of axonal response to guidance cues. BDNF, NGF, and NT-3 have already been shown to modulate growth cone collapse by a recently identified guidance molecule, collapsin-1 (Tuttle and O'Leary, 1998). That study, however, reported only the effects of these neurotrophins on collapsin-induced growth cone collapse relative to each other, not whether they could directly block the effects of collapsin-1. The ability of neurotrophins to modulate axonal response to guidance cues may be widespread, a conclusion supported by (1) the fact that members of two different neurotrophin families, the Trks and the GDNF-like neurotrophins, can each block MAG/myelin inhibition and (2) the observation that the axonal response to a number of guidance molecules can be switched by altering cAMP levels. Obviously, the response of a particular neuron to neurotrophins is quite complex, especially given the likelihood that any one neuron will be exposed to many neurotrophins and guidance cues at any one time. It remains to be seen whether neurotrophins elevate neuronal cAMP to modulate axonal growth during development. We have shown that the response of DRG neurons to MAG switches during postnatal development and that cAMP-dependent mechanisms can regulate both the promoting and inhibiting response (D. Cai et al., submitted). It is possible that the decreased supply of neurotrophins, accompanied by the decreased responsiveness of neurons to neurotrophins, during development (see Jelsma and Aguayo, 1994) leads to a decline in the neuronal levels of cAMP, permitting MAG/myelin to switch to an inhibitory role in the case of DRG axonal growth. The implication is that axonal sprouting is under MAG/myelin control, as we (Shen et al., 1998) and others (Schwegler et al., 1995) have suggested for the physiological role for MAG/myelin inhibition.

Finally, our demonstration that priming neurons with neurotrophins can overcome inhibition by MAG/myelin can explain the extensive growth of axons into white matter in vivo recently reported by others (Berry et al., 1996; Bregman, 1998). In Bregman's experiments, the neurons are exposed to either BDNF or NT-3 prior to encountering myelin. As we show, priming with either of these neurotrophins blocks inhibition by MAG/myelin, although one neurotrophic effect appears to be through a cAMP-dependent pathway, while the other is through a cAMP-independent pathway. In experiments of Berry and his coworkers (Berry et al., 1996), the retinal ganglion cell body is exposed to a number of neurotrophins (NGF, PDGF, CNTF, and GDNF) secreted by Schwann cells in the peripheral nerve explant. The authors suggest that retinal ganglion cells downregulate receptor(s) for myelin inhibitors after exposure to these neurotrophins. Instead, our studies here suggest that these neurotrophins elevate cAMP levels in the ganglion neurons, thereby blocking the intracellular inhibitory effects of MAG and myelin. Recently, Davies et al. (1997) reported that adult DRG neurons implanted into the adult CNS extended long axons through white matter. In their studies, some of the neurons underwent an equivalent to priming by being cultured overnight with NGF before implantation. In addition, because they took great care to minimize glial scarring, which undoubtedly encourages growth, there was also little or no damage to myelin. The implanted DRG axons would then encounter intact myelin and grow over the outer surface, which has no MAG but does have a growth-promoting molecule termed MOG (Turnley and Bartlett, 1998). Others have implanted transfected fibroblasts secreting NT3 or BDNF into lesion sites in the spinal cord (Schnell et al., 1994; Grill et al., 1997; McTigue et al., 1998). However, in those experiments either limited or no regeneration into white matter was observed, perhaps because the neuron/axons were not primed before encountering myelin. The studies of Bregman (1998) and Berry et al. (1996) in vivo, together with our study here, demonstrate two major points: (1) neurotrophins exposed to either the growth cone (Bregman's studies) or to the neuronal cell body (Berry's studies and this report) can affect the response to myelin inhibitors, and (2) more importantly, they point to a feasible approach to encouraging axonal regeneration after injury in vivo. Bregman and Berry show that it will work in vivo. We now show how it works. The next steps are to determine the optimal neurotrophin profile for different neurons to regenerate under different circumstances—quite likely to be different from the optimal neurotrophin profile for survival—and to develop a practical means of administering them after injury to generate effective regeneration.

Experimental Procedures

Preparation of Myelin

Myelin was purified as described previously (Norton and Poduslo, 1973) from rat CNS white matter. After the final hypotonic shock,

the membranes were centrifuged and resuspended in 10 mM HEPES. The protein concentration of the preparation was determined (Biorad) and used immediately as a substrate in the neurite outgrowth assay.

Isolation of Neurons

The cerebella from two animals was combined in 5 ml of 0.025% trypsin, triturated, and incubated for 10 min at 37°C. DMEM (5 ml) containing 10% FCS was added, and cells were centrifuged at 800 rpm for 6 min. The cells were resuspended to a single-cell suspension in 2 ml of Sato (Progesterone, 20 nM; selenium, 30 nM; putrescine, 100 μM; insulin, 5 μg/ml; BSA, 4 mg/ml; L-thyroxine, 0.1 μg/ml; tri-iodo-thyronine, 0.08 μg/ml) (Doherty et al., 1990). For older DRG neurons, ganglia were removed from two animals and incubated in 5 ml of Sato media containing 0.025% trypsin and 0.15% collagenase type I (Worthington) for 30 min at 37°C. The ganglia were triturated and trypsinization was stopped by adding 5 ml of DMEM containing 10% FCS, centrifuged at 800 rpm for 6 min, and resuspended in Sato (De Bellard et al., 1996).

Priming Neurons with Neurotrophins

Tissue culture dishes (6 cm) were coated 16.6 μg/ml with poly-l-lysine (Sigma) for 30 min at room temperature. Excess poly-l-lysine was washed off with H_2O, and the plates were washed one more time. Isolated neurons in Sato were plated onto the poly-l-lysine-coated dishes at 1×10^6 cells/dish. Where indicated, either BDNF, GDNF, or NGF (all from Sigma) was added at a concentration between 25 and 200 ng/ml in the presence or absence of a protein kinase A inhibitor, KT5720 (Calbiochem), at 200 nM. After culture for various times from 1 to 24 hr, the media was removed, neurons were washed with PBS, and removed with 0.1% trypsin with 5 mM EDTA. Trypsinization was stopped by adding 5 ml of DMEM containing 10% FCS; neurons were centrifuged at 800 rpm for 6 min, resuspended in Sato, and plated immediately onto either MAG-expressing CHO cells, control CHO cells, or purified, immobilized myelin.

Neurite Outgrowth on Immobilized Myelin or Transfected Cells

For myelin membranes, wells of an eight-chamber tissue culture slide (Lab-Tek) were coated with 16.6 μg/ml poly-L-lysine at room temperature for 1 hr and washed with 0.1 M $NaHCO_3$. Rat CNS myelin at 0.5–1 μg total protein/well was dried overnight onto the coated wells and used as a substrate (Shen et al., 1998). Monolayers of control and MAG-expressing CHO cells were grown to confluency in individual chambers of an eight-well tissue culture slide (Lab-Tek). The neurite outgrowth assay was carried out as described previously (Mukhopadhyay et al., 1994; De Bellard et al., 1996; Tang et al., 1997b) by adding 2×10^4 cerebellar or DRG neurons, either primed or not, to the immobilized myelin substrate or to the CHO cell monolayers. Where indicated, db cAMP at 1 mM, BDNF, GDNF, or NGF, each at 200 ng/ml, was added or neurons were incubated with pertussis toxin at 2 ng/ml for 1–2 hr prior to the addition of neurotrophin. After 16–18 hr of culture, the neurons were fixed for 30 min with 4% paraformaldehyde and permeabilized with ice-cold methanol for 2 min. The cells were then blocked for 30 min with DMEM containing 10% FCS and incubated for 4 hr with a rabbit polyclonal antibody against GAP43 (1:4000, from R. Curtis and G. Wilkins, Imperial College, London). Cells were washed three times with PBS-BSA (2%) and then incubated for 30 min at room temperature with a biotinylated donkey anti-rabbit Ig (1:500, Amersham), washed three times, and incubated with streptavidin-conjugated Texas Red (1:300, Amersham) for 45 min. After three more washes, the slides were mounted in Permafluor (Baxter) and viewed with a fluorescent microscope. The length of the longest neurite or the total process outgrowth for each GAP43-positive neuron for the first 180–200 neurons encountered when scanning the slide in a systematic manner was determined using an Oncor image analysis program. Using a video camera, the neuron image was projected to a computer screen. The longest neurite or all process were manually traced and quantitated.

Binding of MAG-Fc to Neurons

Protein A–Sepharose–purified Fc chimeras, either MAG-Fc or MUC18-Fc as a control (Kelm et al., 1994; Tang et al., 1997a) were adsorbed at 30 μg/ml for 3 hr at 37°C to wells of microtitre plates coated for 2 hr at 37°C with anti-human IgG at 15 μg/ml in 0.1 M bicarbonate buffer (pH 9.6). Prior to the binding assay, neurons were vitally labeled with a fluorescent dye, calcein AM (Molecular Probes), by incubating 2×10^6 neurons in 5 ml of 10 μM calcein AM in PBS for 15 min at 37°C before being washed and resuspended in PBS. A suspension (100 μl) of vitally labeled neurons, containing 1–2 $\times 10^5$ cells, was added to each well and allowed to incubate for 1 hr at room temperature. The plates were washed three times with PBS applied to each well under gravity, and the fluorescence was measured in a FluorImager (Molecular Dynamics).

cAMP Assay

For each assay, 2×10^5 cerebellar or 2×10^4 DRG neurons were plated per well of a 96-well dish. The neurons were incubated for at least 6 hr in Sato. Then either BDNF, GDNF, or NGF was added at 200 ng/ml, with and without MAG-Fc at 20 μg/ml as indicated, and incubated for a further 30 min. Where indicated, neurons were incubated with pertussis toxin at 2 ng/ml for 1–2 hr prior to the addition of neurotrophin or neurotrophin and MAG-Fc. Cyclic AMP was measured in neurons using a competitive immunoassay, according to the manufacturer's instruction (Amersham).

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