Neuron, Vol. 34, 895–903, June 13, 2002, Copyright ©2002 by Cell Press

Spinal Axon Regeneration Induced by Elevation of Cyclic AMP

Jin Qiu,[1,5,6] Dongming Cai,[1,5] Haining Dai,[2] Marietta McAtee,[2] Paul N. Hoffman,[3] Barbara S. Bregman,[2] and Marie T. Filbin[1,4]
[1]Biology Department
Hunter College
City University of New York
695 Park Avenue
New York, New York 10021
[2]Department of Neuroscience
Georgetown University Medical School
3900 Reservoir Road NW
Washington, DC 20007
[3]Department of Neurology
Johns Hopkins School of Medicine
600 N. Wolfe Street
Baltimore, Maryland 21218

Summary

Myelin inhibitors, including MAG, are major impediments to CNS regeneration. However, CNS axons of DRGs regenerate if the peripheral branch of these neurons is lesioned first. We show that 1 day post-peripheral-lesion, DRG-cAMP levels triple and MAG/myelin no longer inhibit growth, an effect that is PKA dependent. By 1 week post-lesion, DRG-cAMP returns to control, but growth on MAG/myelin improves and is now PKA independent. Inhibiting PKA in vivo blocks the post-lesion growth on MAG/myelin at 1 day and attenuates it at 1 week. Alone, injection of db-cAMP into the DRG mimics completely a conditioning lesion as DRGs grow on MAG/myelin, initially, in a PKA-dependent manner that becomes PKA independent. Importantly, DRG injection of db-cAMP results in extensive regeneration of dorsal column axons lesioned 1 week later. These results may be relevant to developing therapies for spinal cord injury.

Introduction

It is well established that while the adult mammalian PNS can regenerate after injury, the CNS does not (Schwab and Bartholdi, 1996). Rather than being simply a difference in the intrinsic growth capacity of the two systems, this difference in regenerative ability is due largely to differences in post-injury changes in the cellular environment. In the PNS, debris—including the damaged myelin—is cleared. Schwann cells become permissive for regrowth, and no glial scar forms (Scherer and Salzer, 1996). In the CNS, the situation is very different. Myelin debris is removed only very slowly, and within hours of injury the glial scar begins to form (McKeon et al., 1995). Inhibitors of regeneration in myelin along with proteoglycans of the immature scar then prevent growth immediately after injury (Huang et al., 1999). Ultimately, regeneration is physically "locked in" by the impenetratable barrier of the mature glial scar by astrocytes that proliferate, change morphology, and form interdigitizing processes (Fitch and Silver, 1999).

The effect of different environments is evident in the behavior of peripheral neurons that have two branches, one central and one peripheral (Bradbury et al., 2000). One axon from dorsal root ganglion (DRG) neurons extends to the periphery, and the other, from the same cell body, enters the spinal cord where it joins the dorsal column and ascends to the brain. When the peripheral branch is cut, regeneration can occur. In contrast, when the axon that runs from the same cell body is cut after it has joined the dorsal column in the CNS, there is no regeneration. What is of particular interest is that if the peripheral branch is lesioned first, followed by cutting the dorsal column axons either 1 or 2 weeks later, these central axons now regenerate to a significant extent (Neumann and Woolf, 1999). Clearly, this conditioning, peripheral lesion is inducing molecular changes in these neurons such that their central axons can now not only grow but actually grow through an environment that is typically hostile and inhibitory. Previously, we reported that by elevating neuronal cAMP levels, inhibition by both a myelin-specific inhibitor of regeneration, myelin-associated glycoprotein (MAG), and by myelin in general, was overcome. We suggested that elevation of cAMP serves as a general mechanism to block all myelin inhibitors simultaneously (Cai et al., 1999). Here we ask if cAMP plays a role in the ability of dorsal column axons to regrow as a consequence of a previous conditioning peripheral lesion.

Results

A Conditioning Peripheral Lesion Overcomes Inhibition by MAG and Myelin

We first wanted to determine if the improved growth capacity following a conditioning lesion allows the DRG axon to overcome inhibition by MAG and by myelin in general. Figure 1 shows, as we reported before (Mukhopadhyay et al., 1994) that P19–P25 DRG neurons grown on CHO cells transfected to express MAG extend processes that are about 50% shorter than when grown on control CHO cells not expressing MAG. This inhibition by MAG is completely blocked if the peripheral branch of the DRG is lesioned either 1 day or 1 week prior to removal of the neurons from the animals and cultured on MAG (Figure 1). Indeed, by 1 week post-injury, in addition to overcoming inhibition by MAG, there is a general improvement in growth as neurites from these lesioned neurons on control CHO cells are between 80% and 100% longer than from uninjured nerve. A similar effect is seen when the neurons are grown on myelin as a substrate. As reported before (DeBellard et al., 1996), DRG neurons from older animals extend either

[4]Correspondence: filbin@genectr.hunter.cuny.edu
[5]These authors contributed equally to this work.
[6]Present address: Centre for Neuroscience Research, Hodgkin Building, Guy's Campus, King's College London, London SE1 1UL, United Kingdom.

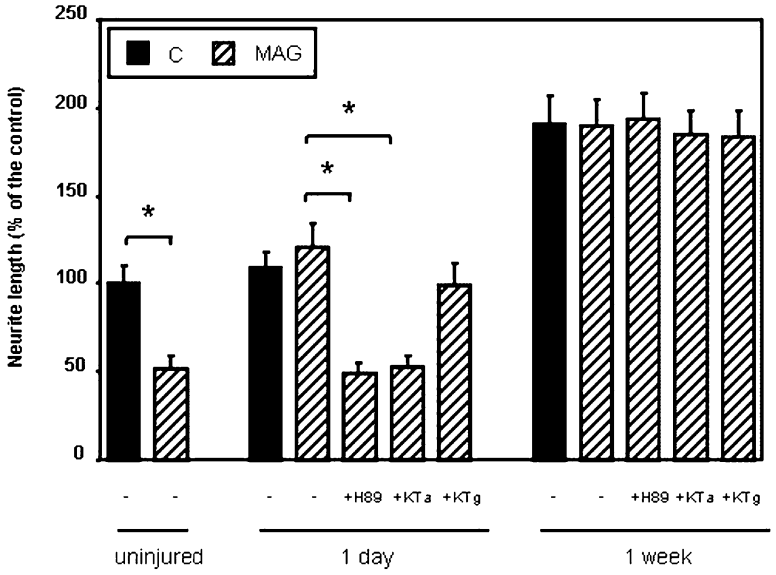


Figure 1. DRG Neurons from Animals with a DRG Peripheral Nerve Lesion Are Not Inhibited by MAG

Sciatic nerves were unilaterally lesioned 1 day or 1 week prior to when L4 and L5 DRG neurons were removed, dissociated, and cultured overnight on either MAG-expressing CHO cells (striped bars) or control CHO cells (black bars) after which they were fixed and immunostained for GAP43. In each experiment the DRGs from four animals were combined, and the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured (± SEM), for at least four separate experiments. Results are presented as percentage of neurite length of DRG neurons from uninjured nerve grown on control CHO cells. Where indicated, 200 nM KT5720 (KTa), 400 nM H89, or 1 mM KT5823 (KTg), were added during culture. Asterisk (*) indicates results are significantly different, $p < 0.05$.

very short or no neurites at all when grown on myelin (Figure 2). However, 1 day after a peripheral lesion these mature DRG neurons now put out processes on myelin (Figure 2). This improved growth on myelin is even more pronounced 1 week after a conditioning peripheral lesion (Figure 2). These results show that the effect of a conditioning lesion on the ability of DRG dorsal column axons to subsequently regenerate is paralleled by an ability of these same neurons to extend axons on MAG and myelin in general.

A Role for cAMP in the Conditioning Lesion Effect

To determine if the ability of mature DRG neurons to grow on MAG and myelin after a conditioning peripheral lesion is dependent on cAMP, an inhibitor of protein kinase A (PKA), a downstream effector of cAMP, was included in the cultures. As can be seen in Figure 1 and 2, when either of the PKA inhibitors, KT5720 or H89, was included in the cultures, the ability of neurons to grow on MAG and on myelin 1 day after the conditioning lesion is completely abolished. An inhibitor of protein kinase G (PKG) has no effect. In sharp contrast, 1 week after a conditioning lesion neither of the PKA inhibitors have an effect on the good growth on either MAG or myelin (Figure 1 and 2). Consistent with the switch in PKA dependence of growth on MAG and myelin occurring between 1 day and 1 week after a conditioning lesion, a switch is also seen in cAMP levels as measured by cAMP antibody staining. When sections through DRGs are stained for cAMP with a cAMP-specific antibody, only background staining is apparent. By 1 day after a conditioning lesion, all the cells stain brightly for cAMP, and, by 1 week after a lesion, this staining has decreased to control levels similar to ganglia from unlesioned nerves (Figure 3A). In agreement with this immunostaining for cAMP, directly measured endogenous

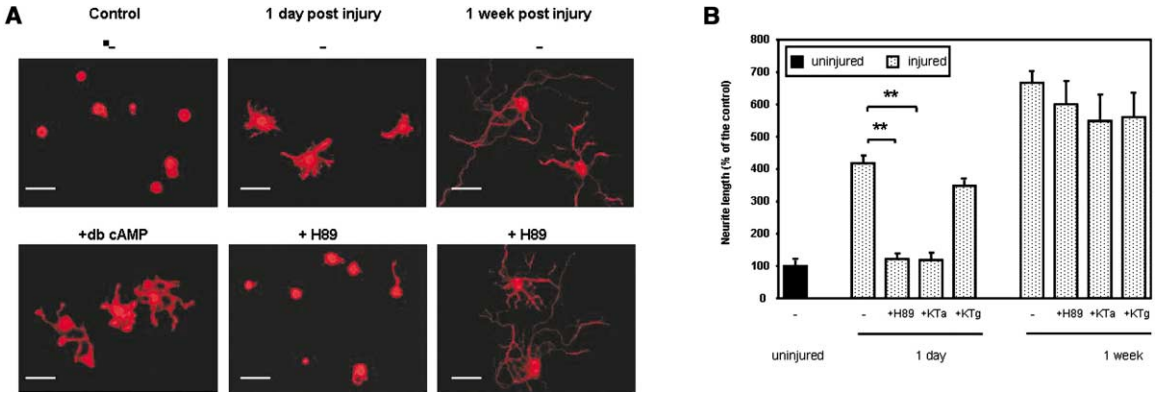


Figure 2. DRG Neurons from Animals with a DRG Peripheral Nerve Lesion Are Not Inhibited by Myelin

Sciatic nerves were unilaterally lesioned either 1 day or 1 week prior to when L4 and L5 DRG neurons were removed, dissociated, and cultured overnight on myelin (stippled bars). After overnight culture, the neurons were fixed and immunostained for GAP 43 (A). Bar, 20 μm. In each experiment, the DRGs from four animals were combined and the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured (± SEM), for at least four separate experiments (B). Results are presented as percentage of neurite length of DRG neurons from uninjured nerve grown on myelin (black bar). Where indicated, 200 nM KT5720 (KTa), 400 nM H89, 1 mM KT5823 (KTg), or 1 mM db-cAMP were added during culture. Double asterisk (**) indicates results are significantly different, $p < 0.001$.

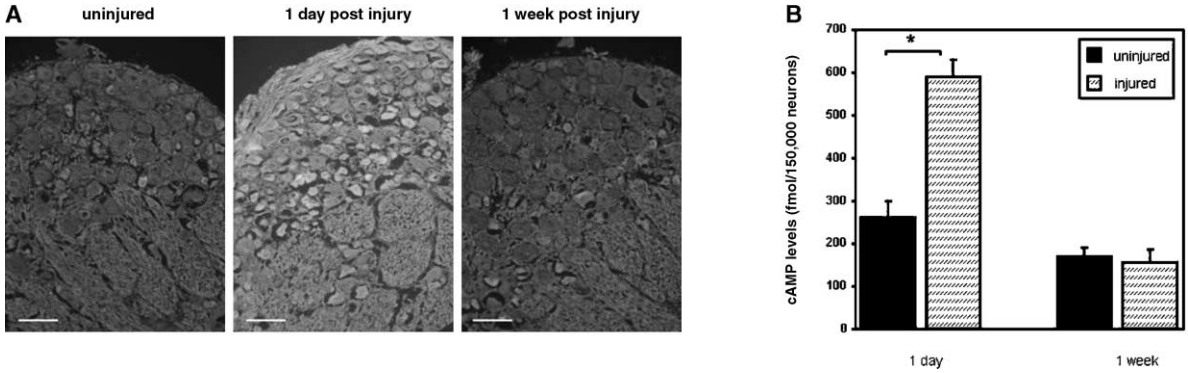


Figure 3. Changes in cAMP Levels in DRG Neurons in Response to a Peripheral Nerve Lesion

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. Bar = 20 μm. (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 (± 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, $p < 0.05$.

cAMP levels in DRG neurons at 1 day post-lesion have increased to about 3-fold higher than in DRG neurons from the unlesioned nerve. Again, by 1 week, the endogenous cAMP levels have dropped back to those of unlesioned nerves (Figure 3B). Therefore, the improved growth of DRG neurons on MAG and myelin subsequent to a conditioning peripheral lesion is initially directly dependent on PKA activation by cAMP but then becomes independent of PKA activation, and this switch in PKA dependence is accompanied by a transient increase in endogenous cAMP levels.

Inhibition of Protein Kinase A Blocks Both the cAMP-Dependent and –Independent Growth

It is possible that the transient increase in cAMP immediately after the sciatic nerve is lesioned is a prerequisite for the eventual PKA-independent growth. That is to say, the increase in cAMP may initiate a cascade of signaling events resulting in and necessary for eventual PKA-independent growth. Alternatively, the later PKA-independent growth may be a completely separate event, independent of the transient elevation of cAMP. To address this issue, and to directly correlate the transient increase in cAMP with both the early PKA-dependent and later PKA-independent growth, an inhibitor of PKA was injected directly into the DRG at the same time as the conditioning lesion was performed. After a single injection of the PKA inhibitor H89, the improved growth on MAG and myelin 1 day later was attenuated but not completely abolished. However, the improved growth on MAG and myelin 1 week after the conditioning lesion was unaffected by a single injection of H89 at the same time as the lesion was performed (data not shown). As only a single injection of H89 was given, it is possible that the short exposure to the PKA inhibitor was insufficient to completely block PKA activation and subsequent downstream signaling for long enough to block the later PKA-independent growth. Because of the anesthesia, animal survival rate is very poor if multiple injections are given over a prolonged period. Instead, following the demonstration that substances delivered intrathecally reach the DRG cell body (Porreca et al., 1999), we placed an osmotic mini-pump to allow delivery of either H89 or saline over the course of 24 hr. After 24 hr of delivery coinciding with the 24 hr post-lesion, the improved growth on MAG and myelin was completely abolished (Figure 4). Importantly, H89 in vivo is not resulting in merely "sick" neurons that extend shorter processes. Addition of db-cAMP in culture reversed the effects of H89 administered in vivo. Also, H89 had no effect on growth on control cells, not expressing MAG, and had no effect on growth from the contralateral, uninjured control nerve that was also receiving H89 (Figure 4A). Intrathecal delivery of H89 also blocked the improved growth of DRG neurons on myelin 1 day post-conditioning lesion (Figure 4B).

After ensuring H89 is still active after 1 week at 37°C (results not shown), H89 was now delivered via minipumps for the entire week between the conditioning lesion and removal of the DRGs from the animals. As before, 1 week after a conditioning lesion, in the absence of H89, MAG did not inhibit growth and, in addition, there was a general improvement in growth of about 80% relative to neurons from uninjured nerve. Delivery of H89 for the entire week following the conditioning lesion blocked the improved growth on MAG cells by about 50% relative to growth of the same neurons on control CHO cells; H89 did not affect growth on control CHO cells (Figure 5). This percent inhibition by MAG is equivalent to the percent inhibition routinely recorded for DRG neurons from unlesioned nerves. Although the improved growth on MAG 1 week after a conditioning lesion is strongly attenuated by intrathecal delivery of H89, reducing it by the typical percent of growth on control cells, it is not reduced to the absolute levels of that of uninjured nerve.

Elevation of cAMP Is Sufficient to Induce Both the cAMP-Dependent and –Independent Growth

The results presented above indicate that a conditioning lesion to the peripheral branch of a DRG nerve induces a transient increase in cAMP in the neuronal cell body, which in turn allows axons to grow on MAG and myelin, eventually in a PKA-independent manner. The question

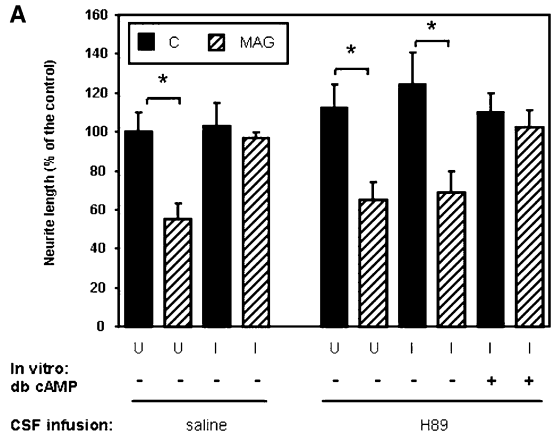


Figure 4. Exposure of DRG Neurons to a Protein Kinase A Inhibitor, H89, In Vivo Blocks the Improved Growth on MAG and Myelin Observed 1 Day Post-Lesion

At the same time as sciatic nerves were unilaterally lesioned, an intrathecal pump was implanted delivering 2 mM H89 or saline, at a rate of 12 μl per day. After 1 day, DRGs from either the injured or uninjured side were removed and plated onto (A) MAG cells (striped bars) or control cells (black bars) or (B) myelin (black bar, uninjured nerve; dotted bars, injured nerve) and cultured overnight before being fixed and immunostained for GAP43. In each experiment, the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured (± SEM), for at least three separate experiments. For each condition, DRGs from four animals were combined. For neurons grown on MAG cells, results are presented as a percentage of neurite length on control cells of DRG neurons from uninjured nerve, delivered with saline. For neurons grown on myelin, results are presented as a percentage of neurite length on myelin of neurons from uninjured nerve delivered with saline. Where indicated, 1 mM db-cAMP was added to neurons in culture. Asterisk (*) indicates results are significantly different, $p < 0.05$.

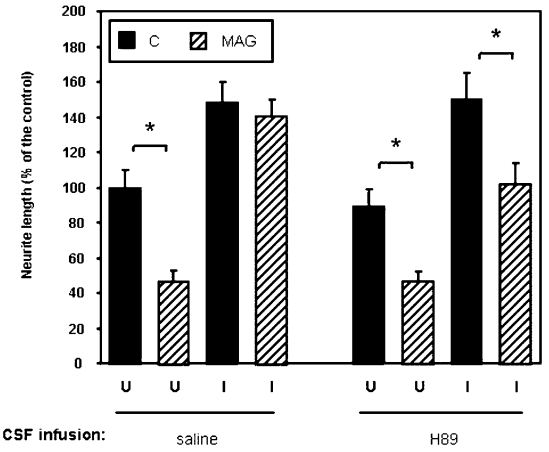


Figure 5. Exposure of DRG Neurons to a Protein Kinase A Inhibitor, H89, In Vivo Attenuates the Improved Growth on MAG Observed 1 Week Post-Lesion

At the same time as sciatic nerves were unilaterally lesioned, an intrathecal pump was implanted delivering 2 mM H89 or saline, at a rate of 12 μl per day. After 1 week, DRGs were removed from either the injured (I) or uninjured side and plated onto MAG cells (striped bars) or control cells (black bars) and cultured overnight before being fixed and immunostained for GAP43. In each experiment, the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured (± SEM), for at least three separate experiments. For each condition, DRGs from four animals were combined. Results are presented as a percentage of neurite length on control cells of DRG neurons from uninjured nerve, delivered with saline. Asterisk (*) indicates results are significantly different, $p < 0.05$.

now is whether an increase in cAMP alone, without a peripheral lesion, is, first, sufficient to induce the ability to grow on MAG/myelin in both a PKA-dependent and -independent manner and, second, to induce subsequent dorsal column regeneration. To test these possibilities, the cAMP analog, db-cAMP, was injected directly into L4 and L5 DRG in vivo. No peripheral nerve-conditioning lesion was performed, and 1 day or 1 week after the injection the neurons were removed and grown on either MAG or myelin (Figures 6A and 6B). It was found that at both 1 day and 1 week after injection of db-cAMP, the inhibition by MAG was completely reversed and growth on myelin was improved by 6-fold, comparable to that following a conditioning lesion (compare Figure 6 with Figures 1 and 2). Importantly, the improved growth is PKA dependent only at 1 day and not at 1 week. As with a conditioning lesion, growth becomes PKA independent after db-cAMP injection (Figure 6). Together, these results demonstrate that elevation of cAMP in the DRG cell body is sufficient to not only overcome inhibition by MAG and myelin when cultured, but also to induce PKA-independent improved growth.

Elevation of cAMP Is Sufficient to Induce Regeneration of Lesioned Spinal Axons

In order to determine whether modulating cAMP levels would increase the ability of central DRG axons to regenerate in vivo, db-cAMP (50 mM, 2 μl; n = 13) or saline (0.9% saline; n = 5) was injected directly into the L5 dorsal root ganglion in vivo, again without a conditioning peripheral nerve lesion. The dorsal column axons were lesioned at the T7 spinal cord segment 1 week after the DRG injections. Regeneration of dorsal column axons was visualized by transganglionic labeling with horseradish peroxidase via the sciatic nerve 1 or 2 weeks after the spinal cord lesion. In all of the animals injected with db-cAMP, there was obvious and extensive regeneration of the dorsal column axons. In sharp contrast, in the saline-treated control animals, although there was

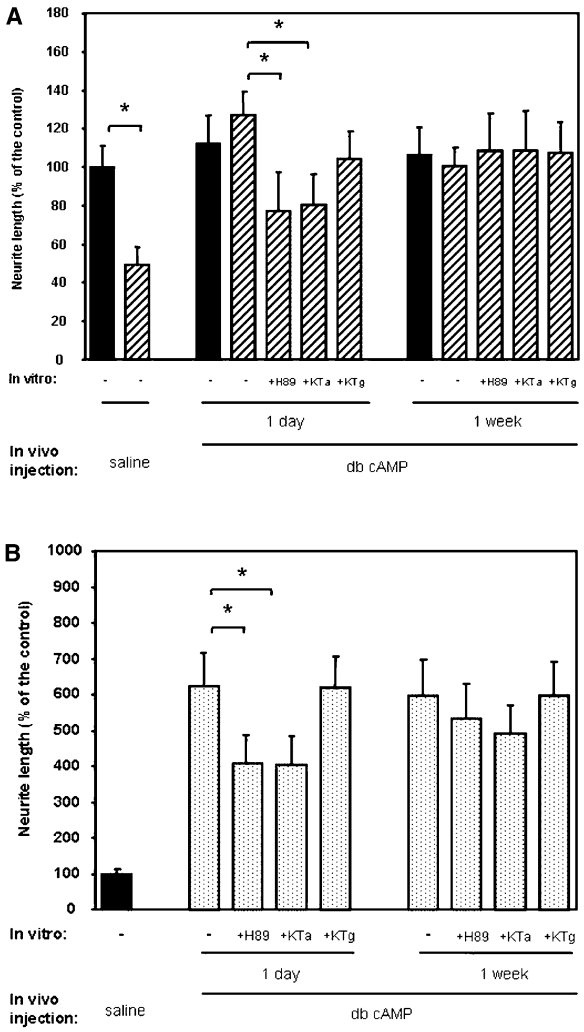


Figure 6. Injection of db-cAMP into DRGs without a Peripheral Lesion Results in Improved Growth on MAG and Myelin 1 Day and 1 Week Later

2 μl of 50 mM db-cAMP or saline was injected directly into L4 and L5 DRGs, without a peripheral lesion and 1 day or 1 week later the DRGs were removed and cultured overnight on either (A) MAG cells (striped bars) or control cells (black bars) or (B) on myelin. In each experiment, the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured (± SEM), for at least three separate experiments. For each condition, DRGs from three animals were combined. Where indicated, 200 nM KT5720 (KTa), 400 nM H89, or 1 mM KT5823 (KTg). Results are presented as a percentage of neurite outgrowth from DRGs injected with saline and grown on control cells (A) or on myelin (B). Asterisk (*) indicates results are significantly different, $p < 0.05$.

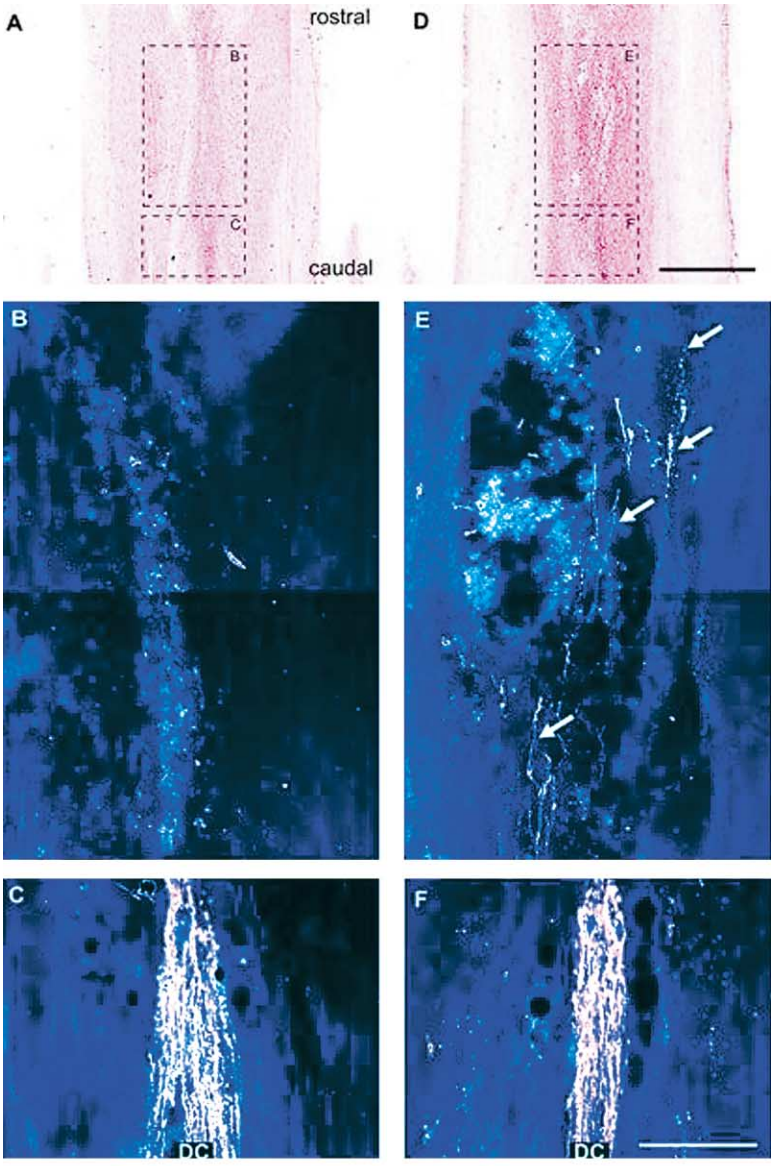
extensive labeling of dorsal column axons within the spinal cord below the level of the lesion, there was no regrowth of dorsal column axons at the lesion site. For all db-cAMP-injected animals, there was both a greater number of labeled dorsal column axons near the lesion site (in gray and white matter) and a greater growth of axons beyond the lesion site compared to the saline-treated animals (Figures 7A–7F). When fiber growth was measured, the growth of the axons was significantly ($p < 0.0001$) greater in the db-cAMP-treated animals (Figure 7G). The distance regenerated from the caudal border of the lesion site ranged from 1.5 mm to 5 mm, and beyond the lesion from 0.6 mm to 0.8 mm, which is equivalent to that seen 1 week after a conditioning lesion (Neumann and Woolf, 1999).

Discussion

The effects of cAMP on regeneration in vivo have been predicted for a number of years (Filbin, 1999). Now we show that indeed it alone can induce regeneration in the mature CNS. Here we report that elevation of cAMP in the cell body is sufficient to induce regeneration of CNS axons. It is of note that Neumann and colleagues report similar findings. Because, in both our study and that of Neumann et al. (2002, this issue of *Neuron*), the treatment is at the cell body, these results are directly relevant to developing a therapy for spinal cord injury; the possibility is now raised of systemic delivery of cAMP-elevating drugs that cross the blood brain barrier. This would obviate intervention at the site of injury, which could result in more damage. In addition, this is the first molecular insight into how a conditioning peripheral lesion can induce subsequent spinal axon regeneration. It is of importance to note that the distances regenerated by dorsal column axons lesioned one week after either a conditioning lesion or db-cAMP injection, are very similar (Figure 7) (Neumann and Woolf, 1999). This greatly strengthens our conclusion that cAMP is sufficient to mimic the effects of a conditioning lesion. However, because only a single DRG, L5, was injected with db-cAMP, the number of dorsal column axons that regenerate are fewer than after a conditioning lesion; when a conditioning lesion is performed, the sciatic nerve is cut, which receives axons from L4, L5, and L6 DRGs. Therefore, a sciatic nerve lesion conditions many more DRG cell bodies and so more dorsal column axons than db-cAMP injection into L5.

From these results, we conclude that elevation of cAMP induces an ability to overcome inhibitors of regeneration in myelin. We suggest that this is what determines whether regeneration occurs or not. It is generally accepted that it is not just a loss of intrinsic growth capacity that prevents CNS regeneration, as CNS axons will regenerate if a favorable environment, such as a peripheral nerve graft, is provided (David and Aguayo, 1981; Richardson et al., 1980). Instead, glial inhibitors appear to be the main culprits in blocking regeneration. Of these inhibitors, immediately after injury and before the glial scar forms, myelin inhibitors are the major players. It was shown that in mice immunized with myelin prior to spinal cord lesion, regeneration occurs and in some cases the scar forms around the regenerated axons (Huang et al., 1999). These findings indicate that there is a window of opportunity for regeneration when only myelin inhibitors are impeding growth, before the glial scar physically locks in the axons.

A role for cAMP in allowing neurons to grow on inhibitors such as MAG and myelin in general and to switch an inhibitory/repulsive effect to promotion/attraction is consistent with what we (Cai et al., 1999), and others (Song et al., 1998), have reported previously in culture. We showed that elevating cAMP in neurons completely



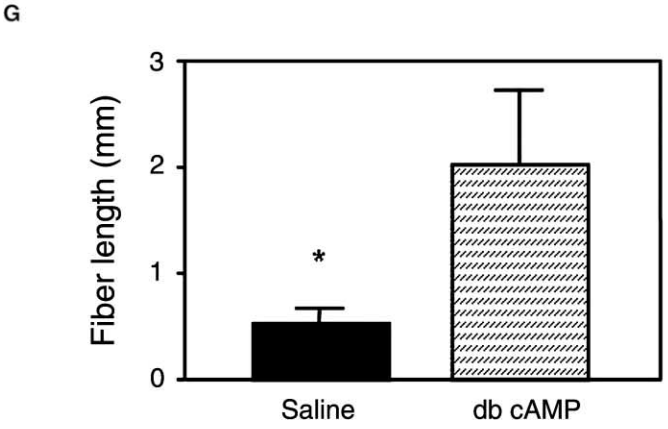


Figure 7. Injection of db-cAMP Induces Regeneration of Dorsal Column Axons In Vivo

L5 DRGs were injected with either saline (A–C) or db-cAMP (D–F) and 1 week later dorsal column axons were lesioned. After a further 1 or 2 weeks, dorsal column axons were labeled transganglionically with HRP. For images (A)–(F), rostral is up and caudal is down.

(A and D) Low-power horizontal Nissl-stained section through the lesion site; saline (A) or db-cAMP (D) injection into L5 DRG. Dorsal column axons were cut at T7 7 days later. Area of enlarged darkfield photomicrographs in (B), (C), (E), and (F) are indicated by the boxes.

(B and E) Darkfield images at the midpoint of the lesion. Dorsal root ganglion axons labeled transganglionically with HRP. (B), saline injection; (E), db-cAMP injection. In (E), arrows indicate axons that traverse the lesion site. There is no regrowth of the central process of the DRG neurons following saline injection (B).

(C and F) Darkfield image caudal to the lesion demonstrates equivalent robust labeling of dorsal column (DC) axons in db-cAMP-injected (F) and saline-injected animals (C).

Scale bars: (A and D), 500 μm; (B, C, E, and F), 150 μm.

(G) Average distance of axon growth (± SD) of the three longest axons for each animal from the caudal border of the lesion site for saline- and db-cAMP-treated animals. Axonal growth was significantly greater ($p < 0.0001$) in db-cAMP-treated animals.

blocked the inhibition by MAG and myelin. In addition, the ability of young neurons to grow on MAG and myelin and to spontaneously regenerate in vivo appears to be another consequence of high endogenous levels of cAMP (Cai et al., 2001). Poo and coworkers showed also that the repulsion of individual growth cones by MAG

and myelin is switched to attraction by elevating cAMP (Song et al., 1998). The results presented here, though, demonstrate cAMP effecting regeneration in vivo in the mature CNS. It is also of note that elevation of cAMP promotes survival of spinal motor neurons (Hanson et al., 1998) and potentiates survival effects of neurotrophins on other types of neurons (Meyer-Franke et al., 1995). Post-lesion elevation of cAMP would then promote both survival and axonal regeneration through an inhibitory environment.

A conditioning lesion improves growth in two phases. In the first phase cAMP is elevated and the improved growth on MAG and myelin is completely blocked by PKA inhibitors. In this early phase, when dorsal columns are lesioned at the same time as the peripheral, conditioning lesion, there is sprouting of dorsal column axons into the lesion site (Neumann and Woolf, 1999). Moreover, Neumann et al. (2002, this issue of *Neuron*) showed that this early phase can be mimicked by db-cAMP injection into DRG 48 hr before the dorsal column lesion. In the second phase, which is underway by 1 week post-conditioning lesion, cAMP levels have dropped back to control levels even though growth on MAG and myelin actually increases and now is PKA independent. If dorsal column axons are now lesioned at this time, 1 week post-conditioning lesion, there is substantial regeneration through, around, and some beyond the lesion site (Neumann and Woolf, 1999). From a number of observations, it can be concluded that the second, more pronounced PKA-independent phase of growth is dependent on the transient elevation of cAMP. First, direct injection of db-cAMP into the DRG, without a conditioning lesion, is sufficient to trigger the later PKA-independent phase of growth. Second, injection of db-cAMP is also sufficient to result in regeneration of dorsal column axons lesioned 1 week after the injection. At an equivalent time after a peripheral lesion, all regeneration is now PKA independent. Finally, administration of a PKA inhibitor at the same time as the conditioning lesion blocks completely the improved growth on MAG and myelin 1 day later and attenuates growth after one week. The inability of a PKA inhibitor to block completely the improved growth observed a week later is likely to be due to methodological limitations. We do not know if PKA activation is completely blocked, and it is possible that the threshold of PKA activation required to trigger the second phase of growth may be much lower than for the first phase. Partial block of PKA activity would then be sufficient to block completely the first phase of growth but not the second phase. The 1 week effect is nonetheless attenuated by H89. Taken together, the data strongly suggest that elevation of cAMP is sufficient to bring about both the early PKA-dependent and the later PKA-independent growth. We do not know if cAMP is the only factor that contributes to this improved growth. However, the similarity in the distance of dorsal column axon regeneration induced by db-cAMP injection and a conditioning lesion (Neumann and Woolf, 1999), suggests that it is indeed largely responsible.

The two-phase effect of both a conditioning lesion and db-cAMP injection on improved growth suggests that the transient increase in cAMP is initiating two separate molecular events. A distinction between the two phases may be a requirement by the late, but not the early phase, for transcription and protein synthesis to occur (Smith and Skene, 1997). It is possible that while cAMP is elevated and PKA activated, the activated PKA has a direct effect on the cytoskeleton. One candidate for mediating this effect could be the small G protein, Rho. McKerracher and her coworkers have shown that inactivation of Rho will induce regeneration on and through myelin, both in vitro and in vivo (Lehmann et al., 1999). Consistent with this suggestion, it has been reported that PKA can directly affect activation of Rho (Lang et al., 1996). For this early phase of growth, ongoing activation of PKA would be required. A direct effect of PKA on the cytoskeleton may also account for rapid reversal of turning of axons to a variety of guidance cues when the cAMP/PKA pathway is manipulated (Song et al., 1998). As well as directly affecting the cytoskeleton, activated PKA could simultaneously influence transcription (Montminy, 1997). The proteins synthesized would then go on to affect growth directly, and their effect, once synthesized, would now be independent of PKA. For such a mechanism to account for late growth, only a single burst in transcription would be required, but the newly synthesized proteins would need to persist for a relatively long time. Indeed, it has been reported that the dorsal columns of mice overexpressing the two growth-associated proteins GAP43 and CAP26 that are localized and enriched in the growth cone, will spontaneously sprout after lesioning even in the absence of a conditioning lesion (Bomze et al., 2001).

Finally, the aim of these studies is to elucidate the molecular consequences of a peripheral conditioning lesion that allows axons to grow through the hostile environment of the CNS and then to induce the same changes in all CNS axons to encourage them to regenerate. Identification of cAMP as a key player in this sequence of events is a major step in this direction.

Experimental Procedures

Peripheral Nerve Lesion

P18 rats were anaesthetized by inhalation of isofluorene, and unilateral sciatic nerve transection was performed at mid-thigh level. Before implantation, osmotic mini-pumps (Alzet) were filled with either 2 mM H89 (Calbiochem) or saline, attached to a cannula, and then incubated at 37°C. Lumbar spinal cord was exposed by laminectomy between L5 and L6. A prefilled cannula was inserted through the atlanta-occipital membrane along the dorsal surface of the cord until the tip rested between L4 and L5. Either H89 or saline was delivered at 0.5 μl per hr.

Neurite Outgrowth

L4 and L5 DRG's were removed and dissociated, and the neurite outgrowth assay performed as previously described (DeBellard et al., 1996). 5×10^4 or 2×10^4 neurons were added to a myelin substrate or to monolayers of transfected CHO cells, respectively. Where indicated, KT5720 (200 nM), H89 (400 nM), KT5823 (1 mM), or db-cAMP (1 mM) (Calbiochem) was added to the cultures. After overnight incubation, cultures were immunostained for GAP43 as described before (Mukhopadhyay et al., 1994). The length of the longest neurite of a group of randomly selected 180–200 neurons was measured using an Oncor image analysis program. Neurite lengths were compared between groups using the Student's t test.

Immunoassay for cAMP

L4 and L5 DRGs were dissociated as previously described (DeBellard et al., 1996), and cAMP was measured immediately using a competitive immunoassay, according to the manufacturer's instruc-

tions (Amersham). In each well, 2×10^5 DRG neurons were used. Each experiment was performed in quadruplet and repeated at least six times.

Immunostaining for cAMP

L4 and L5 DRGs were removed and immediately fixed in 5.5% acrolin for 1 hr at room temperature, then emerged in increasing concentrations of sucrose (from 10% to 30%) at 4°C until reaching equilibrium, embedded with optimum cutting temperature (OCT) mixture, and cryosectioned at 5 μm. Sections were stained for cAMP using rabbit polyclonal antiserum as described (Wiemelt et al., 1997).

Dorsal Root Ganglion Injections

Sprague-Dawley rats (female, 5 weeks old) were anesthetized with chloral hydrate (400 mg/kg, IP). A laminectomy was made at the level of the lumbar enlargement, to expose the left L4 and L5 dorsal roots and associated ganglia. Where indicated, L4 and L5 or only the L5 dorsal root ganglion was injected with 2 μl of H89 (2 mM), db-cAMP (50 mM), or 0.9% saline. Injections were made slowly over 15–20 min, and the pipette was left in place for an additional 3 min before it was removed. When two injections were carried out, the gap between injections was 4 hr.

Dorsal Column Lesions

1 week after DRG injection into L5, the spinal dorsal columns were lesioned. Briefly, each rat was anesthetized (400 mg/kg of 4% aqueous chloral hydrate). Techniques previously described (Bregman et al., 1993) were then used to expose the dorsal column at the thoracic level T6-8. Irridectomy scissors were used to cut the dorsal columns bilaterally. A small pledglet of gelfoam was placed into the lesion site, and the muscles overlaying the lesion sewn together with 6/0 surgical suture. The skin was stapled, and the rats returned to a warming cage to recover. All rats were then maintained with food and water ad libitum in the animal care facility.

Axonal Tracing

At 1 week or 2 weeks after dorsal column lesions, the left flank was shaved and prepared for surgery. A fine incision was made in the skin, and the thigh muscles were excised to expose the left sciatic nerve. The nerve was transected using fine iridectomy scissors, and the lesion was confirmed under high power of a Zeiss dissecting microscope. The proximal end of each cut nerve was then exposed to a mixture of WGA-HRP and β-cholera toxin HRP (1% β-cholera toxin HRP, and 10% WGA-HRP in sterile saline).

Sacrifice and Sectioning

Animals were sacrificed 2–3 days after the application of the HRP tracer. Rats were perfused intracardially with a solution of 0.9% saline with heparin, followed immediately by an ice-cold perfusate of 1.5% gluteraldehyde, 1% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The dorsal column lesion sites, as well as the corresponding DRGs were carefully dissected out under a dissecting microscope, and the tissues were transferred to a 10% sucrose phosphate buffer solution, followed by a 30% sucrose phosphate buffer solution. Sections were collected on a Leica frigocut 2800N cryostat, cut longitudinally at 30 μm. Sections were stained using a standard HRP staining protocol (Mesulam, 1976).

Analysis

The lesions were reconstructed serially to ensure that the dorsal column axons were interrupted completely. The HRP labeling within the dorsal column caudal to the spinal cord lesion was examined. In all of the animals included in this study, there was equivalent transganglionic labeling of dorsal column axons in the spinal cord segments caudal to the lesion in saline and db-cAMP animals. A subset of the 24 rats generated for this study ($n = 9$ total) were used for quantitative analysis of fiber length past midline of lesion. The last nine rats in this experimental series were selected for quantitative analysis, and measurements were completed prior to any fading of the HRP label. Fiber length was determined by observation through a Zeiss microscope, using the micrometer installed as part of the eyepiece. The caudal border of the lesion was determined on Nissl-stained sections, and measurements of axonal growth were made from adjacent uncounterstained tissue. The length of the fibers crossing from the caudal border of the lesion was measured, and the three longest axons for each animal were averaged together.

Acknowledgments

We thank Dr. Arthur McMorris and Dr. Anthony Weimelt for providing the cAMP antibody, Dr. Roger Persell for critically reading this manuscript, and Dr. Lloyd Williams for his assistance with the image analysis. This work was supported by grants from the New York State Spinal Cord fund, the NIH (NS 37060 to M.T.F., NS19259 to B.S.B.), the National Multiple Sclerosis Society, the Paralysed Veterans of America (P.H.), and core facility grants from the Research Centers for Minorities Institute-NIH and Specialized Neuroscience Research Programs-NIH (NS41073). J.Q. was a fellow of the NMSS.

Received: January 28, 2002
Revised: April 23, 2002

References

Bomze, H.M., Bulsara, K.R., Iskandar, B.J., Caroni, P., and Skene, J.H. (2001). Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. Nat. Neurosci. *4*, 38–43.

Bradbury, E.J., McMahon, S.B., and Ramer, M.S. (2000). Keeping in touch: sensory neurone regeneration in the CNS. Trends Pharmacol. Sci. *21*, 389–394.

Bregman, B.S., Kunkel-Bagden, E., Reier, P.J., Dai, H.N., McAtee, M., and Gao, D. (1993). Recovery of function after spinal cord injury: mechanisms underlying transplant-mediated recovery of function differ after spinal cord injury in newborn and adult rats. Exp. Neurol. *123*, 3–16.

Cai, D., Shen, Y., De Bellard, M., Tang, S., and Filbin, M.T. (1999). Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. Neuron *22*, 89–101.

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B.S., and Filbin, M.T. (2001). Neuronal cyclic amp controls the developmental loss in ability of axons to regenerate. J. Neurosci. *21*, 4731–4739.

David, S., and Aguayo, A.J. (1981). Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science *214*, 931–933.

DeBellard, M.E., Tang, S., Mukhopadhyay, G., Shen, Y.J., and Filbin, M.T. (1996). Myelin-associated glycoprotein inhibits axonal regeneration from a variety of neurons via interaction with a sialoglycoprotein. Mol. Cell. Neurosci. *7*, 89–101.

Filbin, M.T. (1999). The benefits of adding insult to injury. Neuron *23*, 2–4.

Fitch, M.T., and Silver, J. (1999). Beyond the glial scar. In CNS Regeneration, M.H. Tuszynski and J.H. Kordower, eds. (San Diego, CA: Academic Press), pp. 55–88.

Hanson, M.G., Jr., Shen, S., Wiemelt, A.P., McMorris, F.A., and Barres, B.A. (1998). Cyclic AMP elevation is sufficient to promote the survival of spinal motor neurons in vitro. J. Neurosci. *18*, 7361–7371.

Huang, D.W., McKerracher, L., Braun, P.E., and David, S. (1999). A therapeutic vaccine approach to stimulate axon regeneration in the adult mammalian spinal cord. Neuron *24*, 639–647.

Lang, P., Gesbert, F., Delespine-Carmagnat, M., Stancou, R., Pouchelet, M., and Bertoglio, J. (1996). Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J. *15*, 510–519.

Lehmann, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebok, A., Leclerc, N., Tigyi, G., and McKerracher, L. (1999). Inactivation of rho signaling pathway promotes CNS axon regeneration. J. Neurosci. *19*, 7537–7547.

McKeon, R.J., Hoke, A., and Silver, J. (1995). Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp. Neurol. *136*, 32–43.

Mesulam, M.M. (1976). The blue reaction product in horseradish

peroxidase neurohistochemistry: incubation parameters and visibility. J. Histochem. Cytochem. *24*, 1273–1280.

Meyer-Franke, A., Kaplan, M.R., Pfrieger, F.W., and Barres, B.A. (1995). Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron *15*, 805–819.

Montminy, M. (1997). Transcriptional regulation by cyclic AMP. Annu. Rev. Biochem. *66*, 807–822.

Mukhopadhyay, G., Doherty, P., Walsh, F.S., Crocker, P.R., and Filbin, M.T. (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron *13*, 757–767.

Neumann, S., and Woolf, C.J. (1999). Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron *23*, 83–91.

Porreca, F., Lai, J., Bian, D., Wegert, S., Ossipov, M.H., Eglen, R.M., Kassotakis, L., Novakovic, S., Rabert, D.K., Sangameswaran, L., and Hunter, J.C. (1999). A comparison of the potential role of the tetrodotoxin-insensitive sodium channels, PN3/SNS and NaN/SNS2, in rat models of chronic pain. Proc. Natl. Acad. Sci. USA *96*, 7640–7644.

Richardson, P.M., McGuinness, U.M., and Aguayo, A.J. (1980). Axons from CNS neurons regenerate into PNS grafts. Nature *284*, 264–265.

Scherer, S.S., and Salzer, J.L. (1996). Axon-schwann cell interactions during peripheral nerve degeneration and regeneration. In Glial Cell Development: Basic Principles and Clinical Relevance, K.R. Jessen and W.D. Richardson, eds. (Oxford: Bios Scientific Publishers), pp. 165–185.

Schwab, M.E., and Bartholdi, D. (1996). Degeneration and regeneration of axons in the lesioned spinal cord. Physiol. Rev. *76*, 319–370.

Smith, D.S., and Skene, J.H. (1997). A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. J. Neurosci. *17*, 646–658.

Song, H., Ming, G., He, Z., Lehmann, M., McKerracher, L., Tessier-Lavigne, M., and Poo, M. (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. Science *281*, 1515–1518.

Wiemelt, A.P., Engleka, M.J., Skorupa, A.F., and McMorris, F.A. (1997). Immunochemical visualization and quantitation of cyclic AMP in single cells. J. Biol. Chem. *272*, 31489–31495.