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Regeneration of Sensory Axons within the Injured Spinal Cord Induced by Intraganglionic cAMP Elevation

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Summary

The peripheral branch of primary sensory neurons regenerates after injury, but there is no regeneration when their central branch is severed by spinal cord injury. Here we show that microinjection of a membrane-permeable analog of cAMP in lumbar dorsal root ganglia markedly increases the regeneration of injured central sensory branches. The injured axons regrow into the spinal cord lesion, often traversing the injury site. This result mimics the effect of a conditioning peripheral nerve lesion. We also demonstrate that sensory neurons exposed to cAMP in vivo, when subsequently cultured in vitro, show enhanced growth of neurites and an ability to overcome inhibition by CNS myelin. Thus, stimulating cAMP signaling increases the intrinsic growth capacity of injured sensory axons. This approach may be useful in promoting regeneration after spinal cord injury.

Introduction

The peripheral and central (spinal cord) axonal branches of adult primary sensory neurons differ fundamentally in their response to injury: the peripheral branch regenerates after injury, but the central branch, which courses in the dorsal columns of the spinal cord, does not (Ramon y Cajal, 1928). The failure of the injured central branch to regenerate likely has multiple causes, including the presence of glial barriers and of inhibitory molecules (e.g., in CNS myelin) (Benfey et al., 1985; Fawcett and Asher, 1999; Fitch and Silver, 1997; Schwab and Caroni, 1988; Schwab and Bartholdi, 1996) and the lack of some growth-promoting molecules at the injury site (Jakeman and Reier, 1991), as well as an inadequate intrinsic growth capacity of the injured neurons (Fawcett, 1992). Attempts have been made to enhance the intrinsic capacity of adult sensory neurons to grow. For example, the extent of neurite outgrowth and the rate of regeneration of the central branch of primary sensory neurons into a peripheral nerve graft can be enhanced when a peripheral nerve lesion is performed at the same time (Richardson and Issa, 1984; Richardson and Verge, 1986; Oudega et al., 1994). Moreover, we previously demonstrated that a conditioning peripheral nerve lesion made at different times prior to a dorsal column lesion, which severs the central branch of large diameter sensory axons, results in regeneration of those fibers into and beyond the dorsal column lesion site (Neumann and Woolf, 1999).

In the present study, we attempted to enhance the intrinsic growth capacity of sensory neurons without injuring the peripheral branch. We hypothesized that the conditioning peripheral lesion increases the intrinsic growth capacity of the injured central axons through a signaling cascade in the cell bodies in these neurons in the DRG. Clues to relevant signaling pathways have come from several in vitro studies. For example, we previously found that one of the components of myelin that inhibits axonal regrowth in mammals, myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), also repels embryonic *Xenopus* spinal axons, but manipulations that stimulate cAMP signaling in these axons converts this repulsion to attraction (Song et al., 1998). Filbin and colleagues have also shown that elevated levels of cAMP secondary to neurotrophin exposure enhance the ability of a variety of neonatal mammalian axons, including primary sensory axons, to grow on substrates of both MAG and myelin (Cai et al., 1999, 2001), which normally inhibit axon regrowth. In the present study, we therefore asked whether exposing sensory neurons in the DRG to a membrane-permeable cAMP analog, db-cAMP, could enhance the regeneration of the central (spinal cord) branch of primary sensory neurons. Based on our previous finding that a dramatic regenerative response is achieved by priming the DRG cells with a conditioning peripheral lesion prior to the dorsal column injury (Neumann and Woolf, 1999), we examined specifically whether exposure of the DRG cell bodies 48 hr prior to the injury could stimulate regeneration. To help interpret the effects we saw, we also asked how the growth behavior of neurons was altered by this treatment, by subsequently removing neurons treated in vivo and culturing them on both permissive and nonpermissive substrates in vitro. Our results imply that exposure to db-cAMP can alter the growth state of these neurons both in vivo and in vitro and may provide a useful tool to stimulate regeneration in the injured spinal cord in vivo.

Results

Dorsal Column Tracing and Lesion

In our in vivo experiments, we used the dorsal columns to study the regenerative capacity of CNS axons. We focused specifically on medium and large diameter sensory neurons with cell bodies located in the L4, L5, and L6 DRG in the lumbar spinal cord. These neurons

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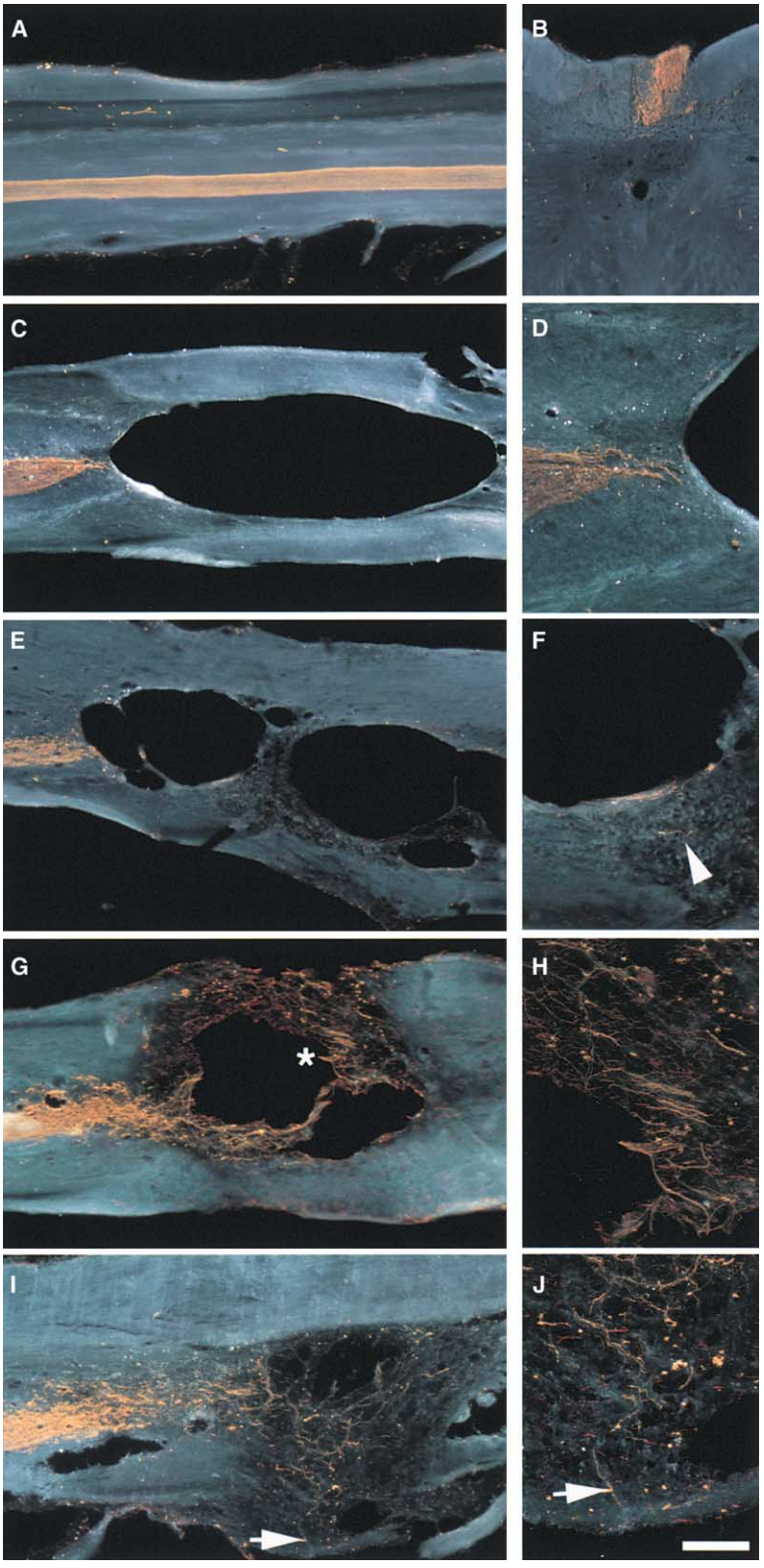

Figure 1. Regeneration of the Central Branches of Sensory Neurons In Vivo after Intraganglionic Injection of db-cAMP

All panels (except [B]) show images from horizontal sections through the thoracic spinal cord of control or operated animals. Rostral (anterior) to the right, caudal (posterior) to the left. To visualize the central branches of sciatic sensory axons coursing in the dorsal columns, these axons are labeled by transneuronal transport of B-HRP injected into the sciatic nerve (B-HRP reaction product appears pink in dark field optics).

(A) In an unoperated animal, the fibers traverse the spinal cord in a tight bundle.

(B) Transverse section through the caudal hindbrain showing termination of the sciatic dorsal column fibers in the gracile nucleus in an unoperated animal. Extensive labeling in the gracile nucleus was observed in all uninjured animals, whereas no labeling was observed in any of the injured animals (data not shown), confirming the completeness of the lesion in each case.

(C and D) Horizontal section through the lesion site taken from a noninjected animal. The sciatic dorsal column fibers do not enter the lesion site.

(E and F) Horizontal section through the lesion site taken from a saline-injected animal. The majority of fibers stop at the lesion site, but a few grow around the cyst ([F], arrowhead). This example illustrates the maximum growth that we observed in saline-treated animals. Note that only very few fibers are present in the lesion.

(G–J) Horizontal sections through the lesion site taken from db-cAMP-injected animals showing regenerating fibers. Many sciatic dorsal column axons regrow into the lesion site; some grow extensively around and on the walls of a cyst (G and H), while others grow toward the surface of the spinal cord ([I and J], arrow). Asterisk in (G) denotes area magnified in (H).

Scale bar: 250 μm for (A), (C), (E), (G), and (I); 50 μm for (B), (D), (F), (H), and (J).

possess both a peripheral branch that innervates the hindlimb as well as a central branch that courses in the gracile fasciculus (the most medial component of the dorsal columns) and terminates in the gracile nucleus in the medulla (Figure 1B). We visualized axons in the gracile fasciculus by injecting an anatomical tracer (the β subunit of cholera toxin conjugated to horseradish peroxidase [B-HRP]) into the sciatic nerve. This tracer is taken up selectively by medium and large diameter myelinated axons (Robertson and Grant, 1989) and transported ret-

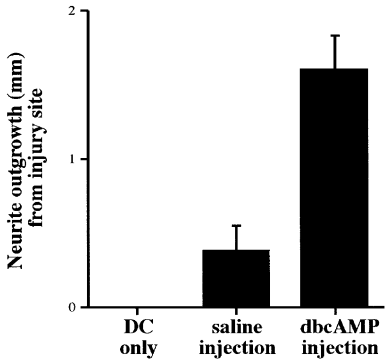

Figure 2. Length of the Longest Regenerating Sciatic Dorsal Column Axons Measured from the Proximal Site of the Lesion

No growth was observed in the lesion site in noninjected animals (n = 6). A small number of fibers grew in the saline-injected group (n = 5; Figure 1F), but only for a short distance. Extensive regeneration of sciatic dorsal column injured axons occurred only in the db-cAMP-injected group (n = 6).

rogradely to cell bodies in the DRG and from there anterogradely into the axons of the ipsilateral gracile fasciculus; collateral branches of these axons can also be identified in the gray matter of the L4-6 lumbar spinal dorsal horn and projecting to Clarke's nucleus in the upper lumbar and lower thoracic segments (LaMotte et al., 1991). Importantly, above thoracic segment 8 (T8), the axons coursing in the dorsal columns are tightly fasciculated and unbranched (Figure 1A). Using microscissors, we transected the dorsal columns bilaterally at the level of the T6-7 thoracic segments, between the dorsal root entry zones, under halothane anesthesia. Ventrally, the lesions extended down to the central canal, so that the dorsal columns were transected in their entirety. In some cases, the central canal was also damaged, leading to the development of cysts that extended proximal and distal to the lesion.

Regeneration of Damaged Dorsal Column Fibers into the Lesion Site after Injection of db-cAMP into DRGs

The regenerative response was assessed 6–8 weeks after dorsal column lesion. Consistent with previous results, we found no signs of regeneration of the injured axons in the dorsal columns of normal, untreated animals (n = 6): in all animals, fibers abruptly stop at the lesion site, and most labeled axons formed a growth cone-like swelling (end bulbs; data not shown), which never penetrated the injury site (Figures 1C and 1D and Figures 2). Proximal to the lesion, the dorsal column tract appears as a tight bundle of fibers. This baseline pattern reflects the fact that axonal regeneration does not normally occur in the CNS (Ramon y Cajal, 1928). Similar results were observed in rats in which the L4/L5 DRGs had been injected with saline (1.5 μl in each ganglion) 48 hr prior to dorsal column lesion: virtually all the fibers stopped dead at the dorsal column lesion, and the density of end bulbs in these rats was similar to noninjected animals (data not shown). We did observe some axons entering the lesion site, but they were very few in number (Figures 1E and 1F) and very short (Figure 2). This small amount of growth may result from injury during injection mimicking the effect of a conditioning lesion (see Discussion). In contrast to the results from these control groups, when we microinjected L4/L5 DRGs with db-cAMP (1.5 μl of a 10 mM solution injected in each ganglion) 48 hr prior to dorsal column lesion, we observed a robust regenerative response. In all cases analyzed (n = 6), massive numbers of damaged fibers penetrated and regenerated into and across the lesion site (Figures 1G–1J). As shown in Figure 2, the length of the fibers was much longer than observed in controls. A prominent feature of the regenerating fibers was a loss of the fasciculation typical of intact dorsal column fibers (Figures 1H and 1J). Some of the fibers in the lesion site grew along cysts (Figures 1G and 1H). Others grew in the same plane as the lesion, toward the surface of the spinal cord, i.e., at a right angle to the normal trajectory of the axons (Figures 1I and 1J). Occasionally, the regenerating axons entered adjacent dorsal roots that were injured during the surgical interruption of the dorsal columns (data not shown). In addition, in this group, sprouting proximal to the lesion site was evident (Figure 1G and 1I), which contrasts with the control groups in which such sprouting was absent. Although substantial growth of many damaged fibers was found within the lesion site in all animals, no regrowth was observed beyond the lesion. Interestingly, although this contrasts with the effects of a conditioning peripheral lesion made 1 week prior to the central lesion, the extent of regeneration observed here is comparable to that observed following a conditioning lesion made at the same time as the central lesion (Neumann and Woolf, 1999).

Enhanced Outgrowth of Neurites on a Semipermissive Substrate after Injection of db-cAMP into DRGs

These results illustrate that injection of db-cAMP into DRG can, at least in part, recapitulate the growth-promoting effects of conditioning sciatic nerve lesions. To begin to address the mechanism through which db-cAMP alters the regenerative capacity of DRG axons, we turned to an in vitro analysis of the effects of such manipulations on the growth capacity of DRG neurites.

It has been well documented that stimulating cAMP signaling in vitro can enhance the growth capacity of both embryonic and adult sensory neurons cultured on a permissive substrate (Rydel and Greene, 1988; Andersen et al., 2000). Those studies examined bulk populations of sensory neurons, whereas our in vivo studies focused on axons projecting in the dorsal columns, which are the myelinated axons of medium and large diameter DRG neurons. To address directly whether this subpopulation is responsive to cAMP stimulation, we used antibody N-52 to neurofilament 200 to selectively mark these cells (Lawson and Waddell, 1991; Shaw et al., 1986). We plated sensory neurons dissociated from L4 and L5 DRG on poly-D-lysine-coated dishes and treated them with forskolin to stimulate cAMP production. At 18 hr, we observed little growth from either control or forskolin-treated cells (data not shown). After 48 hr, however, whereas control neurons displayed only moderate growth (Figures 3A and 3B), there was extensive outgrowth from neurons cultured with 3 or 30 μM

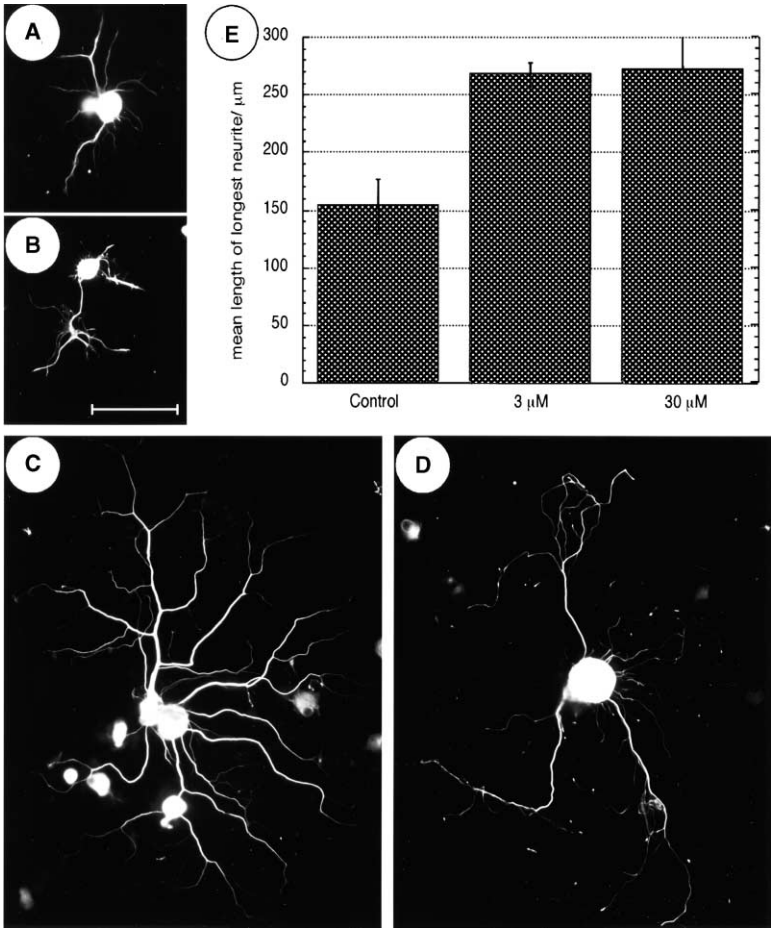

Figure 3. Forksolin Treatment Enhances Axon Outgrowth from Medium and Large Diameter Adult Sensory Neurons

DRG cells were plated on poly-D-lysine coated dishes with or without forskolin for 48 hr, then fixed and stained for N-52. Control cells show only moderate outgrowth (A and B), while forskolin-treated cells have long axons (C and D). (E) The response to forkskolin treatment, quantified as the length of the longest N-52+ neurite per cell, saturates by 3 μM. The mean length values were obtained from three independent experiments. Scale bar: 100 μm.

forskolin (Figures 3C–3E) ($p < 0.004$, Student's t test). Thus, stimulation of cAMP signaling in vitro can increase the growth capacity of medium and large diameter N-52+ adult sensory neurons.

We next examined more specifically whether the in vivo manipulation we had performed, the injection of db-cAMP into DRG, also results in an enhancement of the growth properties of the neurites of those neurons. For this, we unilaterally injected the L4 and L5 ganglia with db-cAMP; 48 hr later, the rats were killed, the injected and the contralateral noninjected (control) L4 and L5 sensory ganglia were separately pooled and dissociated, and the neurons were plated on poly-D-lysine. As a further control, we cultured neurons from ganglia injected with saline. In a previous study, Smith and Skene showed that when control adult sensory neurons are cultured on a permissive substrate, there is a 20–24 hr lag before they extend neurites in a significant way, but that this time can be shortened if neurons are subjected to peripheral nerve injury 2–7 days before plating (Smith and Skene, 1997). We therefore chose to examine the neurons after 18 hr in culture, to determine whether, as with the conditioning lesion, the pretreatment with db-cAMP could affect the timing of onset and/or rate of extension of the axons.

As expected, little outgrowth was observed after 18 hr from N-52+ neurons from noninjected ganglia (Figures 4A, 4E, and 4F). A slight response of neurons to saline injection was observed: the number of neurons with neurites (defined as processes > 30 μm) increased from 6.5% ± 0.7% (noninjected) to about 18.9% ± 2.2% (saline injected; Figures 4B and 4E) ($p < 0.02$, Student's t test). However, the distribution of neurite lengths among neurite-bearing neurons was not markedly changed (Figure 4F). The slight difference between control and saline-treated groups parallels the slight difference observed in vivo (Figures 1 and 2). In contrast to the very poor growth observed in these control groups, after 18 hr we observed extensive growth of N-52+ neurites from neurons treated in vivo with db-cAMP (Figure 4C), as 49.7% ± 4.3% of neurons now had neurites (Figure 4E) ($p < 0.0001$ for comparison with either saline treatment or control). Furthermore, the length of the neurites was significantly increased: for example, the fraction of all N-52+ neurons in the cultures with neurites greater than 250 μm in length increased from 0.1% ± 0.05% and 0.85% ± 0.2% for control or saline-treated conditions, respectively, to 6.5% ± 0.7% for the db-cAMP-treated condition ($p < 0.0003$). This increase did not merely reflect the fact that more neurons had neurites in the db-cAMP-treated case, but also reflected the fact that neurites that extended from these neurons were longer than in controls. This was shown by the analysis of Figure 4F, in which we focused only on neurons with neurites (processes > 30 μm), a procedure that will tend

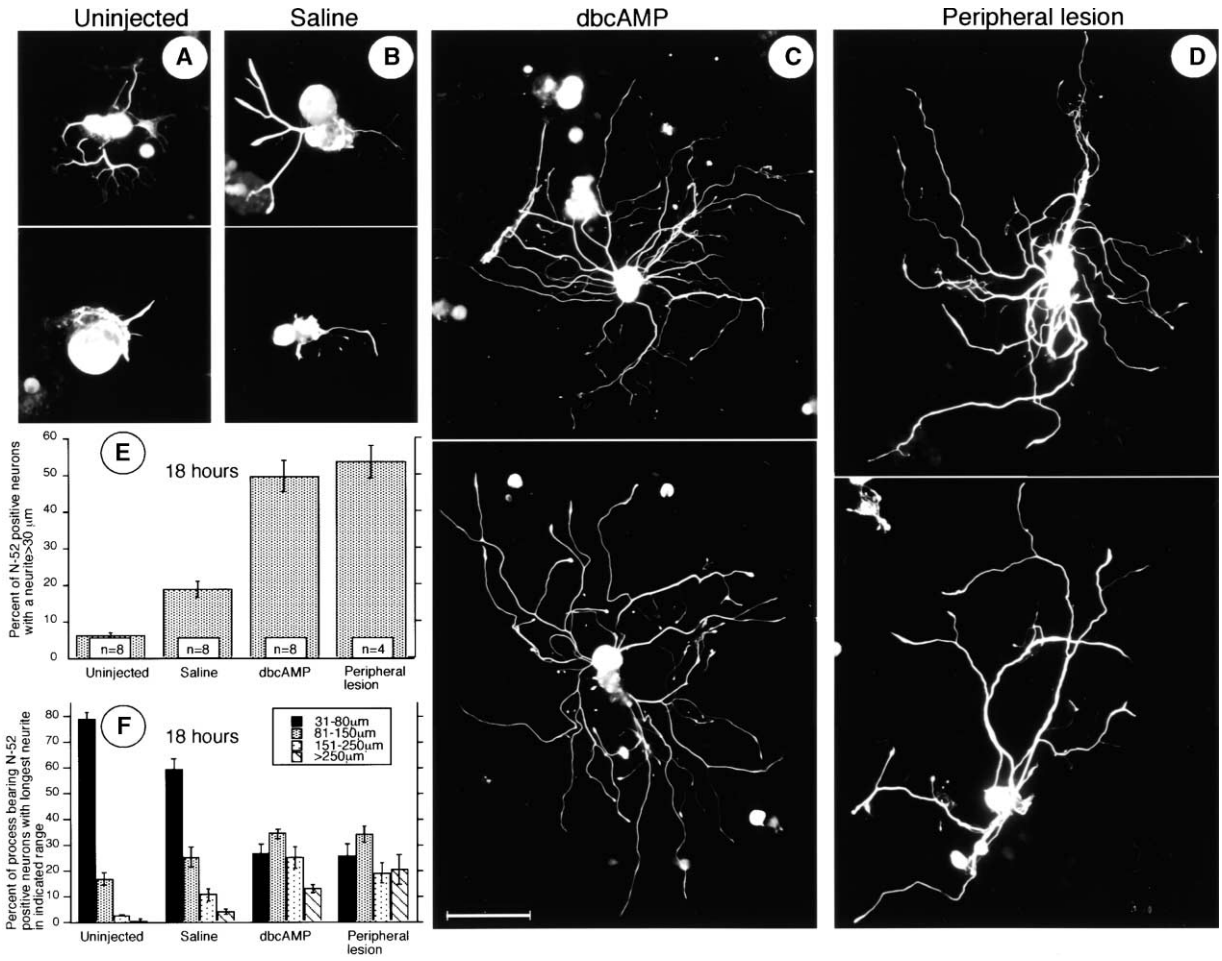

Figure 4. Injection of db-cAMP into DRGs In Vivo or a Conditioning Peripheral Nerve Lesion Enhances Neurite Outgrowth on a Permissive Substrate

(A–D) L4 and L5 DRG were injected unilaterally with 1.5 μl of 10 mM db-cAMP or saline. 2 days later, the DRGs were removed, dissociated, and plated on poly-D-lysine-coated dishes. After 18 hr, cells were fixed and stained for N-52. DRG neurons obtained from noninjected (n = 8) (A) or saline-injected (n = 8) (B) ganglia have either no or only very short neurites at this time point. In contrast, many DRG neurons from db-cAMP-injected DRG (n = 8) (C) or from DRG in which a conditioning peripheral nerve lesion had been performed (n = 4) (D) extend far and often have numerous axons.

(E) Percent neurons with N-52+ neurites (process > 30 μm) under different conditions.

(F) Distribution of neurite lengths among neurons with neurites >30 μm under different conditions (n = 4).

Scale bar, 100 μm.

to overemphasize the contributions of the small number of neurons with neurites in control and saline-treated conditions. Even within these populations of neurite-bearing neurons, a significant shift in the distribution of lengths was observed following db-cAMP treatment, with a significant decrease in the fraction in the 31–80 μm range ($p < 0.002$ for db-cAMP treatment compared to either control or saline-treated), and a concomitant rise in the fraction with lengths in the 151–250 μm range and the >250 μm range (Figure 4F) ($p < 0.004$ for the number in either of those length bins when compared to control or saline-treated; Figure 4F). Thus, the db-cAMP treatment increases both the number of process-bearing cells at 18 hr, as well as the lengths of neurites of those cells.

Our finding that a large fraction (50%) of the neurons exposed to db-cAMP extended neurites in vitro at 18 hr provides evidence that exposure to db-cAMP, like the conditioning lesion, can put these neurons into what Smith and Skene have termed a state of "competence for axonal growth" (Smith and Skene, 1997). To permit a more direct comparison of the effects of db-cAMP treatment to that of a conditioning lesion, we performed conditioning lesions of the sciatic nerve, and 48 hr later we cultured neurons from L4 and L5 DRG. After 18 hr, similar numbers of neurons with neurites were observed following a conditioning lesion (53.7% ± 4.3%) and following db-cAMP treatment (49.7% ± 4.3%, Figure 4E) ($p > 0.4$). Again, when we focused just on neurons with neurites greater than 30 μm in length, there was also a significant increase in the lengths of neurites compared to control and saline-injected groups (Figures 4D and 4E), including a significant increase in the number of neurons with neurites greater than 250 μm (Figure 4F)

($p < 0.003$, when compared to either control or saline-injected). Although the trend appeared to be that neurite lengths were slightly greater after the conditioning lesion treatment than after the db-cAMP treatment, the differences between the two groups were not significant ($p > 0.14$ for each neurite length bin).

Enhanced Outgrowth of Neurites on an Inhibitory Substrate after Injection of db-cAMP into DRGs

Because the environment in which sensory axons regenerate after CNS injury is inhibitory, we next asked whether cAMP pretreatment in vivo could also subsequently enable the neurons to extend neurites on an inhibitory substrate. Dissociated L4/L5 sensory neurons obtained from rats injected as above with db-cAMP were plated on a substrate of poly-D-lysine and myelin, a well-known inhibitory substrate (Schwab and Caroni, 1988). Because normal growth is significantly attenuated on the myelin substrate, in these experiments, we assessed neurite outgrowth after 36 hr rather than after 18 hr in culture. At 36 hr, virtually no outgrowth was observed from noninjected N-52+ neurons (Figures 5A, 5E, and 5F). As on a permissive substrate, when N-52+ neurons from saline-injected neurons were grown on a myelin substrate, we also observed a very slight but statistically significant increase in the number of neurons with neurites, from 4.6% ± 1.0% to 8.5% ± 1.3% (Figures 5B and 5E) ($p < 0.02$), although not a detectable change in the length of the neurites of cells with neurites greater than 30 μm (Figures 5F). In contrast to the poor growth observed for those two sets of neurons, N-52+ neurons from DRG injected with db-cAMP showed extensive growth on myelin (Figure 5C), with a marked increase in the number of neurons with neurites to 29.4% ± 4.3% (Figure 5E) ($p < 0.0001$ for comparison to either control or saline-treated cases). There was also a significant increase in the length of the neurites. For example, among all N-52+ neurons, the fraction with neurites greater than 150 μm increased from 0.6% ± 0.2% or 1.2% ± 0.2% for control or saline-treated, respectively, to 10.0% ± 1.1% for the db-cAMP-treated neurons ($p < 0.0003$ in both cases)—an 8- to 16-fold increase. As in the case of growth on a permissive substrate, this increase did not merely reflect the fact that more neurons had neurites, but also reflected the fact that neurites that extended from these neurons were longer than in controls. This was shown by the analysis of Figure 5F, in which we focused just on the neurons with neurites greater than 30 μm. Even within those populations, there was also a statistically significant increase (more than double) in the fraction of neurons with neurites greater than 150 μm in the db-cAMP-treated case compared to control or saline-injected ($p < 0.005$ in both cases). As was perhaps expected, similar results were obtained for neurons derived from rats subjected to a conditioning sciatic nerve lesion, when cultured on myelin: we observed a statistically significant increase in the number of neurons with neurites greater than 30 μm to 33.1% ± 3.8% (Figures 5D and 5E) ($p < 0.0001$ when compared to either control or saline-injected) and a statistically significant increase in the length of neurites (Figure 5F) (e.g., among neurons with neurites, the fraction with neurites greater than 150 μm again more than doubled compared to control or saline-injected [$p < 0.002$ in both cases]).

Discussion

Our results show that injection of db-cAMP into adult sensory ganglia prior to lesioning their central processes can endow the treated sensory neurons with the ability to regenerate injured axons into the adult CNS in vivo. Based on our in vitro results, we suggest that there are two key mechanisms through which stimulation of cAMP signaling promotes in vivo regeneration: increasing the intrinsic growth capacity of the axons (manifested by an earlier onset of robust axon outgrowth) and enabling them to overcome the effects of inhibitory factors at the lesion site. (Strictly speaking, in our in vitro studies we showed that the cyclic nucleotide treatment can overcome the inhibitory effect of myelin, and for technical reasons we did not test its ability to overcome inhibition by injury site tissue, but the fact that regeneration occurs through that tissue in vivo supports the possibility that the treatment is overcoming inhibition there as well.) Despite the profuse outgrowth of neurites into the lesion site, in this particular paradigm we did not observe growth rostral to the lesion site, as has been observed when a sciatic nerve lesion is made 1 week prior to the dorsal column lesion. Because we used only one dose of db-cAMP and one time point, it is conceivable that increasing the dose administered or changing the time window might allow the severed axons to grow not just through but also beyond the lesion site. Consistent with this possibility, the magnitude of the regeneration that we observed is comparable to that seen when the conditioning sciatic nerve lesion is made at the same time as the dorsal column lesion (Neumann and Woolf, 1999), supporting the possibility of a dose and time dependency to the effect of the manipulations. Indeed, based on the results reported by Filbin and colleagues (Qiu et al., 2002, this issue of *Neuron*), cAMP-induced growth beyond the lesion site is clearly possible and appears to be related to the dose of cAMP injected and to the time between the injection and the spinal cord injury. We have not assessed whether the cAMP treatment also affects the growth of the peripheral branch in vivo.

The parallels between the effects of db-cAMP injection into DRG and the effects of a conditioning peripheral nerve lesion are striking. Each manipulation increases the intrinsic growth capacity of sensory neurons, such that they extend axons more rapidly when placed in culture. Each manipulation also promotes an increased rate of extension of the axons on a permissive substrate and an ability to counteract the inhibitory effects of myelin. Each of these factors, we believe, contributes to our most important finding, namely that either the conditioning lesion or direct injection of db-cAMP into the DRG allows for profuse regeneration of the central branches of injured sensory axons through a spinal cord lesion site. Based on these parallels, we propose that the effects of the conditioning lesion are mediated at least partly by stimulation of cAMP levels in the sensory neurons, a possibility that needs to be directly tested. Conversely, the fact that in the saline-injected group a few fibers penetrated the lesion site (though only for

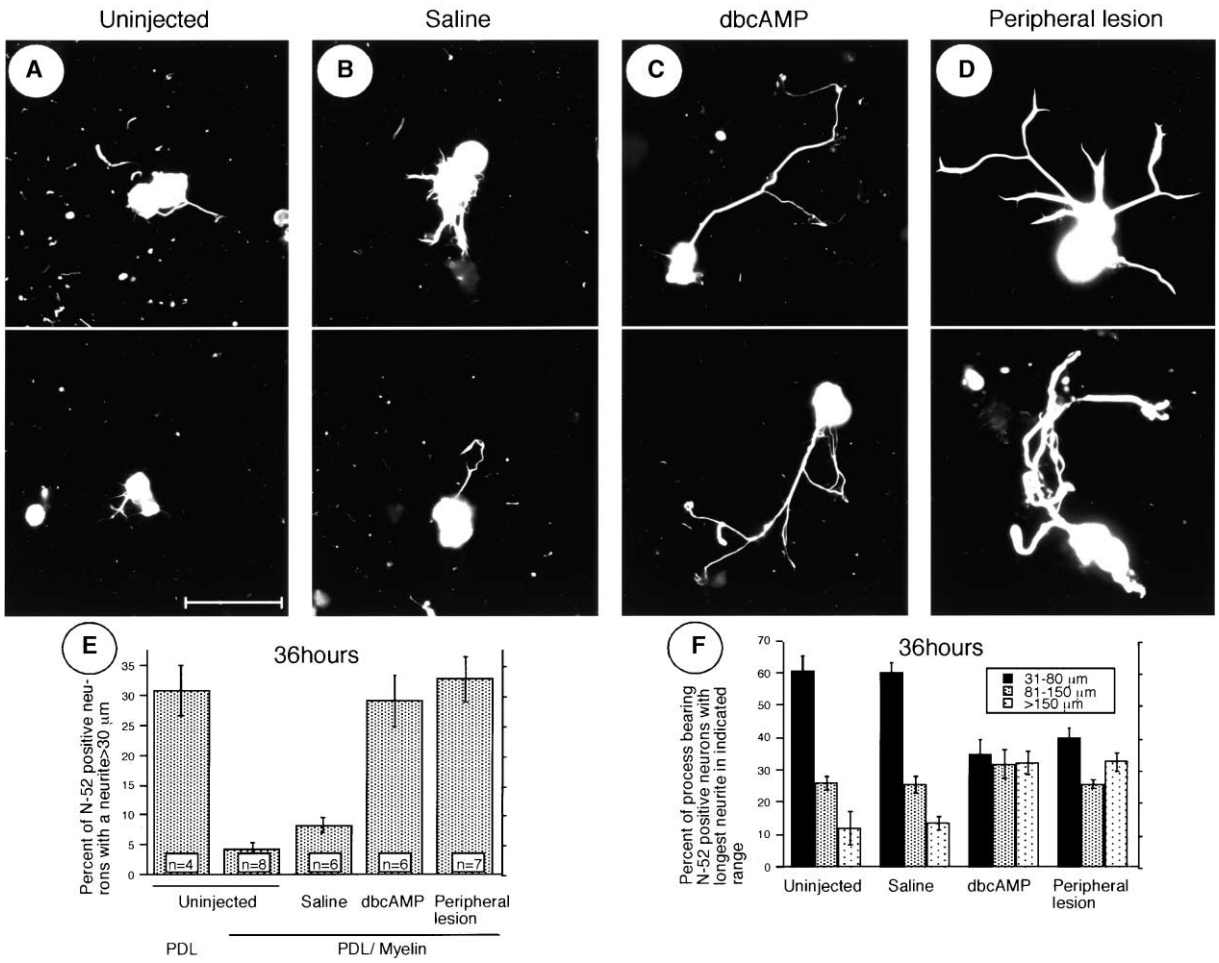

Figure 5. Injection of db-cAMP into DRGs In Vivo or a Conditioning Peripheral Nerve Lesion Enables Axons to Grow on an Inhibitory Substrate

(A–D) L4 and L5 DRG were injected with 1.5 μl of 10 mM db-cAMP or saline. 48 hr later, the DRG were removed, dissociated, and plated on myelin-coated dishes precoated with poly-D-lysine. To establish the inhibitory effect of myelin compared to a permissive substrate, dissociated neurons from noninjected (control) animals were also plated on dishes only coated with poly-D-lysine (n = 4). After 36 hr in culture, cells were fixed and stained for N-52. Whereas noninjected neurons plated on poly-D-lysine alone show robust outgrowth at this time (see [E]), neurons obtained from either noninjected (n = 8) (A) or saline-injected (n = 6) (B) DRG neurons plated on poly-D-lysine and myelin show either no or very little neurite outgrowth. In contrast, many neurons derived from db-cAMP-injected DRG (n = 6) (C) or from DRG in which a conditioning peripheral nerve lesion had been performed (n = 7) (D) show robust axonal growth.

(E) Percent neurons with N-52+ neurites (processes > 30 μm) under different conditions.

(F) Distribution of neurite lengths among neurons with neurites >30 μm under different conditions (n = 4).

Scale bar, 100 μm.

a short distance) suggests that the injection caused a slight injury of the DRG cells that might be tantamount to a slight conditioning lesion (and the response to which might even be mediated by stimulation of cAMP signaling in the cells). It should be emphasized, however, that the slight response seen in the saline-injected group is markedly different from the striking regenerative response observed in the db-cAMP-treated group, in which a substantial number of fibers regenerated extensively into the lesion site in each animal analyzed.

Although we have interpreted our results as if they are due to stimulation of cAMP signaling in the neurons, we cannot, of course, exclude that part of the effect of the in vivo injection could have resulted from effects of the db-cAMP on nonneuronal cells in the DRG, which then somehow indirectly affected the neurons. However, there is every reason to believe that the effect is mediated at least partly, and perhaps entirely, by effects on the neurons themselves. We suggest this because of the previous demonstration that cAMP stimulation in the neurons in vitro (either at the level of individual growth cones or in low-density cultures) can help overcome an inhibitory substrate (Song et al., 1998; Cai et al., 1999, 2001; this study), and because of the evidence that an intrinsic decrease in cAMP levels in a variety of neurons underlies their loss of regenerative ability (Cai et al., 2001). Future studies in which cAMP signaling is specifically modulated in neurons in vivo will help address the contributions of effects on the neurons (cell-autonomous) and effects on nonneuronal cells (nonautonomous), although it will always be difficult to exclude the possibility that even the response to a cell-autonomous

manipulation may yet involve some communication between the altered neuron and its neighboring nonneuronal cells.

In summary, our results provide evidence that manipulation of a specific signal transduction pathway in neurons can promote significant axonal regeneration in the CNS environment in vivo. Although we have focused on the cAMP signaling pathway, it will be important to assess whether additional manipulations of signaling pathways help regeneration further, including stimulation of cGMP signaling, which can help overcome the effects of other repulsive factors such as Slits (Ba-Charvet et al., 2001) and Semaphorins (Song et al., 1998), some of which are known to be expressed at CNS lesion sites (Pasterkamp et al., 1999). In addition, from the perspective of clinical application, it will, of course, be important to determine whether regeneration can be enhanced by stimulating cAMP signaling after spinal cord injury. Nevertheless, our results suggest that stimulating cyclic nucleotide signaling pathways to enhance the growth capacity of injured CNS axons and to overcome the barriers associated with sites of injury may be a useful component of a therapy to enhance axonal regeneration in the CNS. Such an approach will likely be part of a multipronged therapy that includes delivery of growth factors (Blesch et al., 1999; Bradbury et al., 1999; Jakeman et al., 1998; Ramer et al., 2000; Schnell et al., 1994), of blockers of axonal growth inhibitors (Bregman et al., 1995) and/or of glial scar formation (Kalderon et al., 1990; Logan et al., 1994).

Experimental Procedures

Cell Culture

Adult male Sprague Dawley rats (250–350 g) were killed, the L4 and L5 DRGs were dissected and transferred into Hank's buffered salt solution (HBSS) containing 10 mM HEPES. Enzymatic digestion of the DRGs was initiated by placing them into 3% Collagenase type I (Worthington, New Jersey) diluted in HBSS containing 10 mM HEPES and incubating them at 37°C. After 1.5 hr, the DRGs were incubated in saline A containing 0.25% trypsin and 0.02% versene for an additional 25 min at 37°C. Enzymatic digestion was stopped by addition of Leibovitz-15 medium containing 5% horse serum. After sedimentation of the cell clumps, the medium was removed and cells were resuspended in F-12 medium (Gibco) supplemented with N3, 40 mM sucrose, and 0.5% fetal calf serum. Cells were dissociated by mechanical trituration through an unpolished glass pipette (ten times), followed by trituration through a fire-polished pipette (ten times). Cells (2000–3000 per well) were plated into one well per eight-well plate (Labtek) coated with either 1 mg/ml poly-D-lysine (Sigma) diluted in borate buffer solution (DeHoop et al., 1998) alone or poly-D-lysine and, subsequently, 0.5–1 μg myelin per well. Myelin was prepared as described (Colman et al., 1982). Cells were kept in a humidified incubator at 5% CO_2 at 37°C.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde containing 4% sucrose in PBS at room temperature for 15 min. Cells were then quenched with 50 mM ammonium chloride, extracted with 0.1% Triton X-100, and blocked with 5% bovine serum albumin (BSA), incubated with N-52 antibody (Sigma) diluted in 0.03% Triton X-100 and then with a goat anti-mouse antibody conjugated to Cy3 (Jackson) diluted in 3% BSA and 1% goat serum.

Data Acquisition

All the cells per well containing neurites were counted blind to treatment condition using a Zeiss Axiophot and a 20× Plan-Neofluor objective. The number of cells with neurites was obtained by counting all neurons derived from each rat (>1000 cells/rat). For length distribution, approximately 200 cells per animal were randomly selected and their images captured using a Spot 2-RT digital camera (Diagnostic Instruments, Michigan). Axon length was measured using calibrated Spot software.

DRGs Microinjection

Under halothane anesthesia, a small dorsolateral laminectomy was performed in order to expose the left L4-L5 DRGs. Using a recording glass pipette, the DRGs were gently punctured. 1.5 μl of saline or db-cAMP (10 mM) were slowly injected into the DRGs using a 5 μl hamilton syringe with a 30G needle. The wound was then closed, and the rats were allowed to recover. For the in vivo experiments, 48 hr later these rats underwent dorsal column lesions as described above. For the culture experiments, 48 hr later the rats were sacrificed and the DRGs placed in culture conditions as described above.

Dorsal column lesion, peripheral nerve lesion, in vivo axonal tracing, and tissue processing were all performed exactly as described (Neumann and Woolf, 1999).

Quantification of the Regenerative Response

Camera lucida reconstructions of horizontal sections of the lesion site from each animal were used to measure the length of the regenerative response of the sciatic dorsal column axons into the lesion site (Figure 2).

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