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Regeneration of Dorsal Column Fibers into and beyond the Lesion Site following Adult Spinal Cord Injury

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Summary

Regeneration is abortive following adult mammalian CNS injury. We have investigated whether increasing the intrinsic growth state of primary sensory neurons by a conditioning peripheral nerve lesion increases regrowth of their central axons. After dorsal column lesions, all fibers stop at the injury site. Animals with a peripheral axotomy concomitant with the central lesion show axonal growth into the lesion but not into the spinal cord above the lesion. A preconditioning lesion 1 or 2 weeks prior to the dorsal column injury results in growth into the spinal cord above the lesion. In vitro, the growth capacity of DRG neurite is also increased following preconditioning lesions. The intrinsic growth state of injured neurons is, therefore, a key determinant for central regeneration.

Introduction

Primary sensory neurons with cell bodies in the dorsal root ganglion (DRG) possess two branches stemming from a unipolar axon: a peripheral axon that regenerates when injured and a central axon that enters the CNS and does not regenerate on injury (Ramon y Cajal, 1928; Schnell and Schwab, 1990; Xu et al., 1995). The local environments of the peripheral and central axon are strikingly different, most notably in the presence of Schwann cells in the former and oligodendrocytes and astrocytes in the latter, as well as by the composition and organization of the extracellular matrix (Ramon y Cajal, 1928; Bunge et al., 1986; Kleitman et al., 1988). Such differences have been thought to contribute to the failure of axons to grow in the adult mammalian central nervous system (Schwab and Bartholdi, 1996). Providing a permissive growth environment by means of Schwann cell cultures (Li and Raisman, 1994; Xu et al., 1995), olfactory glia (Li et al., 1997), or fetal grafts (Bregman et al., 1989; Iwashita et al., 1994) does increase growth of injured CNS axons. Neutralizing growth cone inhibiting oligodendrocyte myelin proteins also enables axons to circumvent spinal injury (Schnell and Schwab, 1990) and reestablish function (Schnell et al., 1994; Bregman et al., 1995). In addition, transplantation of peripheral nerve grafts results in regeneration of central axons over long distances within the peripheral environment but with very limited regrowth back into the CNS environment (Richardson et al., 1982; Richardson and Issa, 1984; Benfey et al., 1985; Vidal Sanz et al., 1987; David and Aguayo, 1989; Campbell et al., 1992; Zhang et al., 1995; Davies et al., 1997).

Absence of a permissive substrate for growth is not, however, the only factor responsible for the failure of central axons to regenerate (Schwab and Bartholdi, 1996). Three other issues are important: survival of the injured neurons (Coggeshall et al., 1997), the capacity of adult neurons to recapitulate those developmental processes that enable neurite formation and outgrowth, i.e., their intrinsic growth state (Chong et al., 1996), and, finally, the formation of impenetrable barriers at the lesion site (Jakeman and Reier, 1991).

Those DRG neurons whose axons ascend in the dorsal columns provide a useful model system to examine central regeneration. First, these cells do not die after peripheral or central axonal injury (Coggeshall et al., 1997). Second, injuring the peripheral but not the central axons of these cells changes their intrinsic growth state (Schreyer and Skene, 1993; Chong et al., 1994). This can be seen in vitro where an increased growth state can be detected after nerve lesions in dissociated cells or explant DRG cultures (Hu-Tsai et al., 1994; Edstrom et al., 1996; Smith and Skene, 1997). In vivo, inducing peripheral nerve-conditioning lesions at the same time as implanting peripheral nerve grafts immediately adjacent to injured central axons of primary sensory neurons substantially increases the extent and rate of regeneration into the graft (Richardson and Issa, 1984; Richardson and Verge, 1986; Oudega et al., 1994; Chong et al, 1996). Cultured DRG neurons, which are necessarily axotomized in the process of dissociation, grow in CNS white matter in vivo (Davies et al., 1994, 1997), a region normally considered growth repellent (Ramon y Cajal, 1928; Schwab and Bartholdi, 1996). The dorsal columns are also a convenient site to examine the glial reaction at the lesion, which has been thought to be a major factor preventing regeneration in the CNS (Jakeman and Reier, 1991).

To investigate whether a major impediment for central regeneration is the low intrinsic growth capacity of injured adult neurons, we have investigated whether increasing the growth state of sensory neurons by a conditioning peripheral nerve lesion at different times relative to a dorsal column lesion enhances the regeneration of the central axons in the dorsal columns to grow directly into and beyond the lesion site in the spinal cord, sites supposedly nonpermissive for growth.

Results

Sciatic Dorsal Column Fibers

Injection of the tracer B-HRP into the sciatic nerve of naive animals labels the cell bodies of A fibers in the nerve in the L4, L5, and L6 dorsal root ganglia (Woolf et al., 1995) and their central axons, including segmental collateral branches in the dorsal horn (LaMotte et al.,

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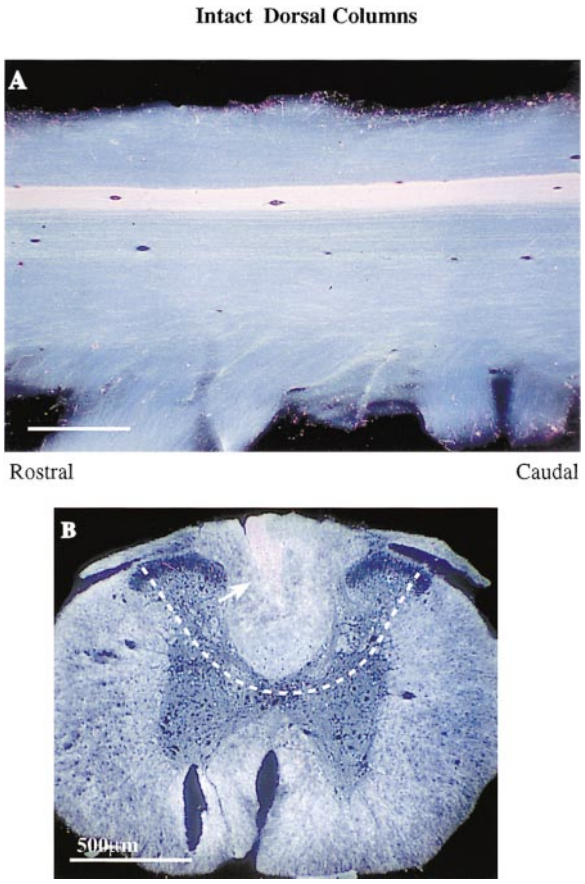

Figure 1. Dark-Field Photomicrographs of the Sciatic Nerve Component of the Dorsal Column Labeled with B-HRP

(A) Horizontal sections through the thoracic spinal cord, showing the pink labeled dorsal column fibers. (B) Transverse section through the thoracic spinal cord showing location of the sciatic dorsal column fibers (arrow). The dotted line indicates the typical bilateral extent of the lesion in experimental animals. Scale bars, 500 μm.

1991; Woolf et al., 1995) and ascending axons of cutaneous and proprioceptive fibers in the dorsal columns (Figure 1). The sciatic component of the dorsal column tract comprises a tight bundle of fibers (Figure 1A), which ascends all the way up the spinal cord in the gracile dorsal funiculus, ipsilateral to the labeled nerve, to the gracile dorsal column nucleus. In addition to segmental collaterals in the L4, 5, and 6 segments of the lumbar spinal cord, collaterals innervating Clarkes nucleus branch off the stem axons in the upper lumbar and lower thoracic segments. Rostral to T8, the tract in the dorsal columns is, though, an unbranched tightly bundled collection of fibers located in a topographically restricted location (Figure 1B).

Dorsal Column Lesions

Lesions were performed by physically sectioning the entire area between the dorsal root entry zones on either side of the T6–7 thoracic spinal cord with an ophthalmic tungsten steel microscissors, producing a physical bilateral dorsal column lesion (see Figure 1B). In the dorsoventral dimension, the lesion extended down to the central canal completely transecting the entire dorsal column. The lesion site could be seen in up to 10 consecutive 50 μm horizontal sections and was associated with a superficial swollen cap of scar tissue bridging the lesion and, in many cases, large CSF-filled cysts that extended proximal and distal to the lesion. Rostro-caudally, the lesion typically extended about 2.5 mm. Rostral to the injury site, the dorsal columns were filled with demyelinated axonal debris.

Failure of Axonal Growth after Dorsal Column Lesions

Six to eight weeks after a dorsal column lesion, no regenerative response of the ascending dorsal column sciatic axons was ever observed—all axons stopped quite dead at the injury site (n = 6) (Figure 2A). Short sparse axonal sprouts were found proximal to the lesion in a few cases, particularly in relation to cysts (Figure 2B), but no growth into or beyond the lesion was detected in any of these animals (Figures 2A and 2B). The injured axons occasionally generated large retraction-bulb-like swellings (Figure 2B). Two animals were investigated 1 year following the dorsal column injuries, and in these, no difference from the 2 month survival time could be detected; all labeled fibers still stopped at the lesion site (Figure 2C). The density of the fiber tract label in these cases was not diminished, indicating that minimal retraction or atrophy of the nonregenerating fibers had occurred.

Regeneration into the Lesion Site after Dorsal Column Section with a Concomitant Sciatic Nerve-Conditioning Lesion

When the dorsal column lesions were accompanied by a simultaneous lesion of the sciatic nerve, the results obtained were dramatically different from those obtained when the dorsal column lesion was performed by itself. In all these cases (n = 10), a massive outgrowth of neurites from the transected dorsal column fibers occurred, proximal to, into, and across the lesion site (Figure 3). Proximal to the lesion site, considerable sprouting occurred, producing a network of mediolaterally oriented fibers growing laterally from the stem axon into the white matter immediately proximal to the lesion. In every animal examined, an invasion of the lesion site was also found, with massive growth of many of the transected fibers directly across the lesion boundary into the substance of the lesion, along the walls of cysts within the lesion, into dorsal roots incorporated into the lesion, and on the surface of the cord beyond the lesion (Figures 3A and 3B). The fibers on the surface of the spinal cord and in the dorsal roots grew rostral to the lesion for up to 6 mm. In contrast, although relatively massive growth was found in all cases within the lesion site, growth into the denervated dorsal columns or into the gray matter within the spinal cord rostral to the lesion was not observed in any of these animals. In many cases, the growth in the lesion was directed in the same plane as the lesion, i.e., at right angles to the normal trajectory of the fibers enabling the fibers to reach the cord surface (Figure 3A). The lesion actually seemed to act not as a barrier, but as a guidance cue to the regenerating fibers. Another prominent feature in all animals was particularly extensive growth on the most superficial portion of the lesion, on its surface, forming

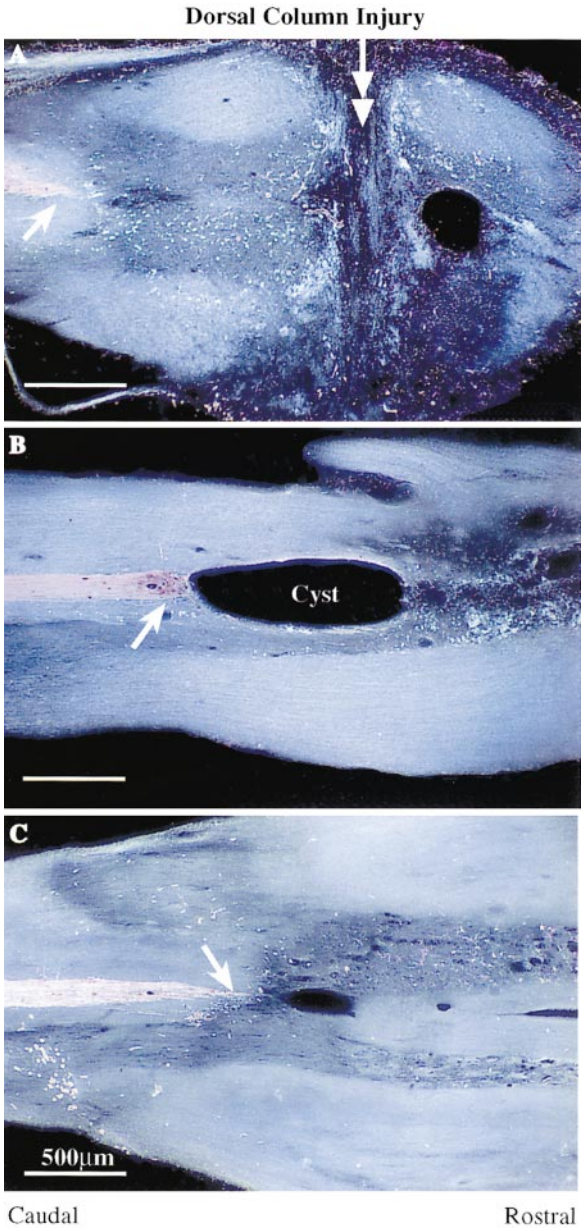

Figure 2. Dark-Field Photomicrographs of Horizontal Sections of the Lesion Site Showing Failure of Regeneration of Injured Dorsal Column Fibers

(A) Absence of regenerative growth into the lesion (double arrow); fibers end blind with minimal sprouting proximal to the lesion site (arrow). (B) Sciatic dorsal column fibers stop at a cyst proximal to the lesion. A few fibers sprout around the cyst but not beyond it into the injury site. (C) Failure of regeneration 1 year after a dorsal column lesion.

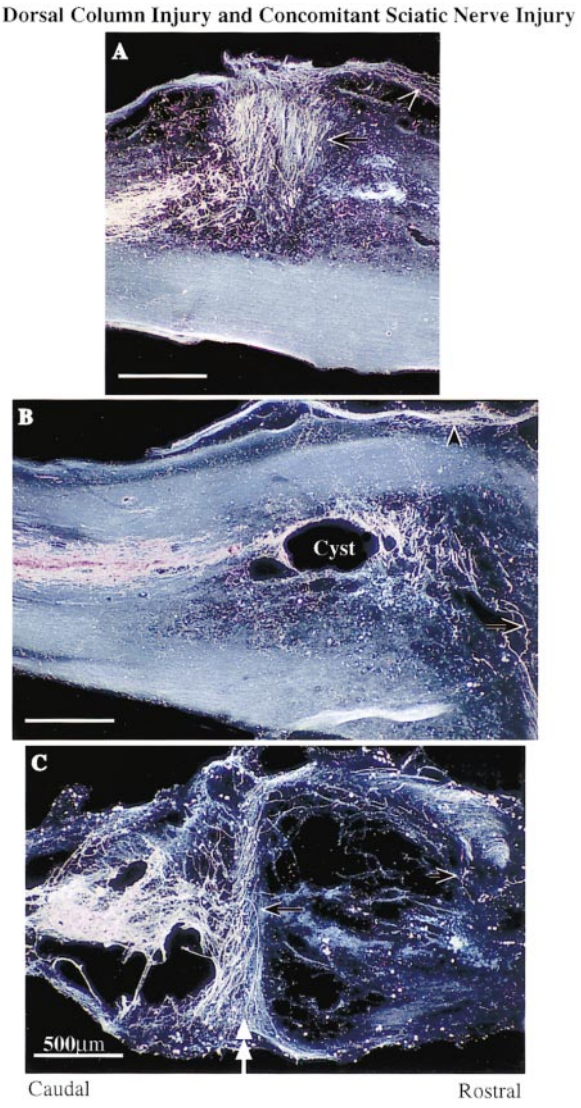

Figure 3. The Effect of a Concomitant Sciatic Nerve-Conditioning Lesion on the Regeneration of Injured Dorsal Column Fibers into the Lesion Site in the Spinal Cord

(A) A horizontal section showing many sciatic dorsal column fibers entering the injury site and growing extensively in the same mediolateral plane as the lesion (black and white arrow), on the surface of the cord and into an injured dorsal root (black and white arrowhead).
(B) A horizontal section from another animal showing many fibers grow into the lesion site around a cyst, deep into the lesion (black and white arrow) and on the surface of the cord (black and white arrowhead).
(C) Section taken from the most superficial part of the lesioned spinal cord in a third animal, showing extensive disorganized growth along the plane of the lesion (double arrow) with some fibers reaching the most rostral edge of the lesion (right black and white arrow) but not growing beyond it.

a fan-like outgrowth in the horizontal plane with a loss of the fasciculation typical of dorsal column fibers (Figure 3C).

Regeneration beyond the Lesion Site with Preconditioning Sciatic Nerve Lesions

To examine whether the timing of the conditioning lesion was critical for the regenerative response, one group of rats had a sciatic nerve lesion performed 1 week before the dorsal column lesion ($n = 8$), another had the conditioning lesion 2 weeks before the spinal cord lesion ($n = 8$), and a third group, 2 weeks after the dorsal column lesion ($n = 6$).

In the animals in which the preconditioning lesion was performed 1 week before the dorsal column injury, two

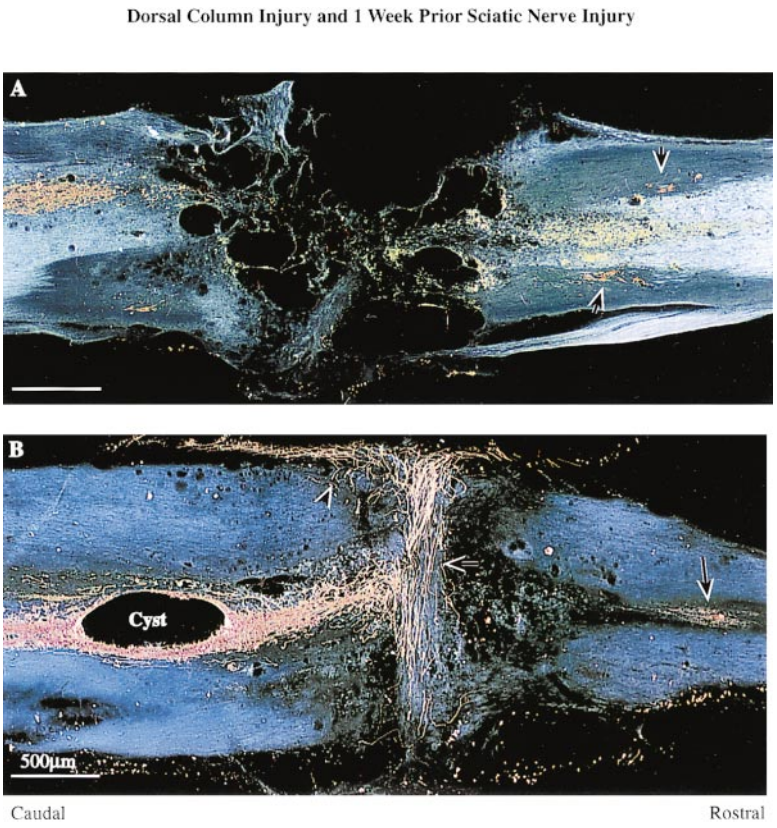

Figure 4. A Sciatic Nerve-Conditioning Lesion 1 Week prior to a Dorsal Column Lesion Results in Regeneration of the Injured Dorsal Column Fibers beyond the Lesion Site in the Thoracic Spinal Cord

(A) In this case, the conditioning lesion resulted in injured fibers regenerating beneath and then beyond the lesion. Axons that have grown beyond the lesion can be seen bilaterally in the gray matter (black and white arrow). (B) This animal shows massive growth both along the plane of the lesion (left black and white arrow), on the surface of the cord (black and white arrowhead), and beyond the lesion into gray matter (right black and white arrow).

distinct patterns of growth manifested. One pattern, present in four of the eight animals examined, consisted of either no or much less growth into the lesion site and on the surface of the spinal cord compared with those animals that had the conditioning lesion at the same time as the dorsal column lesion. Instead, there was substantial growth from the dorsal columns ventrally into the gray matter surrounding the central canal in the midline proximal to the lesion site. This was associated with a growth of many axons both caudally down the spinal cord in the contralateral dorsal horn and rostrally underneath the lesion site along the gray matter around the central canal and then up into the ipsilateral and contralateral dorsal horns rostral to the lesion site. Above the lesion, bilaterally distributed axons could be followed for up to 4 mm beyond the rostral border of the lesion site growing toward the brainstem (Figure 4A). The regenerating fibers rostral to the lesion in these animals tended to avoid the denervated dorsal columns, remaining in the gray matter. The second pattern of growth, in the remaining 50% of the group, included invasion of the lesion site by injured fibers. This pattern was similar to that observed with concomitant conditioning lesions, except in these animals the fibers continued to grow beyond the rostral border of the lesion site into gray matter and, to a more limited extent, the white matter, and ascended up the spinal cord for several millimeters (Figure 4B).

Substantial numbers of fibers managed to traverse the lesion with this preconditioning lesion, but no evidence of terminal label in the dorsal column nuclei was found, so that it is unlikely that significant numbers of axons reached their normal target 6–8 weeks after the lesion. It was also not possible to detect whether the regenerating fibers beyond the lesion formed terminal or collateral synaptic connections with neurons in the gray matter of the dorsal horn.

Preconditioning the sciatic nerve 2 weeks before the spinal cord lesion also resulted in growth into and to a more limited extent beyond the lesion site. Four animals exhibited growth of injured fibers into the lesion site but not beyond (Figure 5A). Dorsal column axons in the other four animals regenerated beyond the lesion site in a pattern similar to that found with the 1 week preconditioning sciatic nerve lesion. In two animals, fibers grew through the lesion and beyond into the gray matter (Figure 5B) or along the border between the gray and white matter. In the other two animals, fibers circumvented the lesion site ventrally and grew rostral to the lesion site in the gray matter or at the border between the gray and white matter (Figure 5C). Conditioning the ipsilateral sciatic nerve 2 weeks after the dorsal column lesion resulted in a failure of any substantial regeneration at the injury site in all the six animals tested.

In order to examine whether the growth observed into and beyond the lesion site was specifically due to increased growth capacity of the severed axons and not a systemic effect of the peripheral nerve injury, one group of animals had concomitant dorsal column and contralateral sciatic nerve lesions (on the right side) ($n = 4$), and another had a contralateral sciatic nerve lesion 1 week prior to the dorsal column lesion ($n = 4$). In both groups, lesioning the contralateral sciatic nerve lesion produced no effect on the pattern of growth of sciatic central axons labeled on the opposite side. The result was identical to that of a dorsal column only lesion

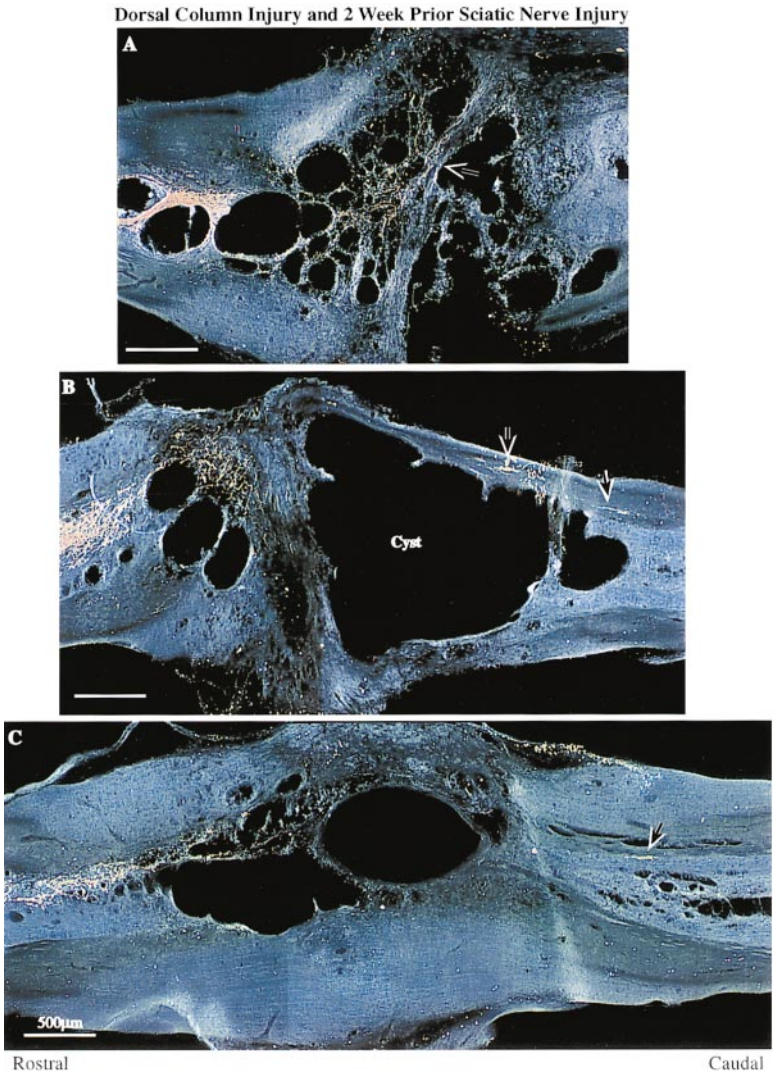

Figure 5. The Effect of a Sciatic Nerve-Conditioning Lesion 2 Weeks prior to a Dorsal Column Lesion on Regeneration of Dorsal Column Fibers in Three Different Animals

(A) Growth of injured fibers into the lesion site (black and white arrow). (B) Growth of injured fibers beyond the lesion site into the dorsal horn gray matter (black and white arrows). (C) Regenerating fibers beyond the lesion site on the border between the gray and white matter (black and white arrow).

with minimal proximal sprouting and most of the dorsal column fibers ending blind at the caudal end of the lesion.

Figure 6 illustrates the differences in the extent of axonal growth from the caudal boundary of the lesion site into the lesion (A) and beyond the rostral extent of the injury site into the denervated spinal cord growing toward the brain (B) for all the different manipulations.

Increased Neurite Outgrowth In Vitro after Preconditioning Sciatic Nerve Lesions

Changes in the intrinsic growth status of DRG neurons were studied by looking at the extent of neurite outgrowth from whole adult DRG explants cultured in the absence of NGF at 1 and 2 weeks following preconditioning peripheral nerve lesions. Neurite outgrowth in

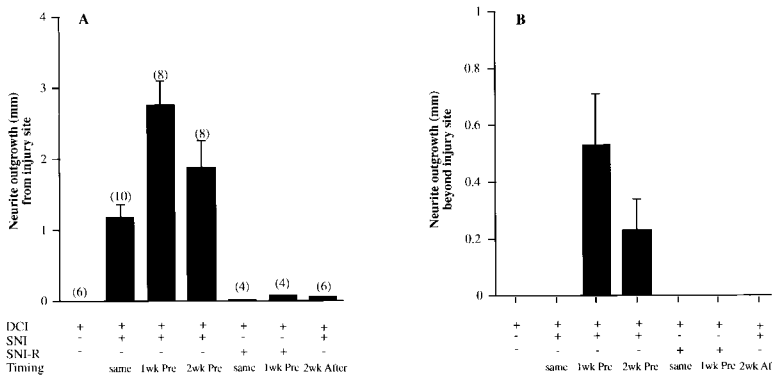

Figure 6. Extent of Regeneration of Injured Dorsal Column Fibers in Response to Sciatic Nerve Injury

(A) Regenerative growth as measured from the proximal site of the lesion. No growth was observed in the dorsal column injury (DCI) only group or in groups that underwent a contralateral right sciatic nerve injury (SNI-R). Regeneration only occurred after an ipsilateral, left sciatic nerve injury. (B) Illustrates growth beyond the injury site. Note growth beyond the injury site was obtained only in the ipsilateral preconditioning groups. n values are given in parentheses over each column.

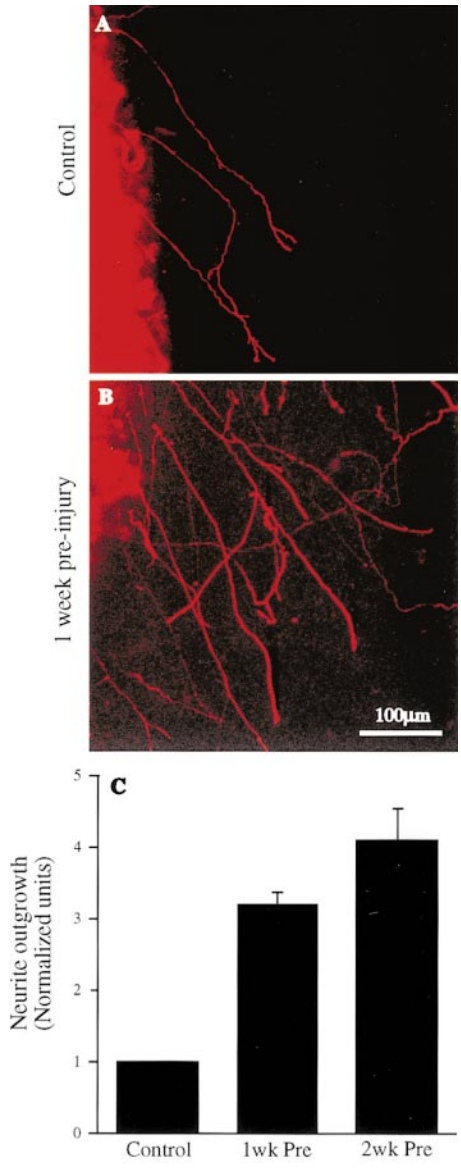

Figure 7. Confocal Photomicrographs Showing DRG Explants Labeled for Rhodamine Phalloidin from a Control Animal and from an Animal with a 1 Week prior Sciatic Nerve-Conditioning Lesion

The number of neurites growing out of the ganglion in the control explant (A) is lower than that from a ganglion after nerve preinjury (B). Note that the nerve preinjury results in an increase in the number of neurites growing out of the ganglion. (C) Neurite outgrowth, measured as total fluorescence intensity in a fixed area adjacent to the explant. Preconditioning lesions at both 1 and 2 weeks result in an increase in neurite outgrowth, compared to the control group.

DRG explants after preconditioning lesions was consistently increased compared to DRG explants from control animals prepared at the same time (Figure 7).

Discussion

Injuring the dorsal columns in the adult rat spinal cord in the absence of any conditioning lesion results in no regeneration or growth of the sectioned dorsal column fibers either proximal to or into the lesion site. These results confirm observations first made by Ramon y Cajal and repeated innumerable times (Schwab and Bartholdi, 1996) that regeneration in the CNS is normally absent or abortive and that the interface with the lesion appears to be an impenetrable barrier. For dorsal column fibers, this lack of a regenerative response is in marked contrast to the growth of their peripheral axons after a peripheral nerve lesion where successful reinnervation of peripheral targets can occur. However, we have now found that a peripheral nerve-conditioning lesion performed at the same time as the dorsal column lesion results in a massive axonal growth into the lesion site and for long distances on the surface of the cord or in Schwann cell-containing environments, such as injured dorsal roots. In addition, we find that a preconditioning peripheral nerve lesion, either 1 or 2 weeks prior to the dorsal column lesion, results in the injured fibers growing within CNS tissue above the lesion for substantial distances toward the brain, either after negotiating a way around, or through the lesioned site. Regeneration in the face of a total lesion of the dorsal columns can, therefore, take place without provision of a new permissive growth environment.

Our in vitro data suggest that improved regenerative response of the central axons is due in part to an increased growth capacity. Peripheral conditioning lesions have been shown before to augment growth of the central axons of DRG cells into peripheral nerve grafts (Richardson et al., 1982; Richardson and Verge, 1987; Ritter et al., 1991; Chong et al., 1996), and now we have shown that altering intrinsic growth status enables injured central axons to grow into and beyond a lesion site within the CNS. This growth is specifically related to changes in the injured fibers since severing the contralateral sciatic nerve resulted in an abortive regenerative response. The lack of a permissive growth environment is not by itself, therefore, an absolute impediment for regeneration, and it is possible to alter DRG cells so that they can acquire the capacity to regenerate through and beyond lesions.

Like the corticospinal tract, which Schwab and colleagues have managed to encourage to grow using an anti-myelin protein antibody (Schnell and Schwab, 1990), regenerating dorsal column fibers prefer to regenerate in the gray matter, although some growth was found in the dorsal columns both below and above the lesion. This latter finding is interesting in the light of the results of Davies et al. (1997), who found that dissociated adult DRG cells can grow in white matter in the adult brain in the absence of tissue injury. Our results differ from theirs in that there is very substantial injury and yet still some growth in the white matter.

A number of different patterns of growth were observed that depended crucially on the timing of the conditioning lesion. In the case of concomitant conditioning lesions, extensive growth directly into the lesion site occurred in all cases. One explanation for such growth might be that Schwann cells infiltrated the lesion during its production. This is highly unlikely, however, because this would take place with or without a conditioning lesion, and no regenerative response was ever detected in the dorsal column lesion only or the contralateral nerve injury group. In addition, we have found no indication of Schwann cell infiltration of the lesion site,

comparing S100 and GFAP immunostaining (data not shown). No growth into the denervated dorsal columns or the gray matter rostral to the lesion was ever found in animals with concomitant nerve lesions. Growth into injured dorsal roots and along the surface of the spinal cord for several millimeters beyond the lesion occurred, however, in many cases. The lack of growth into the denervated dorsal columns may be the consequence either of the formation of an impenetrable substrate barrier at the interface between the lesion site and the denervated tissue due to the inflammatory and glial response to the injury by substrate molecules such as chondroitin sulfate (Pindzola et al., 1993), or to the presence of growth-repellent molecules in the denervated tissue, such as contained in oligodendrocyte myelin (Schwab and Bartholdi, 1996). A key question then is why did a preconditioning lesion permit growth beyond the lesion?

Growth beyond the lesion site toward the brain in animals with preconditioning lesions involved either a circumnavigation by the injured fibers of the lesion via the gray matter or a direct growth into and then beyond the lesion. Growth in the gray matter proximal to the lesion occurred in some cases with fibers growing down the contralateral dorsal horn, an aberrant site and in a direction opposite to normal. What factor determined in an individual animal whether the fibers grew into or around the lesion is not known but may relate to the extent of the physical gap created by the lesion, how quickly the lesion was filled in by cellular infiltrate and extracellular matrix, and the extent of the inflammatory response. If the gap was too extensive to penetrate at the time the fibers started to grow, they may have grown down the only route available to them—ventrally into the gray matter—and then managed to bypass the lesion site.

A conditioning sciatic nerve section was produced after the dorsal column injury was performed to see if it could encourage growth into what would now be an established glial scar. No growth was found in these animals indicating that an established lesion is likely to be a barrier. The preconditioning lesions were performed to see whether sciatic afferents preprimed for growth before the dorsal column lesion produced an even greater growth than a conditioning lesion performed at the same time as the dorsal column section, since it will take a finite amount of time for the injured peripheral fibers to send a retrograde signal from the site of peripheral injury to the DRG, alter transcription, and transport growth-related proteins to the tips of the injured dorsal column fibers at the site of the central lesion. It might be expected that the optimal effect of a conditioning lesion would coincide with the presence of such growth-associated proteins in the axons at the time of the lesion. Having all the molecular machinery already available, as a result of a preconditioning lesion, may enable centrally injured fibers to start to regenerate immediately after a lesion, before inhibitory signals at the lesion site are established. One candidate growth-associated gene is GAP-43, which is upregulated by a peripheral and not a central axotomy in sensory neurons and transported up dorsal column axons (Schreyer and Skene, 1991; Chong et al., 1994). However, in preliminary experiments in transgenic mice overexpressing GAP-43 in DRG and other neurons via a thy1 promotor (Aigner et al., 1995), injured dorsal column lesions failed to regenerate in the way seen with peripheral nerve-conditioning lesions (Neumann and Woolf, unpublished data).

The in vitro explant study shows that preconditioning peripheral nerve lesions increase the capacity of DRG neurons to extend neurites. These results strongly suggest that the increased capacity of the central branches of primary sensory neurons to regenerate in vivo beyond the lesion site is due, at least in part, to an enhanced intrinsic growth state. Increased growth capacity following peripheral axotomy has been observed using dissociated cells (Hu-Tsai et al., 1994; Smith and Skene, 1997) and in DRG explants from young mice (Edstrom et al., 1996). By studying axonal growth in preconditioned DRG explants cultured in the absence of NGF, we were able to detect conditioned growth of non-NGF-responsive fibers, many of which, the TrkB- and C-expressing, will be present in the dorsal columns (Wright and Snider, 1995).

In addition to an increase in growth capacity produced by the peripheral preconditioning lesion, there may also be a reduced detection by the central axons of growth-repellent substrate or soluble inhibitory cues present at the lesion site. This could result from a downregulation of receptors for such molecules in the sensory neurons following the nerve lesion. Nevertheless, the neurites are still likely to be subject to inhibitory influences, and it is the balance between intrinsic growth capacity and inhibitory cues that is likely to determine the extent of regeneration.

Conclusions

The intrinsic growth capacity of DRG neurons is an important factor that contributes to the ability of the central branches of these neurons to regenerate after injury. Injured central axons can grow extensively into the site of injury when primed for growth at the same time as the central lesion. Moreover, regeneration beyond a complete lesion of a central fiber tract is possible in the CNS, when neurons are preprimed for growth. Both findings offer encouraging scope for promoting central regeneration in the adult mammalian nervous system.

Experimental Procedures

Dorsal Column Lesions

Bilateral lesions of the dorsal columns (DC) were performed in adult Sprague-Dawley male rats (250–350 g). Under halothane anesthesia (induction 4%, maintenance 2.5%), a small dorsal laminectomy was performed at the level of the thoracic vertebra to expose the spinal cord. After the dura mater was opened, the dorsal columns were transected under direct vision at T6-T7 using a tungsten steel microscissors. The dorsal columns were cut from one dorsal root entry zone to the other down to the central canal. The wound was closed and the rats allowed to recover for 6 to 8 weeks ($n = 6$) or a year ($n = 2$). Those few animals with incomplete dorsal column lesions were excluded from the study as were the small number where the lesion was sufficiently extensive that no contact between the rostral and caudal margins developed.

Peripheral Conditioning Lesions

Forty rats underwent a dorsal column lesion, as above, and a left sciatic nerve transection. The left sciatic nerve was exposed at mid-thigh level and a ligature was firmly tightened around the nerve distal to its emergence from the greater sciatic notch. The nerve was transected distal to the ligature and the wound closed. In ten rats, the sciatic lesion was performed at the same time as the dorsal

column lesion, in eight it was performed 1 week before the spinal lesion, in eight, 2 weeks before the dorsal column lesion, and in six, 2 weeks after the dorsal column lesion. Peripheral nerve lesions on the right-hand side were performed in eight animals.

Anterograde Neuronal Tracing

Six to eight weeks after the dorsal column lesions the rats were again anesthetized with halothane and the left sciatic nerve exposed. The needle of a 10 μl Hamilton syringe was inserted through an epineurial incision and 1.5 μl of choleragenoid conjugated to horseradish peroxidase (B-HRP) solution injected (List, 1% dissolved in distilled water).

Tissue Processing

Five days after the B-HRP injection, the animals were terminally anesthetized with an intraperitoneal overdose of sodium pentobarbitol and perfused with 200 ml saline (room temperature) followed by 500 ml of 1% paraformaldehyde and 1.25% gluteraldehyde in 0.1 M phosphate buffer. The lower brainstem (for the dorsal column nuclei), the thoracic spinal cord (including a segment below the lesion site and several segments above it), and lumbar 4–5 segments were removed. The thoracic spinal cord was cut horizontally into serial 50 μm frozen sections and stored in sequential wells in 0.1 M phosphate buffer. The brainstem and the lumbar blocks were cut transversely. The sections were processed for peroxidase activity using tetramethylbenzidine (TMB; Sigma) as a substrate and sodium nitroferriyanide as stabilizing agent (Mesulam, 1978). Sections were mounted on gelatinized slides and coverslipped. All sections were analyzed by an observer blinded to the procedures.

Quantification of the Regenerative Response of Sciatic Dorsal Column Fibers

The regenerative capacity of sciatic dorsal column fibers in the different experimental groups was quantified. Camera lucida reconstructions of longitudinal sections of the lesion site from each animal were used to measure the length of regenerating injured dorsal column fibers into and beyond the lesion site.

Explant Preparation and Staining

Lumbar L_4-L_5 DRGs were removed from 10- to 12-week-old adult rats. Whole DRGs were plated into two chamber slides. Each explant was placed on growth factor reduced MATRIGEL (Collaborative Biomedical Products), diluted 1:3 in RPMI. The cultures were maintained in a 37°C, 5% CO_2 humidified incubator for 48 hr. Neurite growth was visualized with phalloidin conjugated to rhodamine (Molecular Probes).

Neurite Outgrowth Quantification—DRG Explants

Photomicrographs of DRG explants stained for rhodamine phalloidin were scanned and analyzed using the NIH image analysis program. After subtracting the background staining from each photomicrograph, the total number of neurites in a fixed area extending from the edge of the DRG was calculated by measuring the fluorescence intensity for each explant. Average fluorescence intensity was then calculated for each animal. In order to correct for the variability between different experiments, results were normalized by dividing the calculated fluorescence intensity for each animal by the average intensity of the control group cultured at the same time ($n = 8$ for each experimental group).

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