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Growth Factors and Combinatorial Therapies for CNS Regeneration

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Abstract

There has been remarkable progress in the last 20 years in understanding mechanisms that underlie the success of axonal regeneration in the peripheral nervous system, and the failure of axonal regeneration in the central nervous system. Following the identification of these underlying mechanisms, several distinct therapeutic approaches have been tested in *in vivo* models of spinal cord injury to enhance central axonal structural plasticity, including the therapeutic administration of neurotrophic factors. While several tested mechanisms apparently enhance axonal growth, more recent, properly controlled studies indicate that experimental approaches to combine therapies that target distinct neural mechanisms achieve greater axonal growth than therapies applied in isolation. The search for combination therapies that optimize axonal growth after SCI continues.

INTRODUCTION

The adult mammalian spinal cord fails to regenerate, whereas the crushed peripheral nerve often successfully regenerates. Several mechanisms have been identified that contribute to the success of peripheral regeneration, including the following: 1) Nervous system growth factors, or neurotrophic factors, are secreted in appropriate physical and temporal gradients to support axonal regeneration after peripheral nerve injury (Terenghi, 1999; Boyd and Gordon, 2003). Schwann cells produce many of these growth factors in the local injured and regenerating milieu (Bhatheja and Field, 2006). 2) Physical bridges form at sites of peripheral nerve injury that “fill-in” the lesion cavity, establishing a permissive physical matrix to support growth (Chernousov and Carey, 2000; Dubovy, 2004). This matrix includes collagen, fibronectin, laminin, Schwann cells and fibroblasts. 3) A set of genes is activated in the nucleus of the damaged peripheral neuron that supports axonal regeneration, including GPA-43, CAP-23, ß-tubulin, and others (Kury et al., 2001; Navarro et al., 2007). 4) Inhibitors to axonal regeneration have not been detected in the injured peripheral nerve to the extent that they are present in the CNS. In the injured central nervous system (CNS), these supportive responses to injury do not occur (Schwab et al., 2006). Further, molecules of two classes have been identified that actively suppress axonal sprouting and regeneration in the CNS: molecules associated with central myelin, including nogo (Buchli and Schwab, 2005), myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), semaphorins, and netrin, and molecules present

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in the extracellular matrix (ECM), particularly the chondroitin sulfate proteoglycan (CSPG) molecule NG2 (Jones et al., 2002; Silver and Miller, 2004; Fawcett, 2006).

Experimental efforts to enhance regeneration of the spinal cord have individually targeted many of the mechanisms listed in the preceding paragraph. These experimental approaches include placement of molecular, cellular or "synthetic" bridges in the lesion cavity (Lakatos and Franklin, 2002; Novikova et al., 2003); stimulation of the injured spinal cord with growth factors (Tuszynski, 2002); "conditioning" of neurons to activate intrinsic genetic programs and proteins related to an active growth state (Neumann et al., 1999, 2002; Qiu et al., 2002; Rossi et al., 2007); and efforts to neutralize myelin- or ECM-related inhibitors (Silver and Miller, 2004; Fawcett, 2006). In many cases, experiments combined two of these approaches by placing cellular transplants in a lesion cavity while simultaneously, for example, administering neurotrophic factors (Lu et al., 2004) or raising cAMP levels (Nikulina et al., 2004; Pearse et al., 2004). Many of these studies reported enhancement of axonal growth and, in some cases, incremental improvement in functional outcome. However, no properly conducted and controlled study to date has demonstrated highly extensive structural or functional recovery after SCI, and in no case has convincing improvement of plasticity and regeneration been demonstrated in a larger animal model.

Indeed, the challenges presented in attempting to achieve recovery of function after spinal cord injury are substantial. The adult human spinal cord contains in excess of 3 million axons projecting rostrally and caudally. The normal patterning of axonal projections during development is established by a detailed and exquisitely orchestrated set of mechanisms that occur both intrinsically within the neuron, and extrinsically in the environment surrounding the growing axon. Within the neuron, genes are sequentially activated that begin the process of axonal elongation, cytoskeletal stabilization, and receptor expression on the tips of growth cones that sense substrates and diffusible signals in the extracellular environment. In turn, the environment influences the extending axon with diffusible molecules such as netrins, semaphorins, growth factors, and extracellular matrix molecules that contribute to attraction or repelling of the growing axon. This array of intrinsic and extrinsic processes that control axon growth occurs in a precisely timed set of events; perturbation of the timing or single components of axonal elongation in the developing nervous system can lead to mistargeting, axonal withdrawal or neuron death.

Clearly, axonal elongation during development depends on "combinations" of events. Both the developing axon and its environment utilize a number of disparate mechanisms to cooperate in an orchestrated series of events that result in growth that is remarkably rapid, accurate and precise. If substantial axonal growth is to be achieved in the injured adult CNS, it seems reasonable to expect that multiple mechanisms will also need to be addressed. The manipulation of any single or pair of factors may be sufficient to detect axonal growth in a rodent model of SCI; however, the probability of achieving growth of sufficient numbers of axons, and over minimal distances of several centimeters in the larger primate spinal cord, will likely require convergence of treatments targeting multiple cellular and extracellular mechanisms. Simple, one-therapy solutions seem unlikely to address this complex problem.

Effects of Growth Factors and Bridges

Several years ago we and others examined the ability of injured adult spinal cord axons to respond to growth factors (Schnell et al., 1994; Tuszynski et al., 1994, 1996; Xu et al., 1995; Bregman et al., 1997; Grill et al., 1997; Kobayashi et al., 1997; Ye and Houle, 1997; Jackeman et al., 1998; Blesch et al., 1999; Bradbury et al., 1999; Liu et al., 1999; Lu et al., 2001; Ruitenberg et al., 2003). Our group introduced nerve growth factor (NGF) into the intact and partially lesioned spinal cord, using techniques of gene delivery. Suspensions of autologous fibroblasts genetically modified to secrete NGF were injected into either the central gray matter

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of the non-lesioned thoracic spinal cord, or were embedded into collagen matrices that were then injected into dorsally hemisected spinal cord lesion cavities of adult rats (Tuszynski et al., 1994, 1996;). We found similar patterns of axonal responsiveness to NGF in both the non-lesioned and lesioned spinal cord: dorsal root ganglion (DRG) nociceptive axons and cerulospinal axons densely penetrated NGF-secreting cell grafts placed in the adult spinal cord (Fig. 1). This finding established the fact that adult axons retain sensitivity to growth factors, that patterns of growth factor sensitivity after injury in adulthood recapitulate developmental patterns of growth factor sensitivity, and that axons grow in rather large numbers when presented with a permissive growth environment containing growth factors. In this set of studies, a “bridge” to support axonal penetration was provided by the cellular graft in the lesion cavity. These results generally supported much earlier findings of Aguayo and colleagues indicating that central axons can extend for essentially unlimited distances in the permissive environment of the peripheral nerve “bridge” (Richardson et al., 1982). Indeed, we now know that peripheral nerve bridges contain substantial quantities of NGF, BDNF, neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF) and glial cell line-derived neurotrophic factor (GDNF) secreted by Schwann cells, which likely stimulate axon growth.

Over subsequent years, a number of groups proceeded to define the spectrum of axonal sensitivity to various growth factors in the injured spinal cord (Table 1). Of note, BDNF supports the growth of a number of neuronal populations that modify the activity of spinal cord motor neurons, including cerulospinal, rubrospinal, raphaepsinal and reticulospinal axons (Kobayashi et al., 1997;Ye and Houle, 1997; Jackeman et al., 1998; Menei et al., 1998;Liu et al., 1999; Lu et al., 2001,2005;Jin et al., 2002;Ruitenberg et al., 2003). Significantly, NT-3 enhances the growth of the most important upper motor neuron population in primates, corticospinal axons (Schnell et al., 1994;Grill et al., 1997; Blits et al., 2003). NT-3 also enhances the growth of ascending dorsal column sensory axons, a model system of great utility in studying both mechanisms and empirical features of axonal elongation in the adult spinal cord (Bradbury et al., 1999;Ramer et al., 2000;Lu et al., 2003;Taylor et al., 2006) (Table 1). These growth factors emerge as the most frequent subjects of therapeutic analysis in models of SCI because of their ability to influence the growth of motor axons, although other growth factors, including insulin-like growth factor -1 (IGF-1), the fibroblast growth factor (FGF) family, and others - remain of considerable potential interest. NGF and GDNF, on the other hand, by promoting growth of nociceptive spinal axons, may risk worsening dysfunctional axonal sprouting after SCI and worsening pain. This point highlights the fact that axonal growth is not a uniformly advantageous phenomenon: aberrant growth of either sensory or motor systems could have deleterious anatomical and functional consequences.

BDNF Effects on Axonal Growth

In a series of studies, several groups reported that BDNF could enhance the growth of supraspinal motor axons into permissive growth milieus placed at sites of SCI. Kobayashi and colleagues reported in 1997 that BDNF promotes the growth of rubrospinal axons into a peripheral nerve graft placed at a site of cervical SCI, and prevents degeneration of the red nucleus cell body in the brainstem (Kobayashi et al., 1997). Further, delayed delivery of BDNF to the red nucleus by one year in this model continued to demonstrate neurotrophic actions, reversing atrophy of neurons in the red nucleus even after this extended time point (Kwon et al., 2002). Liu and colleagues also reported in 1999 that rubrospinal axons regenerated into cellular grafts of autologlous fibroblasts genetically modified to secrete BDNF in a mid-cervical spinal cord lesion site (Liu et al., 1999). They further noted improvement in use of the forepaw on the side affected by the lesion, possibly as a result of local sprouting stimulated by BDNF. Reticulospinal axons, which modulate activity of motor neurons in the spinal cord, also respond to BDNF (Jin et al., 2002) (Fig. 1). Lu and colleagues reported that BDNF did not influence the growth of corticospinal axons in the spinal cord, even though BDNF prevents

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the death of corticospinal neuronal cell bodies when applied to the cortex, most likely because the BDNF receptor trkB was not trafficked from the cortical soma to the spinal axon (Lu et al., 2001). Others had also previously described protective effects of BDNF on cortical neurons (Giehl and Tetzlaff, 1996). In none of these papers was axonal growth beyond the BDNF cell graft or peripheral nerve bridge demonstrated, however. During development, neurotrophic factors classically promote the growth of their responsive axons along chemotropic gradients, with growth maximal at the point of peak growth factor concentration. This appears to be the case in the adult injured spinal cord also: axons readily penetrate BDNF-secreting grafts, but have not been reliably observed to emerge from the grafts again.

NT-3 Effects on Axonal Growth

A related but slightly distinct phenomenon has been observed in assessing responsiveness of corticospinal axons to NT-3. In 1994, Schnell and colleagues reported that a single injection of NT-3 above a spinal cord lesion site, when combined with administration of the Nogo neutralizing body IN-1, significantly enhanced the distance that a corticospinal axon extends below a mid-thoracic spinal dorsal hemisection lesion site (Schnell et al., 1994). Corticospinal axons observed below the lesion site in this experiment might have originated either from: 1) dorsally lesioned corticospinal axons that regenerated through spared host gray matter underlying the dorsal hemisection lesion, or 2) from ventral corticospinal axons, spared by the dorsal hemisection lesion, that subsequently sprouted below the lesion site in response to IN-1 antibody treatment. The single injection of NT-3 protein increased corticospinal axon sprouting above the lesion, and may have slightly increased the density of axons detected below in the lesion. In 1997, Grill and colleagues implanted autologous fibroblasts genetically modified to secrete NT-3 in a mid-thoracic spinal cord dorsal hemisection lesion site (Grill et al., 1997). Interestingly, corticospinal axons did not penetrate the NT-3 secreting cell graft, but did exhibit enhancing sprouting in host gray matter underlying the graft in the lesion site. Sprouting of CST axons occurred in gray matter above, underlying, and 1 mm caudal to the NT-3 graft/lesion site. Rats treated with NT-3 showed partial recovery of locomotion on a horizontal ladder (Grill et al., 1997). This report confirmed that NT-3 can influence injured adult corticospinal axons, and also revealed that corticospinal axons are uniquely repelled by growth into any substrate other than adult gray matter. To the present day, 10 years later, we and to our knowledge others have not clearly succeeded in promoting growth of injured adult corticospinal axons into a substrate placed into the site of an adult spinal cord lesion.

Sensory axons also respond to NT-3 (Bradbury et al., 1999; Ramer et al., 2000; Lu et al., 2003; Taylor et al., 2006). Unlike corticospinal tract axons, however, dorsal column sensory axons successfully penetrate a cell graft placed in a spinal cord lesion site, and their growth into a graft is significantly enhanced by NT-3 (Lu et al., 2003). Like BDNF-responsive descending motor axons, however, ascending dorsal column sensory axons do not emerge from grafts placed in lesion sites to bridge beyond the lesion cavity.

In summary, studies over the past decade clearly demonstrated that injured adult axons retain sensitivity to various growth factors. While mechanistically informative, these findings were of little practical value: axons must grow beyond the lesion site to have a potential for facilitating functional recovery. The observation that corticospinal axons sprout through host gray matter surrounding a dorsal hemisection lesion site was also informative but of limited value in a practical sense: gray matter is usually entirely destroyed at sites of SCI in humans, due its greater vulnerability to ischemia, inflammation and secondary injury. A next step was required.

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Combinatorial Therapies Support Axonal Bridging Beyond Spinal Cord Lesion Sites

In 2004, several groups published findings that combinatorial therapies exert effects in models of SCI that are not fully observed when fewer treatments are applied (Lu et al., 2004; Nikulina et al., 2004; Pearse et al., 2004). Prior to this time, most working in the field expected that treatment combinations would be more potent than individual, incremental approaches to enhancing axonal plasticity and regeneration, but little rigorous and objective data existed to support this belief.

Testing the hypothesis that combinatorial therapies exert greater effects on regeneration than single therapies is not a simple matter. One must control each variable, and if there are more than two combinations to the therapy, then each potential combination must be tested. If there are two therapies, then 3 groups must be tested. If three combinations are tested, then there must be 7 groups. And if 4 potential combinations are tested, then 15 groups must be tested (see Table 2). In our laboratory's models of dorsal column transection, we typically require a sample size of 10-12 animals per group to detect an effect that by convention is statistically significant at the $p<0.05$ level. Thus, testing 3 combinations requires approximately 70-80 rats, and testing 4 combinations requires 150-170 rats. These experiments rapidly become impractical for laboratories with limited personnel, time and financial support.

Nonetheless, as an initial rigorous test of the value of combinatorial therapies, many combinations were subjected to experimental control. We tested the hypothesis that to achieve sensory axonal bridging beyond a C4 spinal cord dorsal column lesion site, the following combinatorial therapies would be required:

1) A cell bridge in the lesion site—In this experiment, we used syngenic bone marrow stromal cells, which establish a "passive" cellular matrix within the lesion site, without producing growth factors or migrating from the lesion site after injection (Lu et al., 2005). These cells integrate well into a lesion site and reestablish cellular connectivity between the proximal and distal ends of the lesion cavity.

2) A growth factor within the bridge in the lesion site—We injected recombinant human NT-3 protein (600 ng) once into the marrow stromal cell suspension in the lesion site at the time of lesion and grafting to provide a trophic stimulus for attracting injured dorsal column sensory axons into the lesion site. Decline in the level of NT-3 protein over time (based on utilization and diffusion) would eliminate it as a *sustained* trophic stimulus that would otherwise risk retaining axons in the lesion site.

3) A growth factor beyond the bridge in the lesion site—To test the hypothesis that a *gradient* of NT-3 beyond a lesion site would elicit sensory axonal bridging not only into, but beyond, the lesion site, one week after the initial lesion, 1000 ng NT-3 was injected into the dorsal column white matter 1.5 mm rostral to the lesion site, to act as a delayed chemotropic stimulus to attract regenerating axons out of the cell graft in the lesion site.

4) A conditioning stimulus to the injured neuron, to "prime" the growth state—Both remote and more recent studies demonstrate that a conditioning (crush) lesion to the peripheral branch of a dorsal root ganglion neuron enhances the sprouting of its central axon after a spinal cord lesion (McQuarrie et al., 1977; Neumann et al., 1999). In contrast, a conditioning lesion of the central branch of a dorsal root ganglion neuron does not enhance growth after either a subsequent peripheral or central lesion. Thus, the peripheral crush "primes" the neuron to enter a growth state. This phenomenon is in part cAMP dependent, and can be replicated by injection of cAMP into the dorsal root ganglion (Neumann et al., 2002; Qiu et al., 2002). The mechanism of central growth activation may be related to upregulation

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of regeneration-associated genes such as GAP-43, C-jun and ß-tubulin, and reduced sensitivity of the injured axon to myelin-associated inhibitors of growth. In our combinatorial study, we tested the hypothesis that the addition of a cAMP injection into the dorsal root ganglion, NT-3 gradients within and beyond a C4 dorsal column lesion cavity, and a cell bridge in the lesion cavity, would support axonal bridging (if a simpler combination did not).

This ambitious experiment manipulated four variables: 1) cell bridge, 2) growth factor within the lesion site, 3) growth factor beyond the lesion site, and 4) cAMP into the dorsal root ganglion cell body. Thus, 16 experimental groups would be required to fully control the experiment and determine the necessity of each component of the combination, as shown in Table 2. We reduced this to a more manageable number based on observations from previous studies over which there would be little disagreement. First, axons cannot extend into the lesion cavity in the absence of a bridging substrate or, in this experiment, the marrow stromal cell graft. In previous experiments in our laboratory and that of all others that we are aware of, working in rat models, axons will not bridge beyond a transection lesion in the absence of a physical substrate for axonal attachment in the lesion cavity. Thus, this consideration allowed exclusion of all groups that did not contain a MSC graft, groups 9-15 in Table 2. We retained group 16, a no-treatment lesion control, to provide a baseline for injury severity in the absence of treatment. We were left with 9 groups to test.

Of these 9 remaining experimental groups, we eliminated another 3 groups as potentially non-essential control conditions. Group 6 from Table 2 would receive a graft and only NT-3 beyond the lesion site. If group 3 (graft + NT-3 in the graft + NT-3 beyond the graft) did not show bridging axons, then group 3 would not either. Indeed, this proved to be the case at the conclusion of the experiment, and group 6 was not needed as a control. Group 7 from Table 2 would receive a graft, NT-3 in the lesion site, and cAMP into the DRG: we hypothesized that in the absence of a neurotrophic gradient beyond the graft, axons would not extend for considerable distances beyond the graft. Group 8 from Table 2 would receive a graft, NT-3 beyond the lesion site and cAMP into the DRG: because the distal NT-3 injection is performed one week after the original lesion, we reasoned it unlikely that axons would extend into the lesion site in sufficient numbers in this group to represent a quantitative "pool" for potential emergence from the graft. While findings of the experiment suggested that the exclusion of the latter two control groups (7 and 8) were likely justified as results below indicate, rigorous proof would require completion of experiments in these groups.

Thus, the experimental design ultimately examined five groups of animals, and 12 animals per group, for a total of 60 subjects, plus lesioned, non-grafted controls. At the conclusion of the experiment, we found that axons bridged beyond the lesion cavity only in the full treatment group that received a cell bridge, NT-3 within and beyond the lesion site, and cAMP priming of the injured neuron (Fig. 2). None of the remaining groups exhibited bridging axons, although in some groups (bridge + cAMP; bridge + NT-3 within and beyond the lesion site) axons extended to the interface of the graft with the rostral host spinal cord.

The risk of misinterpreting spared axons for regenerating axons looms large in all spinal cord studies. In this experiment, we confirmed that lesions were complete by sectioning the nucleus gracilis in every animal: no spared axons reaching the nucleus were observed. Further, axons observed to extend beyond the lesion site in this experiment emerged directly across the graft-host interface (labeled clearly with the reporter gene green fluorescent protein, GFP), and frequently from the center of the graft rather than only the most dorsal or ventral aspects of the lesion site, where spared axons are most likely to be located. Finally, axons extending beyond the lesion site had an irregular and often circuitous course, unlike linear intact axons. For these reasons, it appeared that axons truly bridged beyond the lesion cavity (Fig. 2).

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Two other reports published in 2004 suggested further that combination therapies can generate more extensive anatomical or behavioral plasticity than individual therapies (Pearse et al., 2004; Nikulina et al., 2004). Interestingly, both of these studies, as well as our own, included cAMP augmentation of the neuronal growth state as a component of a combination therapy to improve outcomes in models of SCI. Augmentation of the neuronal growth state remains a major focus of several current efforts in SCI research, including attempts discover novel genes related to enhanced central plasticity and regeneration (Costigan et al., 2002).

CONCLUSION

Reports continue to emerge supporting the concept that combination therapies elicit significantly greater degrees of axon growth than individual therapies (Houle et al., 2006; Tan et al., 2006; Massey et al., 2007). Combinations of bridges, growth factors, degrading enzymes of the extracellular matrix, and myelin neutralization will require systematic but focused examination to design a combinatorial approach most meritorious of testing in larger animal models. The practical hurdles to this approach are not unsubstantial, but must be overcome to generate approaches that may ultimately benefit the human condition.

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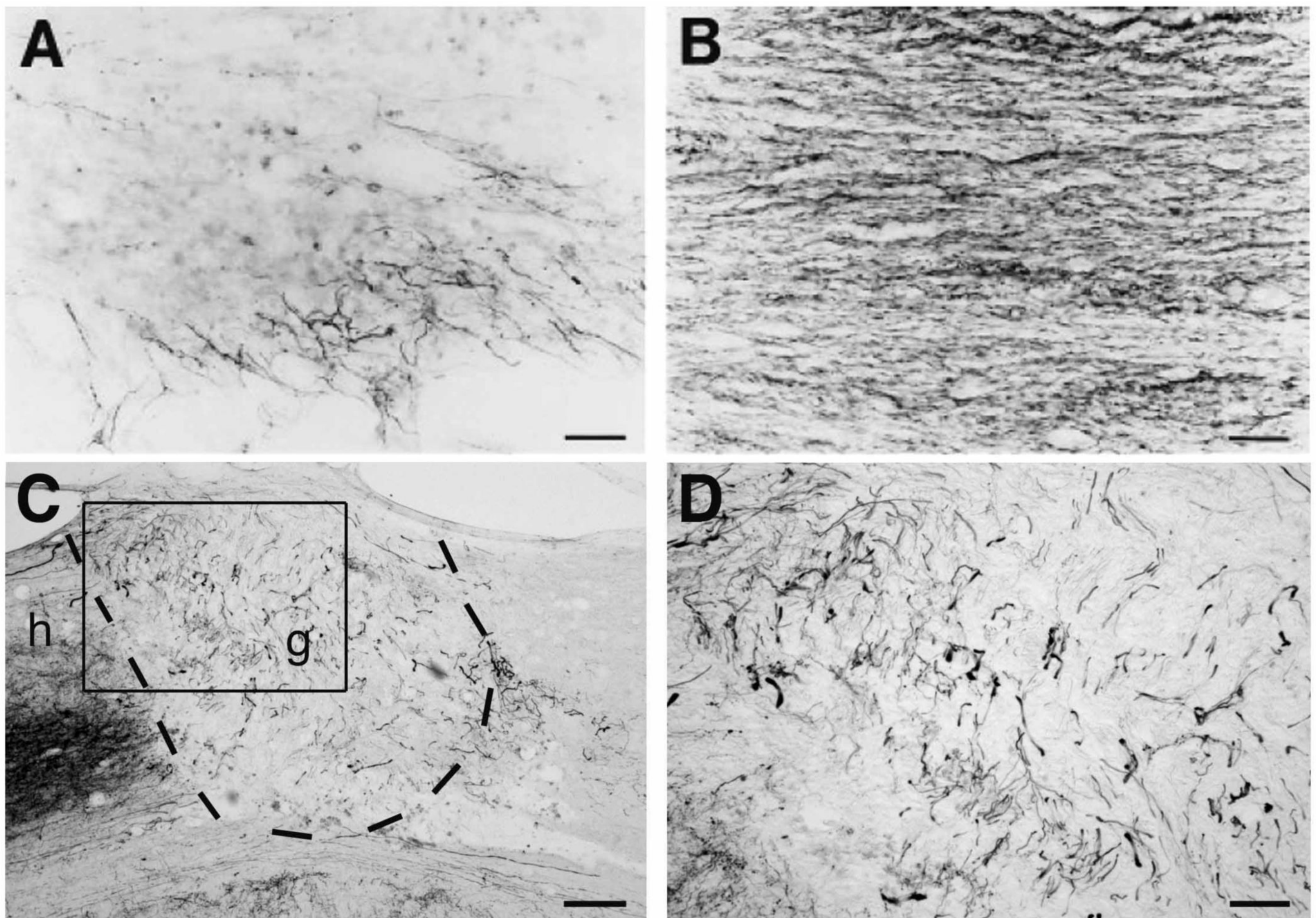

Figure. 1.

Spinal cord axon growth is induced by grafts of NGF- or BDNF-secreting cell grafts. (**A**) Neurofilament immunolabel shows modest axon penetration into control fibroblast graft, whereas (**B**) NGF-secreting graft is densely penetrated by axons 3 mo post-injury (see Table 1 for list of responding axons). (**C**) BDA-labeled reticulospinal axons also extensively penetrate a BDNF-secreting bone marrow stromal cell graft (outlined by dashed lines) placed into site of mid-cervical injury. g, graft; h, host; graft shown 3 mo post-injury. (**D**) Higher magnification of boxed areas from panel C, demonstrating BDA-labeled reticulospinal axons in BDNF-expressing bone marrow stromal cell graft. Scale bar, A-B, 15 μm; C, 210 μm; D, 80 μm.

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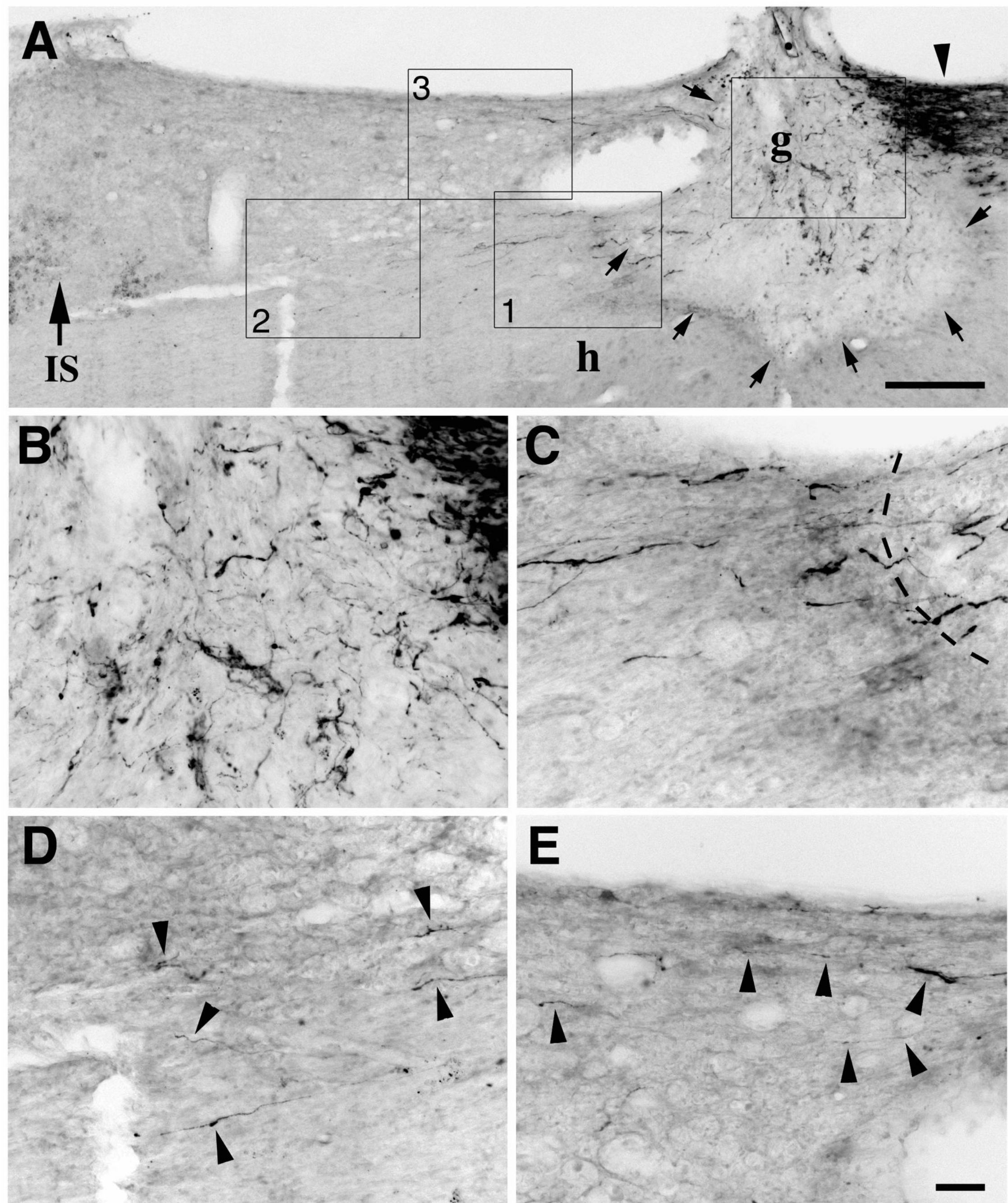

Figure 2.

Sensory axons regenerate beyond spinal cord lesion sites after combined administration of intraganglionic cAMP and axonal application of NT-3. **(A)** Lower magnification view of sagittal section of spinal cord illustrating CTB-labeled dorsal column sensory axons approaching lesion site (arrowhead on upper right), MSC graft in lesion cavity (**g;** arrows indicate host/graft interface), and region rostral to lesion site (left side of figure). Large arrow and **IS** indicate rostral **I**njection **S**ite of NT-3. **(B)** Boxed area of graft: CTB-labeled sensory axons penetrate graft in lesion site. **(C)** Higher magnification of box 1 from panel A, demonstrating crossing of CTB-labeled axons from graft into host white matter beyond the graft. Dashed lines indicate host/graft interface. This crossing occurs at a point well away from

dorsal or ventral lesion regions, reducing likelihood that axons were spared by lesion. Lesion completeness was confirmed by failure to observe CTB-labeled sensory axons in medulla in lesioned subjects. **(D)** Higher magnification of box 2 from panel A, demonstrating several varicose CTB-labeled axons extending 0.5 - 0.7 mm beyond lesion site (arrowheads). **(E)** Higher magnification of box 3 from panel A, showing additional axons extending under the dorsal aspect of the spinal cord beyond the lesion site. Scale bars = 280 μm (**A**), 44 μm (**B-E**). (From Lu et al., J Neurosci 2004; 24:6406).

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Table 1

Sensitivity of Spinal Cord Axons to Growth Factors

Growth Factors	Injured Axons	References
NGF	nociceptive spinal axons	Tuszynski et al., 1994,1996
		Ramer et al., 2000
	cerulospinal axons	Tuszynski et al., 1994,1996
BDNF	rubrospinal axons	Kobayashi et al., 1997
		Ye and Houle, 1997
		Liu et al., 1999
	raphespinal axons	Bregman et al., 1997
		Menei et al., 1998
	coerulospinal axons	Menei et al., 1998
	reticulospinal axons	Ye and Houle, 1997
		Jin et al., 2002
	vestibulospinal	Jin et al., 2002
	local motor axon	Lu et al., 2001
	local sensory axon (CGRP)	Lu et al., 2001
NT-3	corticospinal axons	Schnell et al., 1994
		Grill et al., 1997
	dorsal column sensory axons	Bradbury et al., 1999
NT-4/5	local motor axons	Blesch et al., 2004
	coerulospinal axons	Blesch et al., 2004
	reticulospinal axons	Blesch et al., 2004
	propriospinal axons	Blesch et al., 2004
GDNF	local motor axons	Blesch et al., 2003
	propriospinal axons	Blesch et al., 2003
	dorsal column sensory axons	Blesch et al., 2003
	nociceptive spinal axons	Ramer et al., 2003

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Table 2

Controls for Combinatorial Growth Factor/cAMP Therapy

A. Groups to be assigned:	
MSC	Marrow stromal cell
NT-3	NT-3 in lesion site
NT-3 Beyond	NT-3 beyond lesion site
cAMP	cAMP injection into DRG

B. Hypothetical experimental groups for full control:
1) **MSC**
2) **MSC** + **NT-3**
3) **MSC** + **NT-3** + **NT-3 Beyond**
4) **MSC** + **NT-3** + **NT-3 Beyond** + **cAMP**
5) **MSC** + **cAMP**
6) **MSC** + **NT-3 Beyond**
7) **MSC** + **NT-3** + **cAMP**
8) **MSC** + **NT-3 Beyond** + **cAMP**
9) **NT-3**
10) **NT-3** + **NT-3 Beyond**
11) **NT-3** + **NT-3 Beyond** + **cAMP**
12) **NT-3** + **cAMP**
13) **NT-3 Beyond**
14) **NT-3 Beyond** + **cAMP**
15) **cAMP**
16) **No MSC, no NT-3, no NT-3 beyond, and no cAMP**

Note: Underlined groups were included in final experiments.

Controls for NT-3 or cAMP injections consisted of vehicle solution injections into the respective target sites (see Lu et al., J Neurosci 2004; 24:6402-6409).