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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Neurotrophin Gradients and Axon Growth After Spinal Cord Injury

A dissertation submitted in partial satisfaction of the requirements for the

degree Doctor of Philosophy

in

Biology

by

Laura Anne Taylor

Committee in charge:

    Professor Mark H. Tuszynski, Chair
    Professor Nick Spitzer, Co-Chair
    Professor Darwin Berg
    Professor Armin Blesch
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2007

The Dissertation of Laura Anne Taylor is approved, and it is acceptable in quality and form for publication on microfilm:

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University of California, San Diego

2007

DEDICATION

This work is dedicated to my friend, Julee Sucharda. Your strength and courage as you faced a possible spinal cord injury and recovered from severe spinal column damage provided inspiration for my studies of spinal cord injury.

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ABSTRACT OF THE DISSERTATION

Neurotrophin Gradients and Axon Growth after Spinal Cord Injury

by

Laura Anne Taylor

Doctor of Philosophy in Biology

University of California, San Diego, 2007

Professor Mark H. Tuszynski, Chair

Professor Nick Spitzer, Co-Chair

In order for axon regeneration to result in functional recovery after spinal cord injury (SCI), axons must grow beyond lesion sites and form synapses with neuronal targets in distal host tissue. We tested the hypothesis that neurotrophic factor gradients established distal to lesion sites in adult rats would promote growth of sensory axons into and beyond a site of injury and support synapse formation in denervated targets. First, gradients of neurotrophin expression were established rostral to cell-filled C3 dorsal column lesion sites using delivery of NT-3-expressing lentiviral vectors. Sensory axons regenerated for short distances beyond lesion sites in

animals treated with NT-3, but not control vectors. Long distance axon growth along neurotrophin gradients was not observed. However, when conditioning lesions, which are thought to initiate an overall growth program in neuronal cell bodies, were combined with NT-3 gradients beyond lesion sites, significantly more axons regenerated over greater distances into distal host tissue than when either treatment alone was given.

We next tested the hypothesis that combinatorial treatment would promote growth of sensory axons originating from hindlimb dorsal root ganglia across C1 lesion sites into the nucleus gracilis, the termination site for these axons. Animals subjected to high cervical lesions and combinatorially treated with conditioning lesions and NT-3 exhibited significantly more axons within the nucleus gracilis than animals receiving control lentiviral vectors. Ultrastructural examination of nucleus gracilis tissue indicated the presence of possible synaptic contacts formed by regenerated axons in the denervated target. These data demonstrate that combined stimulation of a neuronal growth program (conditioning lesions) and provision of a positive stimulus at the level of the growth cone (NT-3) can promote long distance axon growth in the inhibitory environment beyond a spinal cord lesion site. In addition, combinatorial treatment can be used to guide axons to appropriate target regions and may support formation of synaptic contacts.

CHAPTER 1

Introduction

Human Spinal Cord Injury

Severe spinal trauma causes debilitating effects in humans with only a small percentage of injured patients making a full recovery. Each year approximately 12,000 individuals sustain spinal cord injuries (SCIs) in the United States. Most SCIs occur during vehicle accidents (46% of injuries), falls (20%), violence (18%) or sports and recreation (11%) (Jackson et al., 2004). These various sources of trauma cause compression, contusion, penetration or maceration of central nervous system (CNS) tissue that results in death of neurons and glia and destruction of axonal connections (Kakulas, 1999). These cellular losses break communication between neurons above and below the injury site, causing the functional impairment associated with SCI. Injured patients may lose voluntary muscle movement, sensation, bladder and bowel control, sexual function, or even the ability to breathe, depending on the nature of the injury. Because CNS neurons fail to regenerate spontaneously, these functional impairments are suffered for a lifetime.

CNS regeneration failure represents a formidable barrier to recovery and consequently, no treatments currently exist for repairing the injured cord. Instead, post-injury care for SCI is limited to procedures aimed at minimizing cord damage that can occur after the initial injury. For example, surgical procedures to stabilize the spine or alleviate pressure on the spinal cord caused by fractured or dislocated

vertebrae or disks are used to help reduce mechanical damage (Fehlings and Perrin, 2006). In addition, techniques for maintaining arterial oxygen and blood pressure may be used in an attempt to reduce secondary cell death at the injury site (Hurlbert, 2006). The pharmaceutical methylprednisolone (MP), a steroid known to reduce acute inflammation (Bartholdi and Schwab, 1995), may also be administered. However, despite its widespread use, the benefits of MP are not clearly established (Bracken et al., 1990; Fehlings, 2001; Qian et al., 2005; Tsutsumi et al., 2006; Leypold et al., 2007). In fact, a recent review of clinical MP use for treatment of SCI concluded that the evidence for harmful side effects of the drug is stronger than the evidence for improved outcomes (Hurlbert, 2006). While current treatments for spinal cord injury are clearly lacking, recent advances in our understanding of the cellular mechanisms that contribute to damage and prevent CNS regeneration have provided new hope that the injured spinal cord may some day be repaired.

Cellular Events in Spinal Cord Injury

        A major effort has been made to elucidate the cellular mechanisms involved in the progression of a spinal cord injury from an initial traumatic event to the chronic injury state. Studies in this area have demonstrated that injury to the spinal cord involves an acute damage phase followed by a long-lasting period of secondary degeneration. During the acute phase, a primary lesion is formed due to mechanical shearing and stretching that damages neurons, support cells and vasculature (Goodman et al., 1979; Wallace et al., 1986; Shi and Pryor, 2002). These events lead to

immediate death of neurons and glia resulting in formation of a primary lesion cavity. During the secondary degeneration phase, which occurs in the days, weeks and months following the primary injury, axons degenerate away from the site of injury and additional death of neurons and glia leads to a progressive enlargement of the primary lesion site (Hagg and Oudega, 2006). In addition, continued death of oligodendrocytes leads to demyelination of spared axon tracts near and at some distance from the lesion (Crowe et al., 1997; Guest et al., 2005; Totoiu and Keirstead, 2005).

Secondary degeneration is caused by a number of factors including ischemia, edema, excitotoxicity, apoptosis and inflammation. Ischemia, caused by damage to the spinal cord vasculature, can lead directly to apoptosis or cause production of reactive oxygen species that damage cell membranes (Chan, 2004; Saito et al., 2005). Edema, which is exacerbated by the inflammatory response, can lead to compression of the spinal cord and additional mechanical damage to CNS cells (Hagg and Oudega, 2006). Over-activation of NMDA or AMPA receptors due to release of excitatory amino acids, such as glutamate, from injured or dead cells leads to excitotoxicity in neurons and glia that survived the initial injury (Park et al., 2004). Finally, cells may become apoptotic due to glucose deprivation, hypoxia (Tanaka et al., 2005), loss of support from surrounding cells, the presence of neurotoxic molecules or other factors (Beattie et al., 2000; Beattie, 2004).

Additional complications are caused by a massive inflammatory response to CNS injury (Jones et al., 2005). In general, inflammation in the CNS is triggered by

resident microglia in the vicinity of the lesion that are activated in response to tissue disruption and vascular damage during the primary injury stage (Tator and Fehlings, 1991; Kreutzberg, 1996; Schnell et al., 1999). Microglia secrete pro-inflammatory cytokines that recruit additional immune cells, including neutrophils and monocytes, to the lesion site. Spinal cord damage is amplified when immune cells secrete neurotoxic factors and produce reactive oxygen species during phagocytosis. Cell death and membrane damage caused by these factors likely contributes to secondary degeneration (Jones et al., 2005). This conclusion is based on studies demonstrating that disabling the immune response after SCI can reduce secondary degeneration after SCI. For example, agents used to block neutrophil accumulation at lesion sites cause reduced cell death and improved functional outcomes after SCI (Pearse et al., 2003). In addition, antibodies that neutralize macrophage-derived TNF-alpha reduce the number of apoptotic cells after CNS injury (Selmaj and Raine, 1988; Lee et al., 2000; Yune et al., 2003). Methylprednisilone, the only pharmaceutical available for treatment of SCI in humans, is thought to reduce inflammation acutely to lessen tissue loss (Pearse et al., 2003). It is important to note that a number of studies have also suggested a beneficial role for inflammatory cells (Schwartz et al., 1991; Arnett et al., 2001; Yin et al., 2003; Yin et al., 2006), suggesting that the role of inflammation in SCI is not a simple one (Bethea, 2000).

Secondary degeneration due to inflammation and other factors is eventually attenuated by formation of a glial scar (Silver and Miller, 2004). During this process, activated astrocytes act in concert with several other cell types to establish a barrier

surrounding the lesion site that consists of astrocytic processes, meningeal cells and extracellular matrix molecules (Reier, 1986). As part of scar formation, activated astrocytes may also work to re-establish the blood-brain barrier (Bush et al., 1999; Preston et al., 2001). Due to glial scarring, regions where tissue has been lost become fluid-filled cysts isolated from spared intact tissue (Hagg and Oudega, 2006). The importance of glial scarring in limiting tissue damage after injury was demonstrated by studies using mutant mice that were genetically altered such that reactive astrocytes could be selectively ablated. After spinal cord injury, mutant mice with ablated astrocytes displayed much larger lesion sites and more cellular degeneration than wild type mice (Faulkner et al., 2004).

Understanding the mechanisms behind the progression of a spinal cord injury, from an initial traumatic insult to secondary degeneration and formation of the glial scar, represent an important line of research in the context of axon regeneration. Mitigation of secondary damage after injury could decrease tissue loss, such that fewer axons would need to be regenerated. Also, if expansion of the primary lesion site could be reduced, the distances over which axons would be required to regenerate could be kept at a minimum. Finally, events such as glial scar formation and degradation of myelin cause deposition or release of factors that can inhibit axon growth. Insight into how these events occur might contribute to strategies for reducing the presence of inhibitors.

Factors Contributing to Regeneration Failure After Spinal Cord Injury

For many years it was thought that injured CNS axons, unlike those of the peripheral nervous system (PNS), did not have the capacity to regenerate (Cajal, 1928). However, in the 1980s Aguayo and colleagues demonstrated the ability of CNS axons to regenerate into peripheral nerve grafts (Aguayo et al., 1981). These findings suggested the intriguing possibility that CNS axons are capable of growth, but are inhibited by components of the CNS environment. Since then it has been established that axons fail to regenerate in the adult CNS due to both extrinsic environmental factors and factors intrinsic to CNS neurons. Extrinsic factors that contribute to CNS regeneration failure include the presence of environmental inhibitors, loss of neurotrophic support, and a lack of growth substrates suitable for axon extension. Intrinsically, CNS neurons lack the ability to activate genetic programs that can result in axon regeneration. Each of these factors is thought to contribute to the overall inability of injured CNS neurons to mount a successful axonal growth response after injury.

I. Environmental Inhibitors

The most well-characterized environmental axon growth inhibitors are associated with CNS glial cells. Early studies indicated that both the glial scar (Rudge and Silver, 1990; McKeon et al., 1991; Davies et al., 1999) and CNS myelin (Schwab

and Caroni, 1988) contain factors that inhibit axon growth. Formation of a glial scar appears to be an important mechanism for protecting intact tissue from uncontrolled degeneration after SCI. However, when regenerating axons encounter the glial scar they exhibit dystrophic growth cones and regenerative growth is aborted (Davies et al., 1999). While the scar probably acts to block regeneration in part by creating a physical barrier to growth, it is now clear that molecular inhibitors expressed by cells comprising the scar play a major role in stalling axon regeneration (Liuzzi and Lasek, 1987; McKeon et al., 1991). In addition, these molecular inhibitors can be expressed beyond the lesion site, such as in regions denervated by an injury, where they may play a role in blocking axon growth (Massey 2007).

Molecular inhibition of axon growth at the glial scar is mediated in large part by proteoglycans, especially those of the chondroitin sulfate proteoglycan (CSPG) family (Canning et al., 1996). Proteoglycans consist of a protein core surrounded by sulphated glycosaminoglycan (GAG) side chains. During development, proteoglycan expression establishes inhibitory barriers that direct axon growth (Bovolenta and Fernaud-Espinosa, 2000) (Snow et al., 1990; Brittis et al., 1992). After SCI, hypertrophic astrocytes, major cellular players in formation of the glial scar, express high levels of CSPGs. Under these conditions, CSPGs potently inhibit axon growth into and beyond spinal cord lesion sites (Sandvig et al., 2004). The mechanistic details underlying CSPG function are largely unknown, but may include direct signaling through neuronal receptors (Dou and Levine, 1997; Koprivica et al., 2005) and/or modulation of growth cone cytoskeletal or membrane components (Tom et al.,

2004), possibly via direct interaction with Rho signaling pathways (Monnier et al.,

2003). It is also possible that CSPGs function as inhibitors by covering growth-

promoting substrates that are part of the normal ECM, such as laminin and fibronectin,

rather than through direct inhibitory signaling events (Zuo et al., 1998).

Chondroitinases, enzymes that degrade GAG side chains, can significantly

reduce axon growth inhibition by CSPGs (Zuo et al., 1998). Thus, these proteins have

been an important tool in understanding CSPG inhibition and application of these

enzymes after human SCI represents a possible strategy for promoting axon growth.

Degradation of GAG side chains using the enzyme Chondroitinase ABC (ChABC),

allows axons to grow over normally inhibitory CSPG substrates in several models of

CNS injury, including SCI (Moon et al., 2001; Bradbury et al., 2002). Importantly,

ChABC application has been used to promote growth of axons beyond spinal cord

lesion sites, presumably by attenuating the inhibitory effect of the glial scar at distal

host/graft interfaces (Chau et al., 2004; Houle et al., 2006). In addition to promoting

growth of injured axons, ChABC delivery can also promote sprouting of spared fibers

after CNS injury (Barritt et al., 2006). This phenomenon has been used to promote

reinnervation of denervated brainstem targets by uninjured axons after SCI and may

be an important mechanism by which degradation of CSPGs could contribute to

recovery of function after SCI (Massey et al., 2006).

Besides scar-derived inhibition, regenerating axons are faced with a number of

molecular inhibitors associated with degenerating and intact CNS myelin. The classic

myelin-associated inhibitors include myelin-associated glycoprotein (MAG)

(McKerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002b) and Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). The most widely studied of these molecules is Nogo-A, one of three Nogo isoforms. Nogo-A is a transmembrane protein including two inhibitory domains that is expressed by oligodendrocytes comprising CNS myelin (Huber et al., 2002). Nogo-A causes inhibition of axon growth through binding to the Nogo-66 receptor (NgR). MAG is also a transmembrane protein and is expressed exclusively by myelinating cells of the CNS (oligodendrocytes) and PNS (Schwann cells) (Filbin, 1995). During CNS injury, MAG is probably released as a soluble fragment, known as dMAG (Tang et al., 2001). Inhibitory signaling by MAG is mediated by NgR and an isoform of NgR called NgR2. GD1 and GT1b gangliosides are thought to facilitate binding of MAG to the axon membrane after which signaling through NgR can occur (Vinson et al., 2001). Finally, the myelin-associated inhibitor OMgp is expressed by oligodendroglia-like cells localized to nodes of Ranvier and is thought to function in this location to limit sprouting of axons (Huang et al., 2005). This protein also signals to inhibit axon outgrowth through binding to NgR.

As mentioned above, the three major myelin inhibitors, OMgp, MAG and Nogo-A, all signal through activation of NgR, which is expressed by a variety of CNS neurons (Sandvig et al., 2004). Thus, the components of NgR-mediated signaling are important targets for attenuating or blocking the effects of myelin-associated inhibition in injured neurons. NgR is an extracellular GPI-linked protein that

promotes intracellular signaling through binding interactions with transmembrane co-receptors including LINGO1 (Park et al., 2005) and either the p75 neurotrophin receptor (p75NTR) (Wang et al., 2002a) or Taj/Troy (Park et al., 2005). Signaling mediated by NgR and these co-receptors causes activation of the small G-protein RhoA leading to growth cone collapse (Walmsley and Mir, 2007). Interestingly, concomitant expression of these signaling components in non-neuronal cells leads to Rho activation in the presence of myelin (Park et al., 2005), demonstrating a major role for this signaling complex in mediating myelin inhibition. A number of studies have now indicated that interruption of intracellular NgR signaling pathways, for example by blocking Rho and its downstream targets (Dergham et al., 2002) or blocking NgR function (Li et al., 2004) promotes regeneration of injured axons.

Genetic studies have been used in an attempt to better understand the role of myelin inhibition after SCI. However, outcomes from these studies have been difficult to interpret. For example, while agents that block function of NgR have been successful at promoting growth of corticospinal (CST) axons after injury (GrandPre et al., 2002), genetic deletion of NgR in mice does not enhance CST regeneration (Kim et al., 2004; Zheng et al., 2005). Genetic deletion of p75NTR also failed to improve regeneration of CST or dorsal column axons after spinal cord injury, although some neurons from these mice could grow over myelin-rich substrates in the brain or optic nerve (Song et al., 2004). Similarly, while antibodies to Nogo-A result in regeneration of injured CST fibers, Nogo knockouts have been reported to exhibit either no regeneration of CST fibers (Zheng et al., 2003), slight regeneration of these fibers

(Simonen et al., 2003) or CST regeneration sufficient to support functional recovery after injury (Kim et al., 2003). Animals with MAG genetic deletion show no regeneration after SCI, despite in vitro studies demonstrating that this protein can potently inhibit axon growth (Bartsch et al., 1995). These differing findings suggest that molecular inhibitors and their receptors probably exhibit redundancy or that some components mediating inhibitory signaling may remain undiscovered. Despite this fact, antibody-mediated neutralization of Nogo-A has been shown to enhance axonal regeneration in several models of spinal cord injury in rodents (Schnell and Schwab, 1990; Bregman et al., 1995; Thallmair et al., 1998) and non-human primates (Fouad et al., 2004). Based on these findings anti-Nogo therapeutics may soon be tested as a possible strategy for reducing the effects of myelin inhibition after human SCI (Thuret et al., 2006).

Adding to the complexity of inhibitory effects after SCI is a growing list of additional molecules capable of blocking axon growth in the context of CNS injury. These factors include semaphorins (Moreau-Fauvarque et al., 2003) ephrins (Benson et al., 2005), and repulsive guidance molecule (RGM) (Hata et al., 2006), all of which act as repulsive guidance cues during development. Semaphorin and ephrin expression is upregulated in astrocytes and fibroblasts after injury and can therefore function as part of inhibition associated with the glial scar (Sandvig et al., 2004). Secreted semaphorins bind to ECM molecules, such as CSPGs and mediate their inhibitory effects through plexin receptors (Sandvig et al., 2004) while ephrins mediate growth cone collapse by binding to Eph receptors located in axonal

membranes. RGM expression is upregulated in myelinated fiber tracts and in the

developing scar after injury and this protein is thought to signal through the neogenin

receptor to inhibit axon growth after SCI (Hata et al., 2006). The complexity of

inhibitory signaling is illustrated by the sheer number of inhibitory molecules that

have been discovered, the differing cell types that express these inhibitors, and the

variety of receptors utilized to mediate axon growth arrest. These intricacies suggest

that attenuating the activities of a vast number of inhibitors might be prohibitively

difficult and highlights the importance of developing strategies aimed at promoting

axon growth *despite* inhibitors.

II. *Loss of neurotrophic support*

Neurotrophic factors are diffusible molecules normally involved in the survival

and development of multiple populations of neurons in the PNS and CNS (Huang and

Reichardt, 2001). Early studies demonstrated that when neurotrophic support is

disrupted *in vitro*, neurons become apoptotic. Moreover, when neurotrophic support is

withdrawn from axons specifically, they retract and degenerate (Campenot, 1977).

Over 30 years ago, researchers suggested the neurotrophic factor hypothesis, which

states that neurotrophic factors expressed by postsynaptic cells are transported to

neuronal cell bodies where they support cell survival during development (Purves,

1986).

While the neurotrophic factor hypothesis was developed based on studies of

the PNS, CNS neurons may also be dependent on trophic signals from post-synaptic

cells. This idea is supported by numerous studies indicating that the application of neurotrophic factors can enhance survival and axon growth after CNS injury (Jones et al., 2001).   An example of this phenomenon occurs after internal capsule lesioning of corticospinal axons, which normally results in death of almost 50% of corticospinal neurons. Application of BDNF results in 100% survival of corticospinal neurons (Giehl and Tetzlaff, 1996). In addition, cholinergic neuron degeneration after transection of axon pathways from the medial septum to the hippocampus can be significantly reduced by application of NGF (Kromer, 1987). When axons are transected during a spinal cord injury, neurons lose contact with post-synaptic cells and therefore may lose trophic support, leading to degeneration of axons and neuron death. However, application of neurotrophic factors to sites of spinal cord injury can rescue neurons and promote axon growth (Blesch et al., 2002).

Expression of neurotrophic factors by injured axons and surrounding cells may be one factor contributing to successful regeneration of PNS axons (Frostick et al., 1998). Following peripheral nerve injury of primary sensory neurons, Schwann cells of the distal stump upregulate expression of a variety of neurotrophic factors that may be involved in the regenerative response of injured neurons (Terenghi, 1999). In addition, injured PNS neurons increase expression of trophic factors and trophic factor receptors that may protect them, as well as nearby neurons and axons, from death and atrophy via autocrine and paracrine signaling. Similarly enhanced neurotrophic factor expression has not been observed after CNS injury (Plunet et al., 2002). In addition to promoting survival, neurotrophins activate signaling pathways that are involved in

axon growth (Lonze and Ginty, 2002; Graef et al., 2003). These observations suggest that loss of neurotrophic support causes death of neurons after SCI and may be a cause underlying failure of de novo axon regeneration in the CNS.

III. Insufficient growth substrates

Injury to the spinal cord results in loss of nervous system tissue. Regions of tissue loss eventually become fluid-filled cysts that do not support axon growth. Studies in which various growth substrates, such as cellular grafts and artificial scaffolds, are used to fill lesion cavities have demonstrated that axon growth can occur into lesion sites if sufficient growth substrates are supplied (Thuret et al., 2006). However, since providing permissive substrates alone generally results in minimal axon regeneration beyond lesion sites, combining these substrates with additional stimuli will be needed to promote significant axon growth.

IV. Lack of intrinsic regenerative potential

While the growth of CNS neurons after injury is clearly inhibited by factors in and around the lesion, it is also thought that CNS neurons lack the ability to sufficiently activate genetic programs that are necessary for axon regeneration. Interestingly, PNS neurons, embryonic neurons, and adult neurons of lower vertebrates can efficiently regenerate axons. A plausible explanation for these distinct

growth potentials is that injury induces unique gene expression patterns in growth competent neurons (i.e. expression of regeneration-associated genes) that are not activated in adult CNS neurons (Harel and Strittmatter, 2006).

Perhaps the most striking evidence supporting this idea comes from studies of dorsal root ganglia (DRG) neurons. DRG sensory neurons project axon branches to both the peripheral and central nervous system. When the peripheral axon branches of DRG neurons are injured, a specific gene expression pattern is activated at the level of the cell soma and regeneration is robust (Costigan et al., 2002; Kubo et al., 2002). In contrast, injury to the central branch activates a different gene set and results in no regeneration (Schreyer and Skene, 1993; Broude et al., 1997). However, central fibers mount an improved regenerative response if peripheral branch lesions occur before central fibers are injured. This phenomenon is known as the conditioning lesion effect (Richardson and Issa, 1984; Neumann and Woolf, 1999). The conditioning lesion effect indicates that activation of appropriate genetic programs may enhance a neuron's intrinsic potential for growth, even in the inhibitory CNS environment.

Developmental studies also support the idea that changes in gene expression affect a neuron's intrinsic capacity for growth. As neurons develop, expression of growth-associated genes, such as GAP-43, is decreased and regenerative capacity is lost (Fitzgerald et al., 1991; Chong et al., 1992; Nicholls and Saunders, 1996). For example, retinal ganglion cells (RGCs) exhibit a marked reduction in regenerative capacity between E16 and E18, which has been associated with dramatically decreasing Bcl-2 gene expression (Chen et al., 1997). High levels of Bcl-2 mRNA

have also been associated with regeneration in peripheral nerves (Merry et al., 1994). Interestingly. Bcl-2 expression is regulated by both CREB and STAT3, which appear to be involved in DRG conditioning lesion-induced regeneration (see below).

Another example of a developmental change that reduces the growth capacity of maturing neurons involves the well-known myelin inhibitor, MAG. MAG is a potent inhibitor of adult DRG axon growth, but actually promotes neurite extension in embryonic and young DRG neurons (Mukhopadhyay et al., 1994). Signaling pathways that elevate levels of cAMP in embryonic versus adult neurons appear to control this switch in response to MAG (Cai et al., 2001). Taken together, these studies and many others have demonstrated that the intrinsic growth potential of peripheral and embryonic neurons is different than that of adult CNS neurons.

Strategies for Promoting Axon Growth after Spinal Cord Injury

Due to the complexity of factors at play to prevent regeneration of axons after SCI, it is generally accepted that a combination of treatments will be needed to achieve meaningful growth of injured axons. A number of strategies for promoting axon regeneration have now been tested, including neutralizing myelin inhibitors, degrading inhibitory ECM molecules, providing growth substrates within lesion sites, providing growth-promoting factors and altering intracellular signaling pathways. Below, the strategies that were combined to promote axon growth in the present work are introduced.

I. *Neurotrophin delivery as a regeneration strategy*

Retrograde neurotrophin signaling functions in regulation of neuronal survival and axon growth (Zweifel et al., 2005). The application of neurotrophic factors to sites of spinal cord injury (SCI) results in improved neuronal survival and allows some axons to regenerate in the inhibitory environment of the injured adult spinal cord. One class of neurotrophic factors, the neurotrophin family of proteins, includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT-4/5 (in mammals). Neurotrophins exist as proneurotrophins, which are then cleaved by proteolysis into their mature forms. All four proneurotrophins bind the p75 neurotrophin receptor (p75NTR) (Reichardt, 2006). After maturation, these proteins can also interact with three Trk receptors while maintaining their ability to bind p75NTR. Distinct axonal populations respond to specific neurotrophins depending on which of the high affinity neurotrophin receptors (TrkA, TrkB or TrkC) they express (Bibel and Barde, 2000). While there is some degree of overlap in binding partners between neurotrophins and Trk receptors, in general NGF binds TrkA, BDNF and NT-4/5 bind TrkB and NT-3 binds TrkC.

Upon Trk receptor or p75NTR binding, neurotrophins activate several intracellular signaling pathways that are implicated in neuronal survival and differentiation, as well as axon outgrowth. Binding to p75NTR initiates signaling through three major pathways. Activation of the NF-kappaB and Jun kinase pathways leads to transcription of genes involved in neuronal survival and apoptosis, respectively (Hamanoue et al., 1999; Kuan et al., 1999). p75NTR signaling also

activates RhoA to reduce growth cone motility. Binding of mature neurotrophins to

Trk receptors initiates signaling through additional pathways. Signaling through the

phospholipase C (PLCγ) pathway and protein kinase C (PKC) in response to TrkR

activation may be involved in synaptic plasticity. TrkR activation of Ras initiates

signaling through the MAP kinase pathway, leading to neuronal differentiation and

neurite outgrowth. PI3 kinase (PI3K) may also be activated by Ras or Gab1 to

promote neuronal survival. Importantly, a major activity of the PI3K pathway is

activation of small G-proteins, such as Cdc42 and Rac, which can promote growth

cone extension by affecting cytoskeletal dynamics. Signaling through PI3K is thought

to be the major pathway mediating neurotropic effects of neurotrophins (Yuan et al.,

2003; Reichardt, 2006). It is interesting to note that neurotrophin signaling through

p75NTR and TrkR have generally opposing roles. Recent studies have demonstrated

that when p75NTR is expressed without concomitant expression of TrkR, apoptotic

signaling can occur.   In contrast, in the presence of Trk receptors, p75NTR apoptotic

pathways are suppressed allowing NF-kappaB signaling through p75NTR to

synergistically contribute to neuronal survival and growth effects mediated by TrkR

(Hamanoue et al., 1999).

A large body of literature suggests that neurotrophins promote neuronal

survival and axonal growth upon delivery to sites of adult CNS injury (Stichel and

Muller, 1998; Jones et al., 2001; Blesch et al., 2002). For example, NGF promotes

regeneration of chronically lesioned thermal and nociceptive axons (Grill et al., 1997a;

Romero et al., 2001).   Application of BDNF-secreting fibroblasts or marrow stromal

cells to sites of spinal cord injury increases survival of corticospinal tract axons and growth of other axons including motor, sensory and coerulospinal fibers (Lu et al., 2001; Lu et al., 2005). Transgenic cellular delivery of NT-3 promotes regeneration of lesioned corticospinal tract axons with associated partial functional recovery in acute (Grill et al., 1997a) and chronic spinal cord injury (Blesch and Tuszynski, 2003). NT-3 also promotes growth of transected ascending sensory axons across the dorsal root entry zone (DREZ) and within the dorsal columns of the spinal cord (Bradbury et al., 1998; Zhang et al., 1998; Bradbury et al., 1999; Oudega and Hagg, 1999; Ramer et al., 2000; Lu et al., 2003; Lu et al., 2004).

In addition to their neurotrophic effects, neurotrophins may provide guidance cues for regenerating axons. During development, growth cones of extending axons sense gradients of diffusible chemoattractive or repulsive factors leading them toward appropriate targets (Tessier-Lavigne and Placzek, 1991; Tessier-Lavigne, 1994). Although a tropic role for neurotrophins during development has not been definitively established, these proteins clearly exhibit chemoattractive effects on growth cones of regenerating and embryonic neurons in vitro (Gundersen and Barrett, 1979; Hory-Lee et al., 1993; Paves and Saarma, 1997; Gavazzi et al., 1999). During development, gradients of neurotrophins have been observed in CNS structures, such as the cingulate and retrosplenial cortexes (Lauterborn et al., 1994).

A number of studies have established a role for neurotrophins in directing target innervation in development of the CNS and PNS, suggesting that these proteins may act as target-derived guidance cues for growing axons (Tucker et al., 2001; Patel

et al., 2003). As one example, during development of the thalamocortical tract, axons from specific thalamic nuclei project into particular regions of the cortex concurrent with expression of NT-3 in these regions. In conditional NT-3 knockout mice these projections do not reach their cortical targets resulting in loss of function (for example, vision). These findings suggest that NT-3 expression by cortical neurons provides short distance tropic cues that are required for growth and synapse formation of particular thalamic projections (Ma et al., 2002). As a second example, DRG proprioceptive neurons express TrkC and their CNS targets (spinal cord motor neurons) express NT-3 (Ernfors and Persson, 1991; Zhou and Rush, 1994), suggesting a possible role for NT-3 as a target-derived guidance cue for these axons. Indeed, in Bax/NT-3 double knockouts proprioceptive fibers are found near motor neuron targets, but innervation does not occur (Patel et al., 2003; Genc et al., 2004). Since Bax knockout neurons do not require NT-3 for trophic support, these studies directly support a tropic role for NT-3 in proprioceptive target innervation. Finally, BDNF delivery to the developing optic tectum and NT-3 delivery to the developing corticospinal tract induce local axon sprouting, suggesting that these factors may normally signal the location of a target (Schnell et al., 1994; Cohen-Cory and Fraser, 1995). Similar to these developmental studies, in many in vivo axonal regeneration studies, axons grew directly toward the source of neurotrophic factor (Houweling et al., 1998; Zhou et al., 2003). Based on all of these studies, it is possible that neurotrophin delivery to sites of SCI, may create a diffusible gradient that guides regenerating growth cones toward the delivery site. Such a gradient may allow axons

to extend through a region that is otherwise inhibitory toward the source of neurotrophic factor.

II. *Ex vivo and in vivo gene transfer*

Neurotrophin delivery to the CNS has been problematic because these proteins do not cross the blood brain barrier. Neurotrophic factors have therefore been delivered to CNS ventricles, intrathecally or by direct protein injections. However, these approaches have several drawbacks. For example, ventricular delivery does not allow for localized delivery while intrathecal approaches achieve only crudely localized targeting. In addition, positioning of intrathecal pumps can damage surrounding tissue (Kwon et al., 2007). Neurotrophin protein can be delivered directly to specific sites with minimal damage, but multiple injections might be required for efficacy due to the short half-life of these proteins in vivo. Delivery of neurotrophins using genetic approaches has emerged as a powerful tool that eliminates some of these problems by providing continuous and localized delivery of therapeutic proteins.

As illustrated by many of the studies mentioned above, one effective means of providing a neurotrophic factor to sites of CNS injury is ex vivo gene delivery. In this approach, cells that have been genetically modified to express a neurotrophic factor(s) are grafted to a lesion site after injury. These grafted cells provide transected axons with a permissive substrate for axonal extension, neurotrophic support and possibly guidance cues (Blesch, 2000; Horner and Gage, 2000; Blesch et al., 2002; Blits et al.,

2002). Several types of cells, such as fibroblasts (Tuszynski et al., 1996; Grill et al., 1997a; Liu et al., 1999a), Schwann cells (Tuszynski et al., 1998), neural stem cells (Liu et al., 1999b) and bone marrow stromal cells (MSCs) (Lu et al., 2005) have been used for ex vivo gene delivery in the lesioned spinal cord. Because bone marrow is relatively easy to access in humans, MSCs are especially attractive as potential autologous cells for grafting into adult hosts. In potential clinical applications, autologous MSCs from individual patients could be cultivated, thereby preventing immune responses against grafted cells.

Ex vivo gene delivery has been useful for delivery of neurotrophic factors to lesion sites. However, if these factors indeed act as guidance molecules for regenerating axons they might also be used to promote axon growth beyond lesion sites. In vivo gene delivery, in which viral vectors directly transduce host cells to allow expression of a particular gene, provides a method for introducing neurotrophic factors to host tissue distal to sites of SCI. Viral delivery of therapeutic proteins has been used in a similar context to promote growth of sensory axons after dorsal root injury. Normally, injured axons cannot cross the inhibitory dorsal root entry zone (DREZ) that connects the dorsal roots to the spinal cord. However, when adenoviral vectors were used to deliver NT-3 (Zhang et al., 1998) or fibroblast growth factor (FGF) (Romero et al., 2001) to spinal cord tissue near injured dorsal roots, axons grew across the DREZ and entered the spinal cord. These studies suggest that expression of growth factors in spinal cord tissue beyond sites of injury might also promote growth of axons across the inhibitory glial scar and into distal host tissue.

Among potentially useful viral vector types, lentiviruses are especially well suited in vivo gene delivery of the CNS for several reasons. Most importantly, lentiviruses effectively transduce nondividing cells, such as neurons. Upon transduction, the viral DNA integrates into the host genome, allowing long-term transgene expression that is superior to that of other vectors (Abdellatif et al., 2006). In addition, the toxicity of lentiviral vectors is limited compared to other viral vectors including adenovirus (Naldini et al., 1996; Blomer et al., 1997; Zhao et al., 2003). Finally, improvements in production and safety, such as development of self-inactivating vectors (Miyoshi et al., 1998), have made lentiviral vectors an attractive tool for in vivo gene transfer. Because of these properties, lentiviruses have already been used successfully to deliver neurotrophic factors for treatment of several animal models of CNS degeneration (Takahashi et al., 1999; Bensadoun et al., 2000; Hottinger et al., 2000; Kordower et al., 2000; Consiglio et al., 2001; Georgievska et al., 2002).

III. Conditioning lesions as a strategy for enhancing intrinsic growth potential

Delivery of neurotrophic factors to the axon tip provides an important stimulus for axon growth, but appears to be insufficient to activate genetic growth programs required to achieve long distance axon growth (see Chapter 2). Thus, neurotrophic factor delivery may need to be combined with additional growth stimuli, such as activation of genetic programs that increase the intrinsic growth potential of the neuron (Lu, 2004). In the case of DRG sensory neurons, activation of genetic growth

programs can be achieved using peripheral nerve conditioning lesions. Peripheral

branch lesions cause upregulation of a number of growth-associated genes, such as

GAP-43 and CAP-43, that are not expressed after central branch lesions (Schreyer and

Skene, 1993). Thus, conditioning lesions appear to enhance the regenerative capacity

of central fibers by the initiation of a genetic growth program in response to the

peripheral nerve lesion, which is then exploited to promote regeneration of centrally

projecting fibers (Smith and Skene 1997).

The mechanisms by which conditioning lesions enhance CNS axon growth are

being intensely investigated and several important components of the response have

now been identified. The best studied of these is modulation of neuronal cAMP levels

by conditioning lesions. Modulation of cAMP affects axon outgrowth by two

mechanisms. Initially, increased cAMP levels lead to growth via a PKA dependent

mechanism, whereas over time the axon growth-promoting effect of the conditioning

lesion becomes PKA independent (Qiu et al., 2002). The initial PKA dependent phase

probably involves direct effects at the growth cone via modulation of Rho GTPases

(Song et al., 1998; Henley et al., 2004). Growth mediated by cAMP over the long

term appears to involve transcriptional changes mediated by direct activation of PKA

and subsequent phosphorylation of the transcription factor CREB. Transcription

initiated by CREB can promote axon growth by contributing to reversal of myelin-

mediated growth inhibition (Gao et al., 2004). The mechanisms by which this reversal

occurs are unknown, but may involve a number of downstream effectors. One

possible effector is arginase 1, an enzyme involved in catalysis of polyamine

production (Cai et al., 2002). Polyamines may be involved in regulation of microtubule assembly and other cytoskeletal events (Kaminska et al., 1992), among other functions, suggesting a possible role for these proteins in affecting axon outgrowth.

In addition to cAMP several other important components of the conditioning lesion response have been identified. Two cytokines, leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) have been shown to be important for DRG peripheral branch regeneration. IL-6 and LIF knock-out mice have demonstrated that these factors may be important components of the conditioning lesion response (Cafferty et al., 2001; Cafferty et al., 2004; Cao et al., 2006). These cytokines are thought to signal via the JAK-STAT signaling pathway, which involves phosphorylation of STAT3 (Levy and Darnell, 2002). Recent studies have shown that the conditioning lesion response requires STAT3 activation, suggesting a possible mechanism by which LIF and IL-6 could mediate growth-promoting effects after conditioning lesions (Qiu et al., 2005). STAT3 is not phosphorylated after lesioning of DRG central branches, whereas robust phosphorylation is observed after conditioning lesions, indicating that this transcription factor could be responsible, in part, for differential gene expression after conditioning lesions. Interestingly, phosphorylation of STAT3 has been associated with neuronal sprouting in the brain (Banan et al., 1998) and a possible downstream target of this factor, SPRR1A, promotes axon outgrowth, possibly via interaction with F-actin (Bonilla et al., 2002).

Additional factors involved in the conditioning lesion effect continue to be identified. Most recently the transcription factor ATF3 was described as a growth-promoting factor after conditioning lesions. Notably, ATF3 enhanced axon growth in vitro over non-inhibitory substrates, but was insufficient to promote axon growth over myelin-associated inhibitors in vitro or in vivo (Seijffers et al., 2006; Seijffers et al., 2007). These findings suggest that some components of the conditioning lesion response act to increase the growth potential of DRG neurons through mechanisms besides attenuating the effects of myelin inhibition. Further investigation of the mechanisms underlying the conditioning lesion may lead to an understanding of important signaling pathways that could be harnessed to increase axon growth therapeutically in the future. In this thesis, conditioning lesions are used to promote the overall growth potential of CNS neurons in combination with neurotrophic factor delivery.

Rationale and Hypotheses

When neurotrophic factors are delivered to spinal cord lesion sites by ex vivo gene delivery, diffusion of the factor from source cells may establish a gradient along which regenerating axons can extend. However, once axons have penetrated the graft, they do not continue to grow into host tissue distally. Upon reaching the source of neurotrophic factor expression, the loss of a growth factor gradient toward which to extend may result in axons remaining in the graft. However, in order for axonal regeneration to result in improved function, axons must regenerate beyond lesion sites

where they have the potential to re-establish connections with surviving neurons beyond the lesion site. The aim of this work is to determine whether neurotrophic factor gradients established in distal host tissue can promote growth of transected axons beyond lesion sites. Furthermore, we sought to determine whether such gradients can be used to promote axonal extension into denervated target regions where regenerated axons have the potential to form new synaptic contacts.

In this thesis, the regeneration of primary sensory neurons mediating proprioception and discriminative sensation from the hindlimbs of adult rats were used as a model system of SCI. Primary sensory neurons reside in lumbar DRGs L4, L5 and L6 (Hebel, 1976) and project peripherally via the sciatic nerve and centrally via the fasciculus gracilis located within the dorsal columns. Most centrally projecting fibers exit the dorsal column within several spinal segments of entry and synapse on motor neurons in the ventral horn of the spinal cord. However, about 15% of fasciculus gracilis axons travel the extent of the spinal cord and terminate predominately in the ipsilateral dorsal column nucleus known as the nucleus gracilis (Smith and Bennett, 1987). Primary sensory neurons express TrkC, the high-affinity receptor for the neurotrophin, NT-3 (McMahon et al., 1994) and are responsive to NT-3 delivery after SCI (Bradbury et al., 1998; Bradbury et al., 1999; Lu et al., 2003; Lu et al., 2004). Therefore, we have chosen to use this neurotrophin for both ex vivo and in vivo gene delivery in experiments designed to test our central hypothesis.

In **Chapter 1** we test the hypothesis that sustained neurotrophin gradients established in and distal to spinal cord lesion sites by ex vivo and in vivo delivery of NT-3 will allow ascending sensory axons to extend into and beyond spinal cord lesion sites.

In **Chapter 2** we determine whether sustained NT-3 gradients combined with peripheral nerve conditioning lesions will increase axon growth beyond lesion sites compared with neurotrophic factor delivery alone.

Finally, in **Chapter 3** we use a combination of conditioning lesions and NT-3 delivery to the nucleus gracilis to test the hypothesis that neurotrophins can guide axons to denervated targets and lead to the formation of new functional synaptic connections.

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CHAPTER 2

**Neurotrophin-3 Gradients Established by Lentiviral Gene Delivery Promote
Short-Distance Axonal Bridging beyond Cellular Grafts in the Injured Spinal
Cord**

ABSTRACT

Neurotrophic factor delivery to sites of spinal cord injury (SCI) promotes axon
growth into but not beyond lesion sites. We tested the hypothesis that sustained
growth factor gradients <u>beyond</u> regions of SCI will promote significant axonal
bridging into and beyond lesions. Adult rats underwent C3 lesions to transect
ascending dorsal column sensory axons and autologous bone marrow stromal cells
were grafted into the lesion to provide a cellular bridge for growth into the injured
region. Concurrently, lentiviral vectors expressing NT-3 or GFP (controls) were
injected into the host cord rostral to the lesion to promote axon extension beyond the
graft/lesion. Four weeks later, NT-3 gradients beyond the lesion were detectable by
ELISA in animals that received NT-3-expressing lentiviral vectors, with highest
average NT-3 levels located near the rostral vector injection site. Significantly more
ascending sensory axons extended into tissue rostral to the lesion site in animals
injected with NT-3 vectors compared to GFP vectors, but only if the zone of NT-3
vector transduction extended continuously from the injection site to the graft; any
"gap" in NT-3 expression from the graft to rostral tissue resulted in axon bridging
failure. Despite axon bridging beyond the lesion, regenerating axons did not continue

to grow over very long distances, even in the presence of a continuing growth factor gradient beyond the lesion. These findings indicate that a localized and continuous gradient of NT-3 can achieve axonal bridging beyond the glial scar, but growth for longer distances is not sustainable simply with a trophic stimulus.

INTRODUCTION

Following spinal cord injury (SCI) in adult mammals, axons do not regenerate and essential neuronal connections are permanently lost. The failure of injured axons to regenerate results from a combination of factors including a lack of permissive growth substrates within lesion sites, the presence of inhibitory molecules that impair axonal outgrowth (Filbin, 2003; Silver and Miller, 2004), lack of neurotrophic support (Tuszynski et al., 1996; Bradbury et al., 1999; Liu et al., 1999a) and the failure of CNS neurons to maximally activate cellular growth programs after injury (Snider et al., 2002). However, the application of neurotrophic factors, such as the neurotrophins, to injured axons or neuronal cell bodies can promote the survival and growth of adult neurons after injury (Schnell et al., 1994; Tetzlaff et al., 1994; Xu et al., 1995; Kobayashi et al., 1997; Ye and Houle, 1997; Grill et al., 1997a; Jakeman et al., 1998; Liu et al., 1999b; Blits et al., 2000; Ramer et al., 2000; Bamber et al., 2001; Coumans et al., 2001; Himes et al., 2001; Lu et al., 2001; Plunet et al., 2002), suggesting that the regenerative capacity of adult CNS neurons may be enhanced.

The transplantation of genetically modified cells expressing neurotrophic factors to lesion sites has been investigated as one means to augment axonal

regeneration after SCI (Menei et al., 1998; Tuszynski et al., 2002; Ruitenberg et al., 2003; Blesch et al., 2004; Lu et al., 2005). Cells transplanted into lesion sites can provide a permissive substrate for axonal extension through lesioned areas, and when transduced to overexpress growth factors, can also provide neurotrophic support for growing axons. While this approach generates relatively robust axonal growth into lesion sites, axons rarely exit the graft to reinnervate the distal host spinal cord. The lack of true axonal bridging following growth factor delivery solely within spinal cord lesion sites is in fact predicted by the observation that axons extend down gradients of diffusible molecules. For example, during nervous system development and in models of regeneration, growth cones extend along gradients of diffusible chemoattractive or repulsive factors (Tessier-Lavigne, 1994). Several studies indicate that neurotrophins and other diffusible proteins may provide such guidance cues for embryonic and regenerating axons (Letourneau, 1978; Gundersen and Barrett, 1979; Gallo et al., 1997; Paves and Saarma, 1997; Ringstedt et al., 1997; Houweling et al., 1998; Zhang et al., 1998; Kirik et al., 2000; Romero et al., 2001; Tucker et al., 2001; Dontchev and Letourneau, 2002; Zhou et al., 2003; Genc et al., 2004; Tessarollo et al., 2004). Thus, provision of neurotrophic factors both within and *beyond* a lesion site could establish an extended gradient enabling axons to grow not only into, but also beyond, cellular grafts in lesion sites.

We have shown previously that transected ascending sensory axons extensively penetrate cellular grafts expressing the neurotrophin NT-3, but that axons do not grow beyond lesion sites (Lu et al., 2003). In addition, if single injections of

NT-3 protein within and beyond lesion sites are combined with cAMP administration, axons will bridge beyond the lesion (Lu et al., 2004). In the present study we examined whether provision of continuous, high levels of NT-3 by lentiviral-mediated delivery beyond a lesion site would promote axonal bridging. We find that axons indeed bridge beyond the lesion if provided a continuous gradient of NT-3, but that long-distance growth is not supported.

MATERIALS AND METHODS

Production of lentiviral vectors:

The vector pLV (Pfeifer et al., 2002) was used to construct NT-3-secreting and control (GFP-expressing) vectors (Figure 1). The NT-3 lentiviral vector was constructed by cloning the complete human NT-3 cDNA plus Kozak consensus sequence into the Bam HI and EcoRI sites of the vector pIRES2-EGFP (Clontech, Palo Alto, CA). The resulting plasmid was digested with NheI and BsrGI to excise the fragment containing huNT-3-IRES-EGFP. This fragment was cloned into the NheI and BsrGI sites of the vector pLV-GFP, thereby replacing the GFP cassette. NT-3 or GFP expression was driven by the CMV/ß-actin hybrid promoter (CAG) (Niwa et al., 1991).

Third generation lentiviral vector plasmids with a split genome packaging system were used for the production of HIV vectors. In vitro and in vivo, transgene expression was initiated from the CAG promoter within 48 hours. VSV-G pseudotyped lentivirus was generated by transient co-transfection of a vector construct

(15 μg) with the VSV-G expressing construct pMDG (5μg) and the packaging

construct (10 μg) into 293T cells, as previously described (Naldini et al., 1996;

Blesch, 2004) High titer stocks of HIV vectors were prepared by ultracentrifugation.

Titers of GFP expressing virus were determined by infection of 293T cells using serial

dilutions. After 48 hours, GFP-expressing colonies were quantified for each dilution

to determine infectious units/ml (IU/ml). Vector stocks were also assayed for p24

antigen levels using an HIV-1 p24 specific ELISA kit (DuPont) as described (Naldini

et al., 1996). NT-3 vector preparations contained 112-134 μg/ml p24 and 4 x 10^7-1.5

x 10^8 IU/ml. Control vector preparations contained 95-112 μg/ml p24 and 1-5 x 10^8

IU/ml.

Isolation of marrow stromal cells (MSCs) to be used as graft cells:

　　　Rat primary marrow stromal cells were isolated according to the method of

Azizi et al. (Azizi et al., 1998). Briefly, Fischer 344 adult female rats were

anesthetized with a combination (2 ml/kg) of ketamine (25 mg/ml), xylazine (1.3

mg/ml) and acepromazine (0.25 mg/ml), decapitated, and tibias and femurs were

dissected. After removing the end of each bone, 5 ml alpha-MEM medium

(Gibco/BRL) medium was injected into the central canal of the bone to extrude

marrow. Cells were cultured in alpha-MEM medium (Gibco/BRL) supplemented with

20% fetal bovine serum (FBS) and antibiotics. Non-adherent cells were removed after

24 hours. Cells were passaged twice and either frozen or transduced with MLV-based

retroviral vectors for the expression of NT-3. Before grafting, cells were thawed and cultured in the same media as above.

Transduction of marrow stromal cells with retroviral vectors prior to grafting:

Syngenic MSCs were genetically modified to produce and secrete human NT-3 using a stable PA317 retrovirus producer cell line as previously described (Grill et al., 1997a). Conditioned media from producer cultures was used to infect MSCs and transduced cells were selected for G418 resistance (100 μg/ml) for 11 days. After selection, cells were grown to confluency and 24 h supernatants were collected for ELISA analysis of NT-3 protein expression (NT-3 Emax ImmunoAssay System, Promega, Madison, WI). Transduced MSCs produced approximately 165 ng NT-3/10^6 cells/24 hours.

BrdU-labeling of marrow stromal cells in vitro:

MSCs were cultured in media containing 2 μm BrdU for 72 hours prior to surgery, with media exchanged every 24 hours. Before collection for surgery, cells were washed 3 times with PBS, isolated by trypsinization followed by centrifugation, and then washed an additional time in 10 ml PBS.

Animal subjects:

Adult female Fischer 344 rats (n = 79) weighing 150-200 gm were used. Institutional, NIH, and Society for Neuroscience guidelines on animal care were

followed. Animals were divided into groups based on the type of cells grafted to the lesion site and the type of lentiviral vector received (Table 1, Figure 1). Graft cells were either naïve marrow stromal cells (**MSC**), or marrow stromal cells transduced in vitro to express NT-3 (**MSC-NT-3**). Lentiviral vectors expressed either GFP only (**Lenti-GFP**), or NT-3 plus GFP (with GFP expressed from an internal ribosome entry site, **Lenti-NT-3,** Supplementary Figure 1). Additional unlesioned animals without graft or lentivirus injections served as controls for immunolabeling or ELISA.

Lesion surgery and vector injections:

Animals underwent a laminectomy at spinal level C2/C3. Dorsal column lesions were made at the caudal aspect of C3 using a Kopf microwire device (Figure 1, Kopf Instruments, Tujunga, CA). After fixation in a spinal stereotaxic unit, a small dural incision was made. The wire knife was lowered into the spinal cord to a depth of 1.1 mm ventral to the dorsal cord surface and 1.1 mm to the left of the midline. The tip of the wireknife was extruded, forming a 2.25 mm-wide arc that was raised to the dorsal surface of the cord, transecting the dorsal funiculus including the ascending (sensory) and descending (corticospinal) axon tracts. To ensure complete axotomy of the entire dorsal column, spinal tissue was compressed against the microwire knife surface using a microaspiration pipette until all visible white matter was transected.

Immediately following the lesion, 2 μl (approx. 75,000 cells/μl) of untransfected MSCs (MSC) or MSCs that had been genetically modified to express NT-3 (MSC-NT-3) were injected through a small hole in the dura mater into the lesion

space. Cells were injected using a pulled glass micropipette attached to a PicoSpritzer II (General Valve, Fairfield, NJ). Lentiviral vectors (2.5 μl) were injected superficially through pulled glass capillaries 2.5 mm rostral to the lesion site into the spinal cord midline at a depth of 0.5 and 1 mm (1.25 μl at each depth), at a rate of 1 μl/min. Pipettes were left in place for 1 min after the injection and were then slowly withdrawn. Overlying muscle layers were sutured and the skin was stapled. Animals were euthanized after four weeks.

Transganglionic labeling of ascending sensory projections:

Animals used for histological analysis (n = 41) received injections of Cholera toxin B (CTB, 1%; 2 μl, List Biologicals; Campbell, CA) into the right and left sciatic nerves three days before euthanasia to label ascending sensory projections.

Measurement of in vivo gradients of NT-3 generated by lentiviral gene delivery:

The ability of lentiviral vectors expressing NT-3 to establish trophic factor gradients within and beyond sites of SCI were examined in the groups indicated in Table 1. Production of NT-3 protein was measured in transverse segments of spinal cords or dissected graft tissue using an NT-3 specific ELISA. Four weeks post-lesion/injection, animals received an overdose of anesthesia and were transcardially perfused with 50 ml ice-cold 0.1 M phosphate-buffered saline (PBS). To obtain isolated graft tissue, the graft region was dissected from the surrounding spinal cord

and immediately frozen. An equivalent region of tissue from the dorsal half of the spinal cord was dissected from intact animals. To obtain transverse spinal cord segments, the spinal cord was rapidly dissected and the lesion center was located under a dissection microscope. A 7 mm long section of spinal cord, extending 5 mm rostral and 2 mm caudal to the lesion center, was carefully and rapidly dissected and immediately frozen. Spinal cords were embedded into TBS Tissue Freezing Media (Triangle Biomedical Sciences; Durham, NC) on dry ice. A cryostat was used to cut spinal cords into 100 μm-thick transverse sections, which were pooled into 1 mm segments (10 adjacent 100 μm segments) for ELISA.

The 1 mm segments, dissected graft tissue, or intact spinal cord segments were weighed, lysed by sonication in lysis buffer (40 μl/mg tissue) containing protease inhibitors (1 mM phenyl methyl sulfonyl fluoride, PMSF) and a protease inhibitor cocktail, (Roche Diagnostics; Mannheim, Germany), centrifuged 10 min. at 14,000 rpm at 4°C, and used for NT-3 ELISA according to manufacturer's instructions (NT-3 Emax ImmunoAssay System, Promega; Madison, WI). For transverse segments, overall levels of NT-3 were compared between the four groups by repeated measures analysis of variance (ANOVA) followed by Fischer's posthoc test. Levels of NT-3 in different 1 mm segments within a group were also compared by Fischer's posthoc tests. For dissected graft tissue and intact spinal cord analyses, groups were compared by ANOVA followed by Fischer's post-hoc test. A significance criterion of $p < 0.05$ was used for all statistical tests.

<u>Immunohistochemical analysis of spinal cord and brainstem sections:</u>

Animals used for immunolabeling were transcardially perfused with 100 ml cold PBS followed by 300 ml 4% paraformaldehyde in phosphate buffer. Spinal cords and brainstems were removed, post fixed overnight in 4% paraformaldehyde, and cryoprotected in 0.1 M phosphate buffer containing 30% sucrose at 4°C. For spinal cords, tissue was blocked in TBS Tissue Freezing Media (Triangle Biomedical Sciences) on dry ice and sagittal sections were cut at 35 μm intervals with a cryostat. Every seventh section was immediately mounted on glass slides for Nissl staining. For brainstems, blocked tissue was cut in the coronal plane at 40 μm thickness and every ninth section was immediately mounted for Nissl staining.

Spinal cord sections were triple-labeled for CTB using streptavidin-HRP light level immunohistochemistry followed by GFP and GFAP fluorescent immunolabeling. The following immunocytochemical markers were used: cholera toxin beta-subunit (goat anti-CTB 1:80,000; List, Campbell, CA), to detect ascending sensory axons; glial fibrillary acidic protein (GFAP, monoclonal 1:1000; Chemicon, Temecula, CA) to label astrocytes; and green fluorescent protein (rabbit anti-GFP 1:750; Molecular Probes, Eugene, OR) to label vector-transduced cells. Immunocytochemical labeling for CTB was performed first with streptavidin-HRP-based light level immunohistochemistry using free floating sections in the following protocol: 1) overnight incubation in primary antibodies at 4ºC, 2) incubation for one hour with biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA) at room temperature, 3) one hour incubation with avidin-biotinylated peroxidase

complex (1:150; Vector Elite Kit, Vector Laboratories) at room temperature, and 4)

treatment for 4 minutes with 0.05% solution of 3.3' diaminobenzidine, 0.01% H_2O_2

and 0.04% nickel chloride at room temperature. Fluorescence immunohistochemistry

for GFP and GFAP was then performed on the same sections according to the

following protocol: 1) overnight incubation in primary antibody, then 2) 2.5 hour

incubation with fluorescent-conjugated secondary antibodies (donkey anti-rabbit

Alexa-488 at 1:150; donkey anti-mouse Alexa 594 at 1:200; Molecular Probes).

Brainstem sections were immunolabeled for CTB using the same streptavidin-HRP-

based light level procedure as described above for spinal cord. Labeled sections were

mounted and coverslipped with Cytoseal 60 mounting media (Richard Allen

Scientific, Kalamazoo, MI).

Quantification of axonal profiles distal to the lesion:

   To determine the number of CTB-labeled axons that extended beyond the

dorsal column lesion site, 1-of-7 35-μm sagittal spinal cord sections triple-labeled for

CTB, GFAP and GFP were examined under fluorescence and transmission light

microscopy by an observer blinded to group identity. GFAP labeling of astrocytes

was used to identify the lesion boundary. The rostral lesion border was defined as the

region where GFAP labeled astrocytic cell bodies were found rostral to the lesion.

Since this border was often irregular, a dorso-ventral line representing the lesion edge

was defined as the *most rostral extent* of the lesion site.

A calibrated reticle eyepiece was used to delineate regions 0-250 μm, 250-500 μm and 500-1000 μm rostral to the defined dorso-ventral line and CTB-labeled axonal profiles were quantified in each region at 200X and 400X magnification. Axons found in GFAP-labeled areas that were caudal to the defined dorso-ventral line were counted and included in the number of profiles from 0-250 μm from the lesion. The distance from the rostral lesion border to the first GFP-expressing cell (indicating the limit of the zone of lenti vector transduction) in the rostral spinal cord was also measured. For animals in which the zone of vector transduction was within 100 μm of the lesion border, the average number of axonal profiles in each region, 0-250 μm, 250-500 μm and 500-1000 μm from the lesion edge, was compared between the four treatment groups by ANOVA followed by Fischer's post-hoc analysis, using a significance criterion of $p < 0.05$. To identify in greater detail the position of axons within the first 250 μm-distance from the rostral graft-host interface, an additional sagittal spinal cord series was analyzed as described above, except that axon numbers within 50 μm segments, rather than 250 μm segments, were quantified rostral to the GFAP border.

Quantification of axonal density and GFP labeling in cell grafts:

In a series of sagittal sections consisting of every 7th 35-μm-thick section, two sections containing the most axon-dense grafts were selected for analysis by an observer blinded to group identity. For each section, 200X magnification photomicrographs indicating: 1) GFAP labeling surrounding the lesion site, 2) CTB-

labeled axonal profiles, and 3) GFP-labeled cells within the lesion site, were obtained

using Magnafire software (Version 2.0, Karl Storz Imaging). Identical light,

microscope, and camera settings were used for all image acquisition procedures.

Using ImageJ software (ImageJ 1.33u, Wayne Rasband, NIH, USA), light-level

images indicating CTB-labeled axonal profiles were converted to black and white

binary images using appropriate threshold settings such that axonal profiles appeared

black and background appeared white. Non-specific labeling of objects other than

axons, such as artifactual spots resulting from tissue processing, was edited from

images as previously described (Lu et al., 2005). Area-matched fluorescent

photographs of GFAP labeling were used to outline the graft/lesion area and outlines

were superimposed onto binary-converted light level images. ImageJ software was

then used to measure black pixels per total pixels in the outlined graft area. In the

same sections, an equivalent procedure was used to measure the density of GFP-

labeled cells within the GFAP-defined lesion border.

Evaluation of lesion completeness:

Incomplete lesions would result in CTB labeling of the nucleus gracilis in the

causal medulla. In all subjects, the medulla was sectioned coronally at 40 μm

intervals and every ninth section was labeled for CTB. Using transmission light

microscopy and 200X and 400X magnification, an examiner blinded to group identity

determined whether axons or terminals were evident. Four intact animals underwent

CTB injections of both sciatic nerves as positive controls for medullary labeling.

<u>Assessment of survival and differentiation of grafted marrow stromal cells</u>:

To examine whether grafted, BrdU-labeled MSCs survived in the lesion site, 1-of-7 spinal cord sections were labeled for BrdU (sheep anti BrdU 1:250; Abcam, Cambridge, MA) in animals that received BrdU-labeled graft cells according to the HRP-based light level procedure described above. To test whether BrdU-labeled MSCs differentiated into cells of a neural lineage, double immunolabeling was performed using the neural markers NeuN, GFAP and Iba (for neurons, astrocytes and macrophages/microglia, respectively) per established protocols (Yang et al., 2006). The following primary antibodies were used: BrdU (sheep 1:200) NeuN (monoclonal 1:250; Chemicon), GFAP (1:1000; Chemicon) or IbaI (rabbit 1:1500; Wako, Richmond, VA). Representative sections from each experimental group were examined using thin-plane scanning confocal microscopy, and putatively double-labeled cells were reconstructed in xy, yx and xz planes.

RESULTS

Injections of lentiviral vectors establish a rostro-caudal gradient of NT-3 in the injured spinal cord.

We tested the ability of lentiviral vectors expressing NT-3 to establish trophic factor gradients within and beyond sites of SCI using the experimental model illustrated in Figure 1. Lentiviral vectors expressed NT-3 and Green Fluorescent Protein (Lenti-NT-3), or GFP alone (Lenti-GFP) in controls (Supplemental Figure 1).

To determine whether exogenous NT-3 formed a gradient beyond spinal cord lesion sites, NT-3 levels were quantified by ELISA in transverse spinal cord segments in four experimental groups (Table 1, groups 1-4). ELISA indicated that overall NT-3 protein levels were higher in subjects that received Lenti-NT-3 injections (Figure 2A) compared to subjects that received Lenti-GFP injections (repeated measures ANOVA $p < 0.001$, Fischer's posthoc analysis $p < 0.01$). In animals that received Lenti-NT-3 injections, gradients of NT-3 were present distal to the lesion site. Peak NT-3 levels were detected in transverse spinal cord segments containing the lentiviral vector injection site (125 ± 50 ng/g and 96 ± 21 ng/g, groups 1 & 2, respectively) and progressively diminished as a function of distance from the injection site toward the lesion.   Animals that received Lenti-GFP injections did not show rostro-caudal gradients, but exhibited consistently low levels of NT-3 in spinal cord segments distal to the lesion site (~0.4 ng/g). These levels are similar to those previously reported in intact rat spinal cord (~0.6 ng/g, (Tokumine et al., 2003) and to those we measured in intact spinal cord (see below). In the two groups that received Lenti-GFP injections, animals that received MSC-NT-3 grafts exhibited two-fold higher levels of NT-3 in the two spinal cord segments that included the lesion area than animals that received MSC grafts (Figure 2B). These levels were substantially exceeded in Lenti-NT-3 injected animals.

The above analysis did not directly measure NT-3 levels within grafts, since 1 mm spinal cord segments contained both graft and surrounding host spinal cord. To accurately measure levels of NT-3 encountered by axons within the graft environment,

ELISA was performed on dissected grafts in separate groups of animals (Table 1). After 4 weeks, MSC-NT-3 grafts produced significantly higher levels of NT-3 compared to the intact spinal cord and to MSC grafts lacking NT-3 expression (Figure 2C), indicating that MSC-NT-3 graft cells expressed supraphysiological levels of NT-3 for the duration of the experiment. Levels of NT-3 in MSC-NT-3 grafts (4 ng/g) were 5-fold lower than levels of NT-3 in rostral spinal cord segments taken from animals that received Lenti-NT-3 injections (> 20 ng/g). Thus, NT-3 levels generated by Lenti-NT-3 vectors were high enough that the presence of NT-3-expressing graft cells did not affect the formation of a rostral gradient. In contrast, levels of NT-3 in rostral spinal cord segments from Lenti-GFP injected animals (0.4 ng/g) were lower than those measured in MSC-NT-3 grafts. Altogether, these data indicate that injection of lentiviral vectors expressing NT-3 can establish a successively increasing neurotrophic factor gradient away from the lesion site that is not achieved if NT-3 is expressed only by graft cells.

Lentiviral NT-3 gene transfer combined with marrow stromal cell grafts support bridging axonal regeneration across spinal cord lesion sites.

We examined whether lentivirally generated NT-3 gradients would support growth of transected ascending sensory axons into and beyond a dorsal column lesion site. CTB-labeled ascending sensory axons were observed beyond the astrocyte-defined lesion border only in animals receiving injections of Lenti-NT-3 vectors rostral to the lesion site (Figure 3A-3C). In addition, high magnification images of

CTB-labeled axons demonstrated that axons topographically associated with regions

of NT-3-expressing vector-transduced cells (Figure 3D-3G).

Axons extending beyond the lesion site appeared to be regenerating rather than

spared axons or axons that had circumvented the lesion site, based on several

observations. First, the morphology of axons extending beyond the lesion site was

circuitous and their course was non-linear, unlike intact axons. Second, axons bridged

beyond the lesion border at all dorso-ventral levels of the grafts, not only at the dorsal

or ventral-most aspect where spared axons might reside (Steward et al.,

2003)(Supplementary Figure 2). Third, no CTB-labeled axonal profiles were

observed ventral to the lesion site or in lateral unlesioned portions of the spinal cord.

Fourth, sectioning of the medulla through the entire extent of the nucleus gracilis

indicated an absence of CTB labeled axons in all lesioned subjects, confirming that

lesions were complete. In contrast, CTB injections in four intact rats (Table 1)

showed the typical distribution of CTB label in the nucleus gracilis (Supplementary

Figure 3).

We quantified bridging axons and axon density within cellular grafts in animal

subjects in which the zone of vector transduction was within 100 μm of the rostral

lesion border (Figure 4, Supplementary Figure 4). Both groups of animals that

received lentiviral vectors expressing NT-3 exhibited axons emerging rostrally from

the MSC graft, while animals that received control Lenti-GFP vectors exhibited

minimal to no axon growth beyond the graft (Figure 4A). Statistically significant

differences in axon bridging compared to control Lenti-GFP injected animals were

observed for up to 500 μm in subjects that received Lenti-NT-3 vector injections combined with MSC-NT-3 graft cells. In addition, more axons were observed at the 0-250 μm distance in this group than in the group that received naïve MSC graft cells combined with Lenti-NT-3 vectors.

Within the graft, axon density was increased by the presence of MSC-NT-3 graft cells and to an even greater degree by injection of Lenti-NT-3 vectors rostrally (Figure 4B). Lenti-NT-3 vector injections combined with MSC-NT-3 graft cells resulted in the highest axon density, significantly higher than both groups that received Lenti-GFP vectors. Among animals that received Lenti-GFP vectors, subjects with MSC-NT-3 grafts exhibited a 9-fold increase in graft axon density compared to subjects that received naïve MSC grafts (Figure 4B), indicating that graft cell expression of NT-3 increases axonal penetration of grafts. Consistent with previous data (Lu et al., 2003), axon density was significantly higher in MSC-NT-3 grafts than in MSC grafts when only Lenti-GFP groups were compared (p = 0.01). Nissl staining and immunohistochemical detection of BrdU-prelabeled MSCs indicated that MSC and MSC-NT-3 cells survived and integrated at the lesion site (Supplementary figure 5). Double labeling for BrdU and neural markers indicated that graft cells did not differentiate into cells of neural lineage.

Regenerating axons extend and turn toward local sources of NT-3.

We observed axon extension beyond the lesion site only when Lenti-NT-3 vectors diffused the entire 2.5 mm distance from the rostral injection site to the

lesion/graft site (13-of-21 Lenti-NT-3 injected animals). In these subjects, Lenti-NT-3 transduced cells were evident within 100 μm of the host/lesion border (Figure 5A-5C). In the majority of these animals some vector transduction of cells within the graft was also observed, but the extent of this transduction did not differ between Lenti-NT-3-injected groups (based on analysis of GFP density in grafts, p = 0.34). When Lenti-NT-3 vector failed to diffuse to the lesion border, axons were not observed beyond the lesion site (Figure 5D and 5E). Control Lenti-GFP injected groups exhibited only rare axons extending beyond the graft, regardless of the distance of Lenti-GFP transduction from the lesion site (Figure 5F-5H). These results suggest that the ability of sensory axons to extend beyond a lesion site is highly sensitive to local availability of NT-3.

When we compared expression of the GFP reporter gene in Lenti-NT-3 injected animals with the topography of sensory axon growth distal to the lesion, axon extension was closely associated with regions of NT-3 expression. For example, when vector distribution was restricted to specific sub-regions of the dorsal columns rostral to the lesion site, axonal profiles preferentially extended into these regions (Figure 5, 6). Thus, regenerating dorsal column sensory axons exhibit specific in vivo extension and directional growth toward local sources of growth factors, similar to findings of in vitro "turning assays" characterizing axonal responses to diffusible molecules (Gundersen and Barrett, 1979; Paves and Saarma, 1997).

Regenerating sensory axons do not extend for long distances despite the presence of extended NT-3 gradients.

The preceding data indicate that regenerating sensory axons require immediate spatial availability of NT-3 adjacent to lesion/graft sites in order to emerge from the inhospitable host/lesion interface, and that regenerating axons will extend preferentially into local regions expressing NT-3. While this indicates that sensory axons are sensitive to NT-3 and exhibit directional growth toward NT-3 sources, axons nonetheless did not continue to grow along the extended gradients of NT-3 that were established in the spinal cord rostral to the lesion. Indeed, the number of axons bridging 200 μm beyond the rostral lesion site was approximately half the number of axons crossing the rostral lesion interface, and this number was further reduced 2-fold at a distance of 300 μm (Supplementary Figure 4). The maximum distance of axon growth in animals receiving Lenti-NT-3 vector was 1000 μm beyond the lesion, whereas a continuing gradient of NT-3 extended beyond this point for an additional 1-1.5 mm (as indicated by ELISA, Figure 2). Instead of continuing growth rostrally, axons sprouted locally, creating a dense network of axonal profiles upon reaching a region of high NT-3 expression rostral to the lesion (Figure 6).

DISCUSSION

The present findings demonstrate sensory axon sensitivity to local availability of growth factors after injury to the CNS. This sensitivity allows axons to emerge from cellular grafts placed in lesion sites. Axons exhibit specific association with local sources of NT-3, such that growth is dependent upon spatial distributions of NT-3 expression beyond a lesion site. While this growth parallels some features of in

vitro "turning assays" that have shown growth cone or neurite extension toward

diffusible chemotropic gradients (Letourneau, 1978; Gundersen and Barrett, 1979;

Ming et al., 1997; Paves and Saarma, 1997; Genc et al., 2004), the growth we

observed in vivo cannot be identified as strictly "chemotropic" in nature because

axons failed to follow gradients of NT-3 that extended for long distances beyond the

lesion cavity.

While axons did emerge from MSC grafts in both groups of animals that

received Lenti-NT-3 vectors, axon growth beyond lesion sites was significantly

greater when NT-3 delivery occurred within *and* rostral to the graft. The functional

significance of NT-3 expression by MSCs in the graft is uncertain, but may have

helped initiate the regenerative response by providing a source of NT-3 located

immediately adjacent to the injured sensory axons at the distal host/graft interface. In

addition, NT-3-secreting MSCs in the lesion cavity actively express NT-3 at the time

of grafting, whereas gene expression from injected lentiviral vectors becomes

detectable no sooner than 24-48 hours post-transduction (Baekelandt et al., 2002). For

these reasons NT-3-expressing MSCs may have enhanced recruitment of axons into

the lesion site, increasing the number of axons in position to respond to the NT-3

delivered at more rostral sites. In support of this hypothesis, MSC-NT-3 grafts

promoted 9-fold more axonal growth into lesion sites than MSC grafts in control

animals that received Lenti-GFP vectors. Alternatively, the greater axon growth in

animals that received Lenti-NT-3/MSC-NT-3 treatment could have resulted from

superior Lenti-NT-3 vector diffusion into the lesion/graft site compared to the Lenti-

NT-3/MSC group. However, quantification of GFP density in the lesion/graft site showed no difference between these groups, arguing against this possibility.

Axon growth extended beyond the lesion site in animals that received Lenti-NT-3 vectors, but did not continue for long distances despite the presence of NT-3 gradients in the rostral spinal cord. There are several potential explanations for the lack of long distance extension. Inhibitory molecules beyond the injured region create an environment that is non-permissive for axonal extension over long distances. Inhibition related to the presence of astrocytes (McKeon et al., 1991), the extracellular matrix (Silver and Miller, 2004) and multiple myelin-associated molecules (Filbin, 2003) may counteract the stimulating influence of growth factors and terminate what might otherwise be continuing growth along a gradient of NT-3. Higher concentrations of NT-3 might overcome these inhibitory influences (Forander et al., 1994). Also, stimulatory factors other than NT-3 may be present in the immediate environment surrounding the lesion site that diminish as distance from the injury increases, thereby depleting the extending axon of a necessary stimulus to support long-distance growth. For example, macrophages accumulating around a lesion may constitute a local but not extended source of diffusible factors to support growth (Yin et al., 2003; Yin et al., 2006).

Alternatively, NT-3 may provide only very short-distance cues for axon growth or in fact signal termination of growth in certain situations, as suggested by studies of nervous system development (Ernfors and Persson, 1991; Chen and Frank, 1999; Tucker et al., 2001; Ma et al., 2002; Mori et al., 2002; Genc et al., 2004).

Previous experiments indicate that during development, DRG axon navigation towards peripheral targets involves dynamic changes in NT-3 expression, such that NT-3 levels are increased in regions where axon tips are growing and decreased in these same regions as axons grow distally. Eventually, NT-3 expression is restricted to final target tissues (Farinas et al., 1996). Thus, sustained NT-3 expression, such as that generated by lentiviral expression, might represent saturating levels that send a "stop" signal for axon growth. A mechanism for decreasing NT-3 expression as axons reach one spatial location may be necessary to allow continued growth into more distant locations. Finally, although we detected an overall rostro-caudal NT-3 gradient by ELISA, lenti-transduced cells expressing NT-3 may create local micro-gradients of NT-3 as diffusion occurs. It is therefore possible that axons extend along micro-gradients for short distances and are unable to detect a more extended gradient.

We recently reported that the combination of NT-3 protein beyond a lesion site, together with augmentation of cyclic AMP levels in the sensory neuronal soma, promoted longer-distance axonal growth than observed in the present study (Lu et al., 2004). This is notable, because the magnitude and persistence of NT-3 delivery beyond the lesion site achieved in the current study likely exceeded by several-fold the amount of NT-3 delivered in our previous study, wherein 1 μg of NT-3 protein was injected *rostral* to the lesion site at a single time point. Yet the previous study achieved greater distances of sensory axonal growth, with axon extension over a 2 mm distance beyond the lesion cavity to the site of rostral NT-3 injection. However, the *density* of axon growth within regions of NT-3 delivery in the present study was

higher than we observed in the previous study. This suggests that cAMP and NT-3 may fundamentally differ in their effects on the growth of injured axons in the adult CNS. Whereas cAMP may alter the intrinsic ability of adult axons to elongate, possibly by upregulating expression of growth-related genes or impairing responses to inhibitory environmental molecules (Gao et al., 2003), NT-3 may act at a more local and terminal level, inducing axonal sprouting and termination (Cafferty et al., 2001). Indeed, the morphology of axons extending densely into regions of NT-3 expression in the present study was fine and highly varicose, suggesting potential attempts to locate neuronal targets. Alternatively, as indicated in the preceding paragraph, the greater amounts of NT-3 delivered in the present study may simply have constituted saturating levels of growth factors to the extending axons, overwhelming the ability of the axon to sense a continued gradient.

The effects of NT-3 observed in the present study raise the possibility that the sole administration of this or other trophic factors at and beyond the injury site may not achieve the goal of long-distance axonal growth after SCI. If the biological role of the growth factor is indeed to provide a recognition signal for target reinnervation, rather than a long-distance growth signal, a modified strategy would be required to promote substantial long-distance axonal regeneration. Combinatorial approaches to facilitate regeneration might be optimal, wherein non-tropic agents recruit and stimulate the elongation of sufficient numbers of axons over biologically meaningful distances to reach an intended target, and trophic factors are administered into the

specific target to induce elaboration of terminals and enhance target reinnervation.

Future studies can be designed to test these possibilities.

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Table 2.1. Experimental groups. Animals were divided into 4 groups (Groups 1-4) based on the type of cells grafted to the lesion site and the type of lentiviral vector injected. Cellular grafts consisted of marrow stromal cells (**MSC**) or marrow stromal cells genetically modified to express NT-3 (**MSC-NT-3**). Lentiviral vectors expressed either GFP only (**Lenti-GFP**), or NT-3 plus GFP (**Lenti-NT-3**). Animals were used for ELISA or immunocytochemical analysis. Two additional groups of animals (Groups 5, 6) received MSC or MSC-NT-3 grafts, but no lentivirus injection, and were used to determine levels of NT-3 in dissected graft segments by ELISA. A final group of animals (Group 7) remained unlesioned, received no graft cells or lentivirus injection and was used as an immunohistochemical control to identify normal CTB labeling or to determine NT-3 levels in naïve spinal cord.

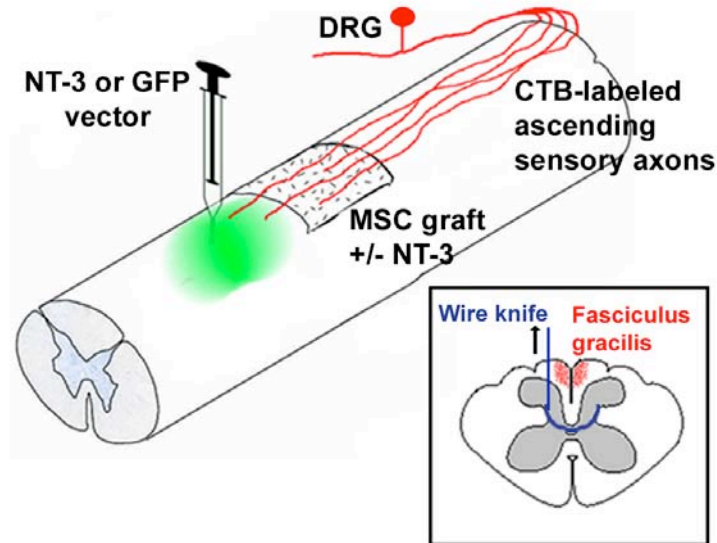| Groups	Cells in lesion site	Lentiviral vector injected rostral to lesion	Anatomical Analysis	ELISA
1	MSC-NT-3	Lenti-NT-3	N=11	N=6
2	MSC	Lenti-NT-3	N=10	N=6
3	MSC-NT-3	Lenti-GFP	N=8	N=6
4	MSC	Lenti-GFP	N=8	N=6
5	MSC-NT-3	No lentivirus		N=6
6	MSC	No lentivirus		N=5
7	No lesion	No lentivirus	N=4	N=3

Figure 2.1. Schematic illustrating experimental design. Adult rats underwent dorsal column wire knife lesions at cervical level 3 (C3) to transect ascending sensory axons (inset). Autologous bone marrow stromal cells (either naive or modified in vitro to express NT-3) were grafted into the lesion site and lentiviral vectors expressing NT-3 or GFP (control) were injected into the spinal cord 2.5 mm rostral to the lesion site. After 4 weeks, animals were killed for ELISA or ascending sensory axons were traced by injection of cholera toxin beta subunit (CTB) into the sciatic nerve and animals were killed 3 days later for histology.
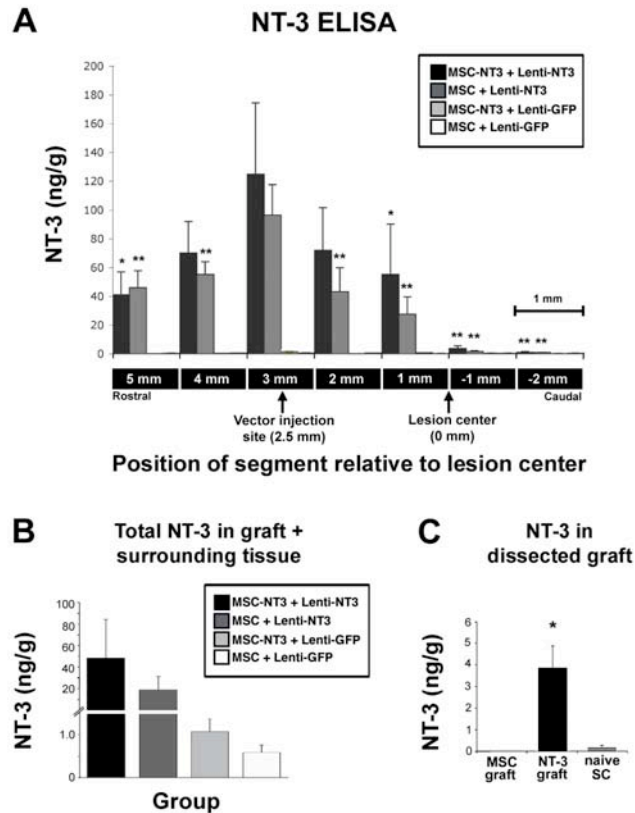
Figure 2.2. Injection of NT-3-expressing lentiviral vectors establishes a gradient of NT-3 in the injured spinal cord. (**A**) 4 weeks after lesion and vector injection, spinal cords were sectioned into 1 mm segments as shown schematically on the x-axis. ELISA of individual 1 mm segments indicated that in animals that received Lenti-NT-3 vector injections, but not Lenti-GFP injections, a gradient of NT-3 was established rostral to the lesion site. For groups that received Lenti NT-3 vector injections, asterisks indicate differences in NT-3 levels between the vector injection site (3 mm segment) and other segments in the same treatment group ($p < 0.001$, repeated measures ANOVA; *$p < 0.05$, **$p < 0.01$, Fischer's post-hoc). Values are mean ± SEM. (**B**) Total NT-3 levels in the two spinal cord segments containing the graft/lesion site, and surrounding host tissue (1 mm and –1 mm), were greatly increased in animals that received Lenti-NT-3 vectors compared to Lenti-GFP vectors. In animals that received Lenti-GFP vectors, expression of NT-3 by genetically modified MSC grafts was evident as a two-fold increase in total NT-3 detected in the 1 mm and –1 mm spinal cord segments. Note that these measures include all host tissue within the spinal cord surrounding the lesion site, diluting amounts of NT-3 produced by NT-3-secreting MSCs in the lesion site. (**C**) Levels of NT-3 were also measured in grafts specifically dissected from the lesion site, undiluted by surrounding host tissue. ELISA on this specific MSC graft tissue showed significantly higher levels of NT-3 in MSC-NT-3 grafted animals compared to MSC grafted animals or intact spinal cord ($p < 0.01$, ANOVA; *$p < 0.05$, Fischer's posthoc).
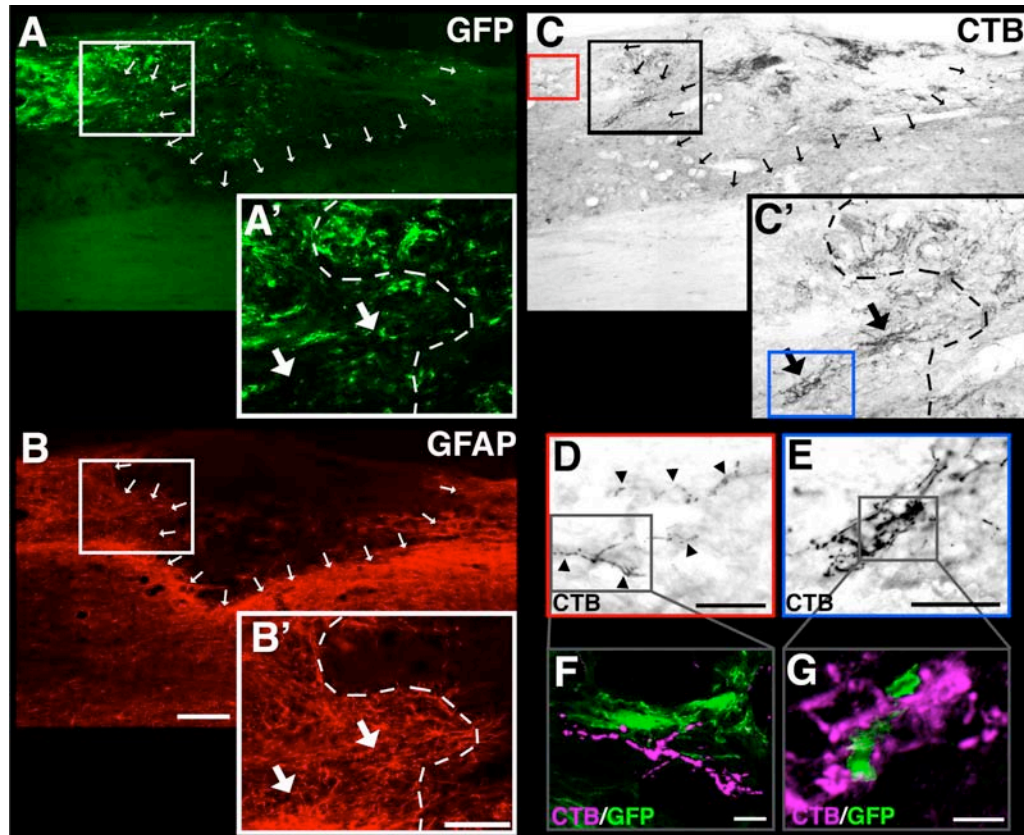
Figure 2.3. Ascending sensory axons extend beyond the graft/lesion site towards NT-3 vector-transduced cells. Triple immunolabeling for (**A**) GFP to label NT-3 vector-transduced cells, (**B**) GFAP to indicate the extent of the lesion/graft site and (**C**) CTB to label ascending sensory axons in a sagittally cut spinal cord 4 weeks after MSC-NT-3 cell grafting and Lenti-NT-3 injection. (**A'- C', insets**) Higher magnification of the rostral host/graft interface shows (**C'**) axons (arrows) growing into (**B'**) GFAP-rich regions beyond the lesion site where (**A'**) vector-transduced cells express NT-3. (**D, E**) High magnification of red and blue boxed areas in **C, C',** respectively. (**F, G**) Higher magnification of boxed regions in **D, E** demonstrate the association of CTB-labeled axons (pseudo-colored purple) with Lenti-NT-3 vector-transduced cells expressing the GFP reporter (green). Arrows in **A-C** and dashed lines in **A'-C'** indicate the graft/astrocyte border. Rostral is to the left, dorsal to the top. Scale bars: A-C, 200 μm; A'-C,' 100 μm; D,E, 50 μm; F,G, 10 μm.
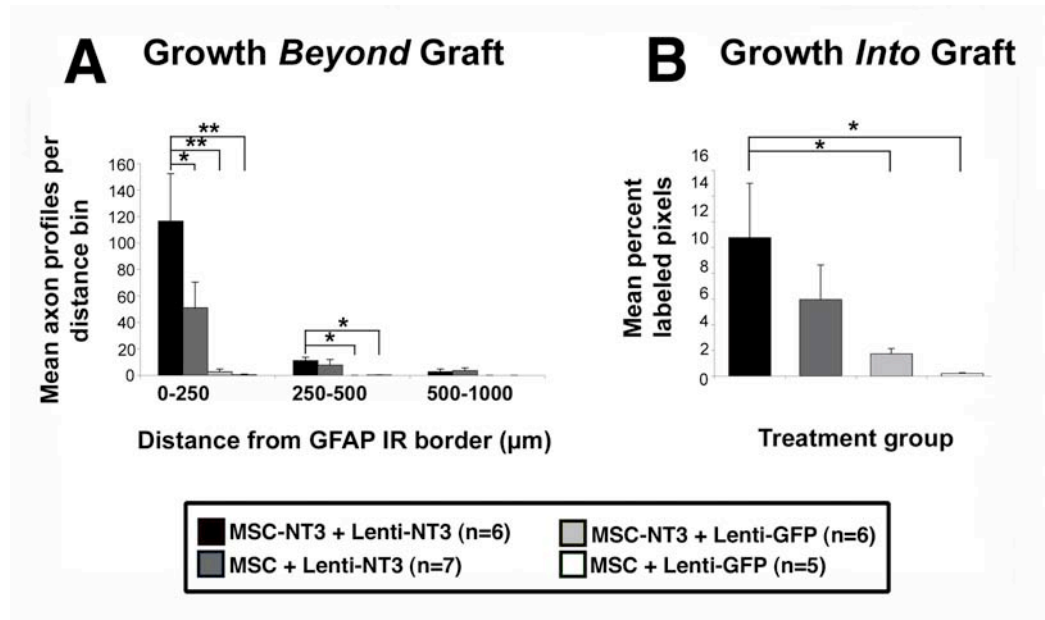
Figure 2.4. Quantification of CTB-labeled axonal profiles rostral to and within the lesion. Animals that received MSC-NT-3 graft cells combined with Lenti-NT-3 injections exhibited (**A**) significantly more axonal profiles beyond the astrocyte-defined lesion border than animals from any other group, and (**B**) significantly higher axonal density within the lesion border than animals that received Lenti-GFP injections (ANOVA, *p < 0.05, Fischer's post hoc analysis). Values are mean ± SEM.

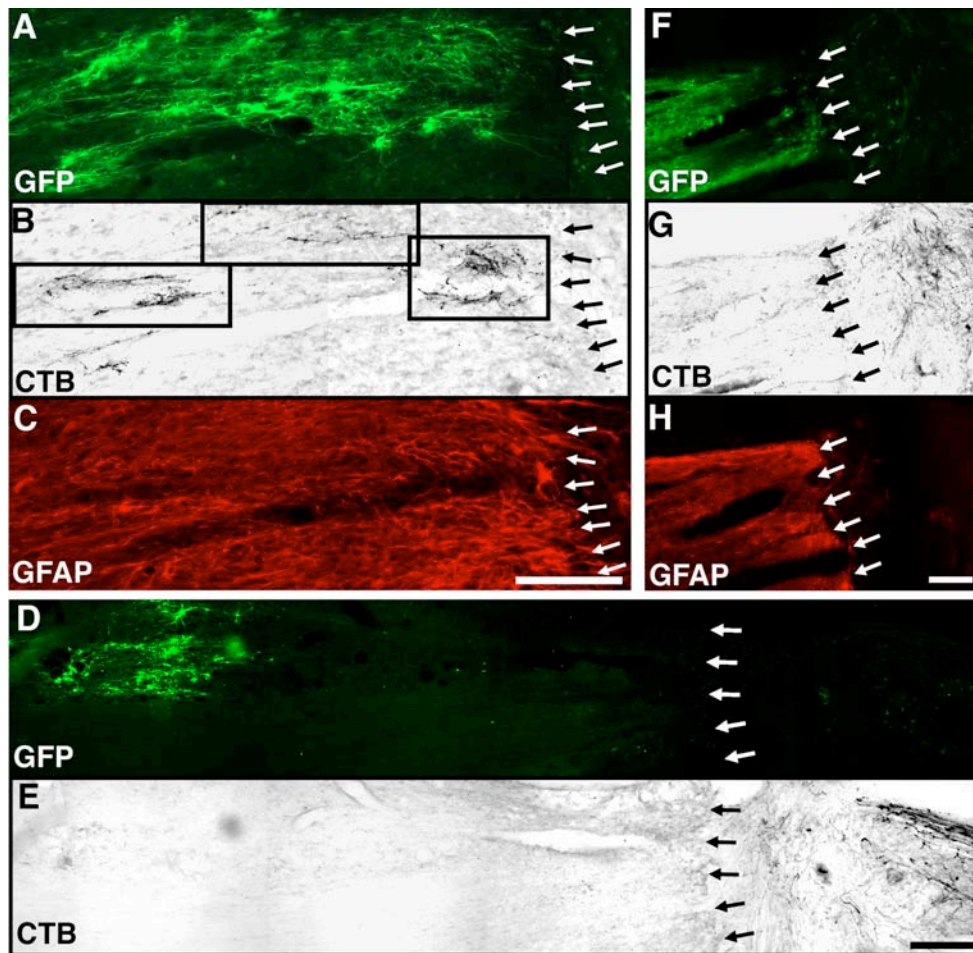**Figure 2.5. Axonal growth beyond the lesion site occurred only when lentivirus-mediated NT-3 expression was located within 100 *µ*m of the lesion border.** Triple immunolabeling for (**A**) GFP, (**B**) CTB and (**C**) GFAP shows that in subjects exhibiting bridging axons, Lenti-NT-3 transduced cells (expressing the reporter GFP) were evident within 100 *µ*m of the host/lesion border. Boxes in **B** indicate regions of high axon density rostral to the lesion site (MSC + Lenti-NT-3 treatment group). (**D**) When the zone of Lenti-NT-3 transduction was distant from the lesion site, (**E**) bridging axons were not observed (MSC + Lenti-NT-3 treatment group). (**F-H**) In animals injected with Lenti-GFP, axons did not extend beyond the graft, regardless of the distance between Lenti-GFP transduction and the lesion site (MSC-NT-3 + Lenti-GFP). Rostral is to the left, dorsal to the top. Scale bars: A-C, 140 *µ*m; D,E, 210 *µ*m; F-H, 100 *µ*m.
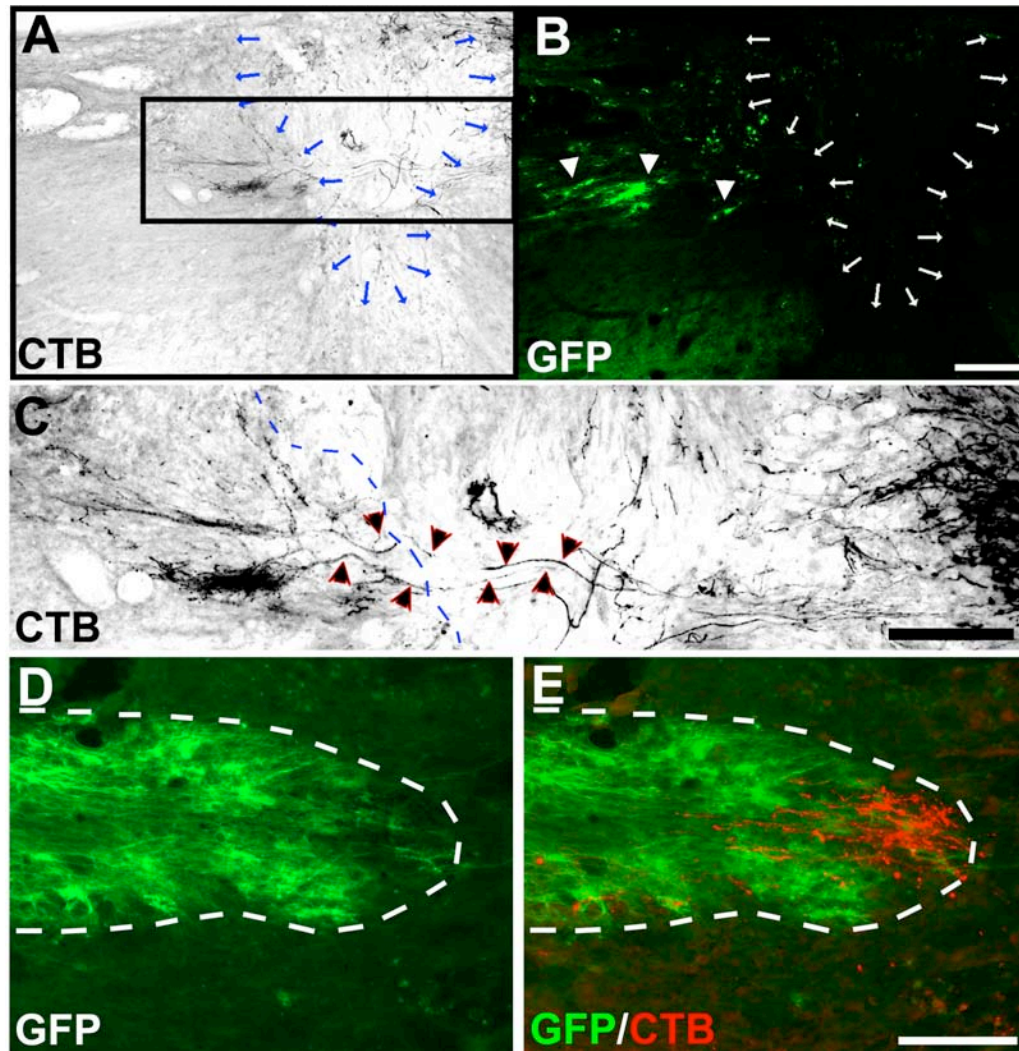
Figure 2.6. The topography of sensory axon bridging beyond the lesion site corresponds to the location of NT-3 lentiviral transduction. (**A**) CTB-labeled axons (box) crossing the lesion extend beyond the lesion border (arrows), toward (**B**) the region of dorsal column white matter where cells have been transduced by Lenti-NT-3 vectors (arrowheads). (**C**) Axons can be traced from the main CTB-labeled tract at the caudal end of the lesion site, across the lesion site, and beyond the graft/host interface (dotted lines). (**D**) Upon reaching areas of high NT-3 expression distal to a lesion site (outlined by dashed lines), (**E**) axons (pseudocolored red) sprout locally. These axons do not extend for long distances within NT-3 expressing tissue. Scale bars: A, B, 200 μm; C, 140 μm; E, F, 100 μm.
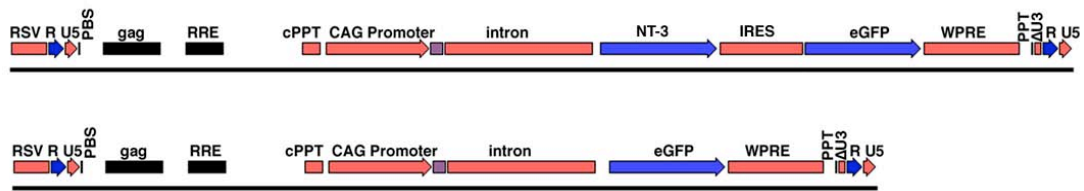
Supplementary Figure 2.1. Maps of lentiviral vectors for NT-3 and GFP expression. Lentiviral construct for the expression of NT-3 (Lenti-NT-3, top) also expressed GFP from an internal ribosome entry site (IRES), allowing vector-transduced cells to be identified by GFP labeling. Control vectors expressed GFP alone (Lenti-GFP, bottom). RSV: Rous Sarcoma Virus promoter/enhancer, PBS: primer binding site, gag: part of the gag coding sequence, RRE: rev response element, cPPT: HIV central polypurine tract, CAG Promoter: CMV enhancer/chicken β-actin promoter, WPRE: Woodchuck posttranscriptional response element.
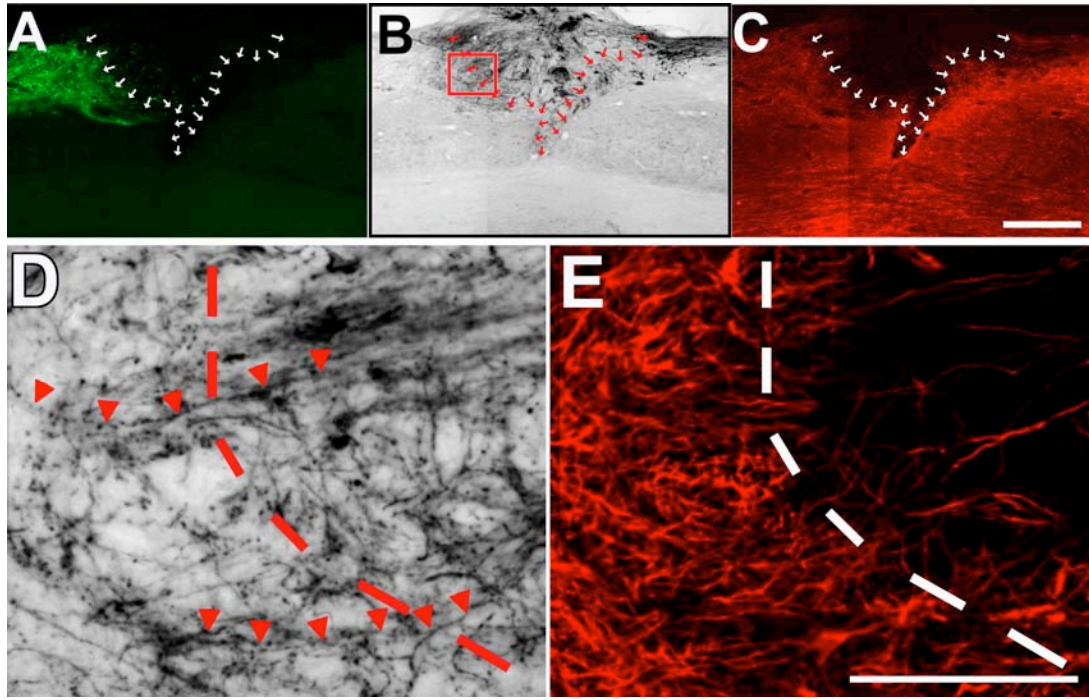
Supplementary Figure 2.2. Axons bridge, rather than circumvent, dorsal column lesion sites. (**A**) A region of extensive vector transduction rostral to a lesion site (indicated by GFP labeling) promotes extension of a large number of (**B**) CTB-labeled axons within the (**C**) GFAP-defined lesion site (outlined by arrows). (**D**) Many axons (arrowheads) cross from the graft (delineated by dashed lines) into a region of (**E**) GFAP labeling, suggesting that axons have bridged the lesion site. Scale bars: A-C, 500 μm; D,E, 100 μm.
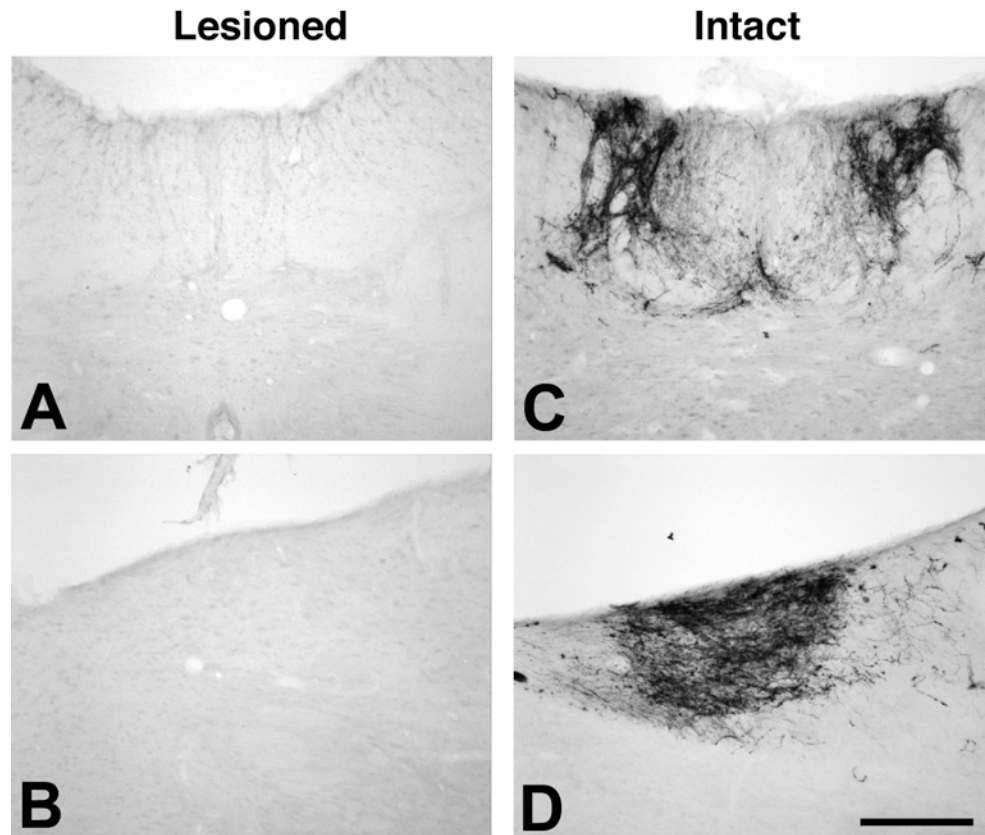
Supplementary Figure 2.3. Lesion completeness. **(A)** Caudal and **(B)** rostral regions of the nucleus gracilis in lesioned animals show an absence of ascending sensory axons in the brainstem, confirming completeness of lesions, while **(C)** caudal and **(D)** rostral regions of the nucleus in intact control animals show robust labeling of untransected axons in the nucleus. Scale bar: A-D, 200 μm.

Axon growth beyond lesion sites
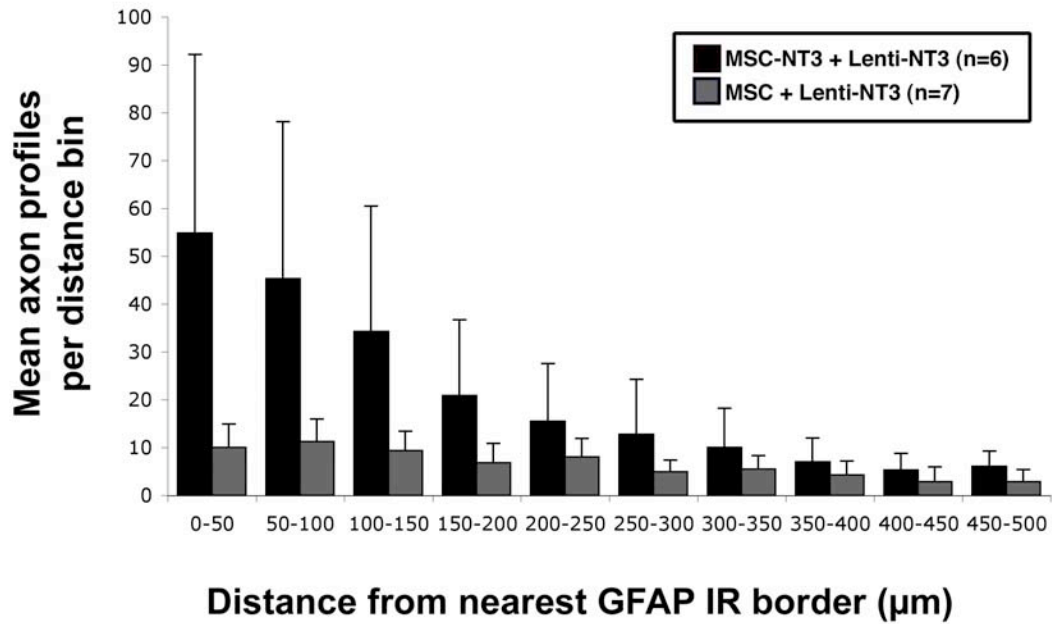
Supplementary Figure 2.4. Quantification of rostral axonal growth over 50 μm intervals in animals that received Lenti-NT-3 injections. The number of axons at each distance from the lesion site decreases with increasing distance from the rostral GFAP-defined lesion border. Values are mean ± SEM.
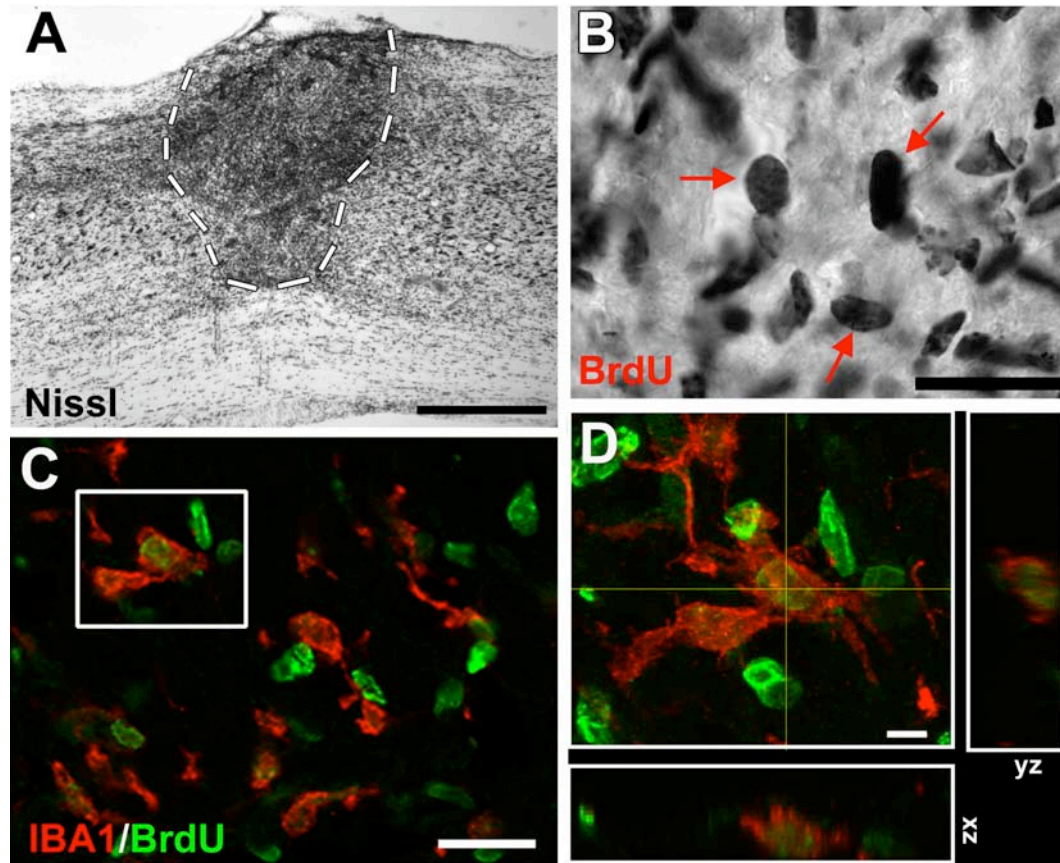
Supplementary Figure 2.5. Characterization of MSC grafts. (A) Nissl staining shows integration of grafted cells and filling of the cavity (outlined by dashed lines). (**B**) Marrow stromal cells pre-labeled with BrdU before grafting and subsequently detected by BrdU immunocytochemistry (arrowheads) within the lesion site, 4 weeks post-grafting. (**C**) The majority of Iba1-labeled microglia/macrophages in the graft (red) did not co-localize with BrdU labeling (green). Occasionally, IBA1-labeled cells contained some BrdU-labeling, representing either MSC differentiation or phagocytosis of graft cells (box). (**D**) High magnification of boxed area in C. In contrast, double labeling for BrdU and GFAP or BrdU and NeuN did not reveal any detectable co-localization (data not shown). Scale bars: A, 500 μm; B, 25 μm; C, 20 μm; D, 5 μm.

CHAPTER 3

Neurotrophin Gradients and Conditioning Lesions Promote Axonal Bridging of Cellular Grafts and Support Long Distance Axon Growth

ABSTRACT

Neurotrophic factors and conditioning lesions of the peripheral branch of dorsal root ganglia (DRG) neurons have been shown enhance the growth capacity of ascending sensory axons after spinal cord lesions. In this study we investigated whether a combination of conditioning lesions to enhance the growth capacity of DRG neurons and lentiviral NT-3 gene transfer could enhance the number and distance of axons regenerating into and beyond a C3 dorsal column lesion site filled with bone marrow stromal cells. Comparison of axonal growth beyond the lesion site between different treatment groups indicated that conditioning lesions alone were ineffective in inducing axonal bridging. Only animals with combinatorial treatment exhibited significantly increased numbers of regenerating axons beyond lesions. In addition, the maximum distance of axonal growth was highest in animals receiving combinatorial treatments. These data suggest that both activation of growth programs at the level of the cell body and signaling at the extending growth cone are necessary components for axonal growth over extended distances in the injured adult CNS.

INTRODUCTION

An abundance of data indicates that regeneration in the injured CNS is limited due to a combination of factors including inflammatory responses (Bethea and Dietrich, 2002), inhibitory extracellular matrix (Silver and Miller, 2004; Yiu and He, 2006), myelin-based inhibitors (Filbin, 2003; Schwab, 2004), a lack of permissive growth substrates at the lesion site (Oudega and Xu, 2006), a lack of sufficient growth stimuli at the axonal tip (Hendriks et al., 2004) and insufficient activation of axonal growth programs at the level of the cell soma (Goldberg et al., 2002; Plunet et al., 2002; Teng and Tang, 2006). Based on these findings it has become increasingly clear that combinatorial treatments will likely be required to address the presence of inhibitory factors and a lack of stimulatory factors for axonal growth.

Primary sensory neurons of the dorsal root ganglia (DRG) are a unique cell type for studies of axonal regeneration because these neurons have bifurcated axons that project to both the peripheral and the central nervous system. While the peripheral branches mount a robust regenerative response after injury, central branches that ascend into the dorsal columns of the spinal cord fail to regenerate (Schreyer and Skene, 1993). Interestingly, a limited degree of regeneration of centrally projecting axons can be elicited if the peripheral branches are injured prior to a central dorsal column lesion (McQuarrie et al., 1977; Richardson and Issa, 1984; Oudega et al., 1994; Chong et al., 1996; Neumann and Woolf, 1999; Neumann et al., 2005). These observations have led to the use of peripheral branch lesions, or "conditioning lesions," to study the cellular signaling pathways that promote axon growth in the

presence of inhibitory substrates. The molecular mechanisms underlying the conditioning lesion are incompletely understood and may involve IL-6 (Cafferty et al., 2004; Cao et al., 2006) and STAT3 signaling (Qiu et al., 2005), transcription factors such as CREB (Gao et al., 2004) and ATF3 (Seijffers et al., 2006; Seijffers et al., 2007), and increases in intracellular cAMP to activate Protein kinase A signaling (Neumann et al., 2002; Qiu et al., 2002; Nikulina et al., 2004). Increases in intra-axonal protein synthesis and degradation may also be involved (Verma et al., 2005).

Regeneration of dorsal column sensory axons can also be enhanced by delivery of specific growth factors to the lesion site. For example, ex vivo neurotrophin delivery to lesion sites by genetically modified graft cells results in significant axonal growth into these lesion sites compared to unmodified cells (Blesch et al., 2002; Blesch and Tuszynski, 2003; Lu et al., 2003). However, this type of delivery rarely gives rise to axonal projections that extend beyond the lesion or into the distal spinal cord. The growth potential of these axons can be further improved by several methods. When viral vectors are used to establish gradients of neurotrophic factors beyond the lesion, the number of axons bridging the lesion site is increased. In this case, growth is mostly restricted to short distances (250 μm) distal to the injury (Taylor et al., 2006). However, elevating cAMP in neuronal cell bodies, which has been shown to mimic the effect of conditioning lesions (Neumann et al., 2002; Qiu et al., 2002), combined with neurotrophin protein injections beyond lesion sites, results in longer distance (~2mm) growth of sensory axons into distal tissue (Lu et al., 2004).

This represents a significant improvement in the distance of axon growth compared to neurotrophin delivery alone, but the number of bridging axons is fewer.

In the present study we tested the hypothesis that conditioning lesions combined with sustained neurotrophin gradients generated by lentiviral NT-3 delivery would increase the number of regenerating axons and the distance of growth beyond lesion sites compared with NT-3 delivery alone. We find that the combined treatment results in significantly more axons beyond the lesion site, and axonal growth over longer distances into distal host tissue compared to single treatments (conditioning lesions or NT-3 gradients) alone.

MATERIALS AND METHODS

Adult female Fischer 344 animals (3-4 months old) were used. To compare the effects of conditioning lesions, distal NT-3 gradients and a combination of these two treatments, adult rats received bilateral conditioning lesions of the sciatic nerve one week prior to dorsal column lesions at cervical level 3 (C3, Fig. 1). On the day of the spinal cord lesion, bone marrow stromal cells (MSC) mixed with NT-3 protein were grafted into the lesion site to provide a substrate for axon growth into the injury site. Then, lentivirus expressing NT-3 or GFP as control was injected into dorsal column white matter 2.5 mm rostral to the lesion. Additional control animals received the same graft and virus injections but no conditioning lesion (Table 1). Axons were traced with cholera toxin B subunit (CTB) four weeks after injury.

Lesion surgeries, grafting and lentivirus injections:

Animals were anesthetized using a combination (2ml/kg) of ketamin (25mg/ml), rompun (1.8mg/ml) and acepromazine (0.25mg/ml). The dorsal funiculus was completely transected bilaterally at C3 using a tungsten wire knife (Kopf Instruments, Tujunga, CA) combined with dorsal tract compression to completely sever axons, as previously described (Lu et al., 2004; Taylor et al., 2006). Primary adult F344 marrow stromal cells (MSCs) were isolated and cultivated as described (Azizi et al., 1998; Taylor et al., 2006). Immediately following the dorsal column lesion, 2 μl (75,000 cells/μl) of MSCs mixed with NT-3 protein (0.5 μg/μl) were injected into the spinal cord lesion site through a pulled glass micropipette using a PicoSpritzer II (General Valve, Fairfield, NJ). Lenti-NT-3-GFP vector and Lenti-GFP vector were generated as previously described (Taylor et al., 2006). Lentiviral vectors (2.5 μl total; titer 100 μg/ml p24, ~1 x 10^8 infectious units/ml) were injected through pulled glass micropipettes, 2.5mm rostral to the lesion site, into the spinal cord midline at a depth of 0.5 and 1mm. Pipettes were left in place for 1 min before withdrawal.

Conditioning lesions surgery:

Some rats were subjected to peripheral conditioning lesions 7 days prior to central nervous system lesion, cell grafting and lentivirus injections. Sciatic nerves were exposed bilaterally at mid-thigh level and firmly compressed with fine jeweler's forceps for 15 seconds.

Axonal tracing, histology and immunohistochemistry:

Dorsal-column sensory axons were labeled transganglionically by cholera toxin B subunit (CTB) injection into the sciatic nerve proximal to the conditioning lesion site (2 μl of 1% solution per sciatic nerve) three days before perfusion, as described previously (Bradbury et al., 1999; Lu et al., 2004; Taylor et al., 2006). Animals were transcardially perfused with 4% PFA, post-fixed overnight, and cryoprotected in 30% sucrose at 4°C. Spinal cords were sectioned sagittally at 35 μm intervals on a cryostat. All sections were immunohistochemically processed free-floating. For visualization of CTB-labeled sensory axons, endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide and non-specific antibody reactions were blocked with 5% horse serum for 1 hr at room temperature. Sections were incubated for 72 hours at 4°C with the primary CTB antibody (goat polyclonal 1:80,000 dilution, List Biological Labs) followed by incubation with a biotinylated horse anti-goat IgG secondary antibody (1:200 dilution, Vector Laboratory Inc.) for 1 hr at room temperature. After 1 hr incubation in avidin-biotin peroxidase complex (1:100 dilution, Elite kit, Vector Laboratories Inc.) at room temperature, diaminobenzidine (0.05%) with nickel chloride (0.04%) was used as a chromagen. In the same sections GFP and GFAP were detected by fluorescence labeling. Sections were incubated overnight with antibodies against GFP (1:1500, Molecular Probes, Eugene, OR) and GFAP (1:1000 Chemicon, Temecula, CA) at 4°C. After washes, sections were incubated with Alexa 488 and Alexa 594 fluorophore-conjugated

secondary antibodies for 2.5 hr at room temperature (1:300, Molecular Probes,

Eugene, OR).

Axonal quantification:

The rostral host/graft interface was outlined using GFAP immunolabeling.

The number of CTB labeled axons crossing a line placed at the interface and 50, 100,

200, 400, 800 and 1600 μm rostral to the interface was counted at 400x magnification

in a series of one-in-seven sections. Observers were blinded to the nature of the

experimental manipulation.

Statistical Methods:

The number of axons crossing lines at 0, 50, 100, 200, 400, 800 and 1600 μm

beyond the lesion was compared between all four groups by ANOVA at each distance.

Differences between individual groups were assessed by Fischer's posthoc analysis.

To determine whether the maximum distance of axon growth differed between

treatment groups, the longest axon from each animal was measured and an average

maximum distance was obtained for each group. Average maximum distances were

compared between groups by ANOVA followed by Fischer's posthoc analysis.

RESULTS

Conditioning Lesions in Combination with NT-3 Enhance Axonal Bridging

Beyond the Lesion Site

To compare the effects of conditioning lesions, distal NT-3 gradients and a combination of these two treatments, the dorsal columns of adult rats were transected, bone marrow stromal cells mixed with NT-3 protein were grafted to the lesion site and lentiviral vectors for the expression of NT-3 or GFP were injected rostral to the lesion (Fig. 1). In addition, half of the animals in each vector group received conditioning lesions 1 week prior to dorsal column lesions, cell grafting and vector injection (Table 1). Four weeks after spinal cord lesions, CTB was injected to sciatic nerves to anterogradely label ascending sensory axons.

An examination of CTB-labeled axons indicated that axonal bridging across the graft into the rostral spinal cord for more than 100 μm occurred only in animals that received cell grafts and NT-3 expressing virus (Fig. 2). In animals that received conditioning lesions, cell grafts, and GFP virus, some axons reached the rostral host/graft interface, identified by GFAP labeling, but virtually no axons extended further in the rostral white matter of the spinal cord. In animals with lentiviral NT-3 delivery without conditioning lesions, a small number of axons extended beyond the rostral host/graft interface and for short distances beyond, as previously reported (Taylor et al., 2006). However, the number of axons bridging beyond lesion sites after NT-3 delivery was increased approximately 8-fold when animals received combinatorial therapy with conditioning lesions, cellular grafts and lenti-NT-3 injections rostral to the lesion site. In combinatorially treated subjects, the mean number of axons extending beyond the lesion site was significantly different from all other groups at distances ranging from 0 μm up to 1600 μm rostral to the lesion (Fig.

3, ANOVA followed by Fischer's posthoc analysis, p<0.001 at 0, 50, 100, 200, 800, and 1200 μm; p<0.01 at 400 and 1600 μm). Axons in combinatorial treatment groups extended a mean maximum distance of nearly 2.5 mm, a distance that is six times greater than the distance measured in animals that received NT-3 lentivirus injections and cell grafts without conditioning lesions (ANOVA followed by Fischer's posthoc analysis, p<0.0001). Crossing axons in animals that received the full treatment were found at all dorsoventral levels within the dorsal funiculus and extended preferentially in white matter rostral to the graft into regions where NT-3 was expressed by virus-transduced cells (Fig. 4). Bridging axons were often found to orient along GFAP positive processes near the lesion site (Fig. 4F) and were frequently associated with blood vessels in distal tissue (Fig. 4G).

DISCUSSION

We show here that initiation of an axonal growth program by peripheral conditioning lesions prior to a spinal cord injury allows dorsal column sensory axons to extend into the inhospitable environment beyond a spinal cord lesion site, and to regenerate over extended distances if an additional growth stimulus is provided by a neurotrophic factor (NT-3). In contrast, conditioning lesions alone promote growth of some axons into autologous cellular grafts placed within lesion sites, but do not support growth of axons into host tissue beyond the lesion. NT-3 delivery alone increased the number of axons extending across lesion sites and into distal host tissue, but failed to elicit long distance growth. Thus, a combination of stimuli at the level of

the cell body and at the distal axon are necessary to substantially increase the number of axons regenerating for long distances within inhibitory white matter rostral to the lesion site.

A number of studies have indicated that conditioning lesions or cAMP signaling can promote growth of axons over inhibitory substrates in vitro. However, in vivo, axons grow mainly in gray matter surrounding lesion sites rather than in white matter in response to these stimuli (Neumann and Woolf, 1999; Pasterkamp et al., 2001; Neumann et al., 2002; Qiu et al., 2002). Thus, despite the activation of a genetic program for growth, axons can still be blocked by inhibitory signals at the level of the extending growth cone. When inhibition is attenuated, for example using antibodies that mask NG2 (an inhibitory chondroitin sulfate proteoglycan) (Tan et al., 2006), axons can extend into white matter after conditioning lesions. These findings support the notion that conditioning lesions alone (i.e. activation of a cellular growth program) are insufficient to promote growth over inhibitors in vivo. However, in the present study, axons grew directly into white matter substrates when both stimulation of the transected distal axon and the cell body were combined. These findings are consistent with those reported by Lu et al. (2004) when cAMP injections to DRGs were combined with NT-3 protein delivery distal to lesion sites. Growth signals (i.e. neurotrophins) provided at the extending growth cone and activation of genetic growth programs (i.e. conditioning lesions or cAMP injections) may provide a mechanism for balancing inhibitory signals while also promoting long distance growth.

While axonal bridging beyond the lesion site was also observed by Lu et al. (2004) using the same lesion model, axon growth responses were substantially higher in the present study. The larger growth response could be attributed to differences between cAMP delivery versus conditioning lesions, or to differences between a single NT-3 protein injection versus sustained NT-3 delivery. Previous studies have indicated that the elevation of cAMP is an important component of the conditioning lesion response (Neumann et al., 2002; Qiu et al., 2002; Hannila and Filbin, 2007). While the use of this treatment is clearly beneficial for axonal growth beyond lesion sites, the conditioning lesion activates several signaling pathways that may increase axon growth beyond that supported by cAMP elevation alone (for example (Seijffers et al., 2006; Seijffers et al., 2007)). A major effort is now underway in our group to compare gene expression in dorsal root ganglion neurons after conditioning lesions versus cAMP elevation in order to understand which signaling pathways are most important for achieving substantial axon growth after injury to the central branches of DRG neurons.

The NT-3 delivery methods in these studies could also account for differences in axon growth beyond the lesion. In the present experiment, NT-3 was expressed over long periods, whereas Lu et al. (2004) delivered a single protein injection that would have degraded relatively quickly. Long-term NT-3 delivery may have provided higher and sustained concentrations of NT-3 that resulted in an increased growth stimulus. Alternatively, since the conditioning lesion and neurotrophin signaling pathways overlap (Teng and Tang, 2006), continuous NT-3 signaling might play a role

in sustaining the conditioning lesion response over long time periods, resulting in more axon growth over longer distances (Neumann et al., 2005). For example, neurotrophins might act to maintain elevated levels of cAMP initiated by conditioning via signaling through the Erk pathway to inhibit PDE4 and block degradation of cAMP (Kaplan and Miller, 2000; Gao et al., 2003; Hannila and Filbin, 2007).

Importantly, the present work clearly illustrates that conditioning lesions combined with lentiviral NT-3 delivery promote regeneration of large numbers of axons for relatively long distances into distal host tissue. Thus, we have developed a model system that uses distal neurotrophin gradients to promote a substantial degree of sensory axon growth beyond lesion sites. Such a system provides a means to study the behavior of axons that have regenerated into a host environment beyond a spinal cord injury. In the subsequent chapter we use this model system to test whether sensory axons that have regenerated into the nucleus gracilis, the normal termination site for these axons, are able to form functional synapses upon reaching denervated neuronal targets.

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Table 3.1. Experimental groups. All animals received C3 dorsal column wire knife lesions and MSC grafts mixed with NT-3 protein. Animals were divided into groups based on whether or not they received peripheral nerve conditioning lesions or no conditioning treatment and whether they received NT-3-GFP-expressing or GFP-expressing lentiviral vector injections rostral to the lesion site.

Groups	Conditioning	Rostral vector	Number of animals
1	No treatment	GFP	n=6
2	No treatment	NT-3-GFP	n=6
3	Conditioning lesion	GFP	n=6
4	Conditioning lesion	NT-3-GFP	n=6
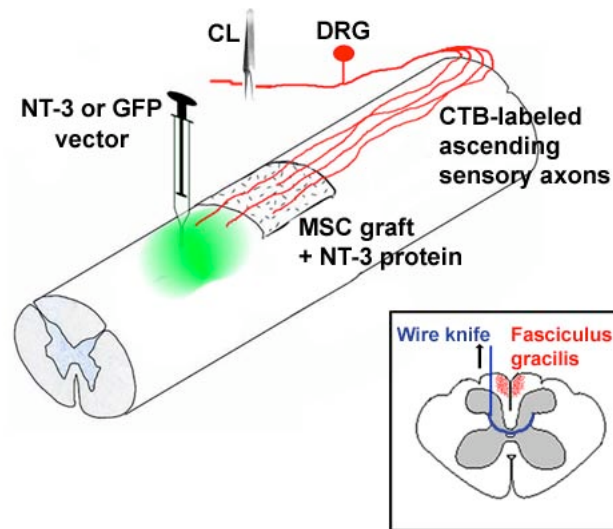
Figure 3.1. Schematic illustration of experimental paradigm. Adult rats underwent dorsal column wire knife lesions at cervical level 3 (C3) to transect ascending sensory axons. Syngenic bone marrow stromal cells (MSC) mixed with NT-3 protein (0.5 μg/μl) were grafted into the lesion site and lentiviral vectors expressing NT-3-GFP or GFP only (control) were injected into the dorsal columns of the spinal cord 2.5 mm rostral to the lesion site in all animals. Some animals underwent conditioning lesions (CL) of the sciatic nerve 7 days before C3 lesions. After 4 weeks ascending sensory axons were traced by injection of cholera toxin beta subunit (CTB) into the sciatic nerve.
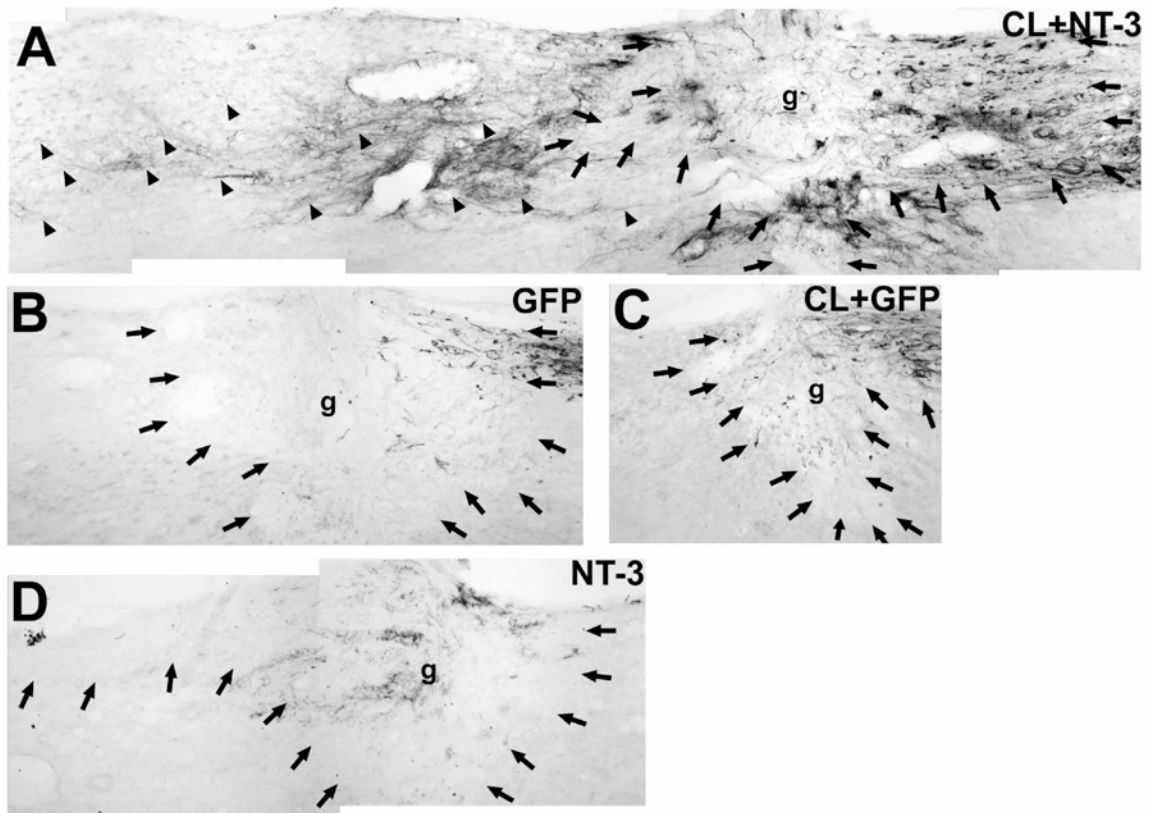
Figure 3.2. A combination of conditioning lesions and lentiviral NT-3 gene transfer results in significant axonal bridging across the lesion site. (A) A combination of NT-3 lentivirus and conditioning lesions results in numerous axons (arrowheads) bridging beyond the lesion (g, outlined by arrows) and extending for long distances in the rostral host spinal cord. (B) In contrast, CTB-labeled ascending sensory axons fail to bridge across the MSC-filled lesion site in control animals that received injections of GFP expressing lentivirus without conditioning lesions. (C) Conditioning lesions (CL) combined with control GFP virus injections promotes growth of only a few axons through the lesion site, but these axons do not continue to extend beyond the lesion into host white matter. (D) Animals that received NT-3 expressing virus rostral to the lesion show bridging axons, but this axonal extension is limited to extremely short distances. Rostral is to the left, dorsal at the top. Scale bars: A-D (in D), 170 μm.
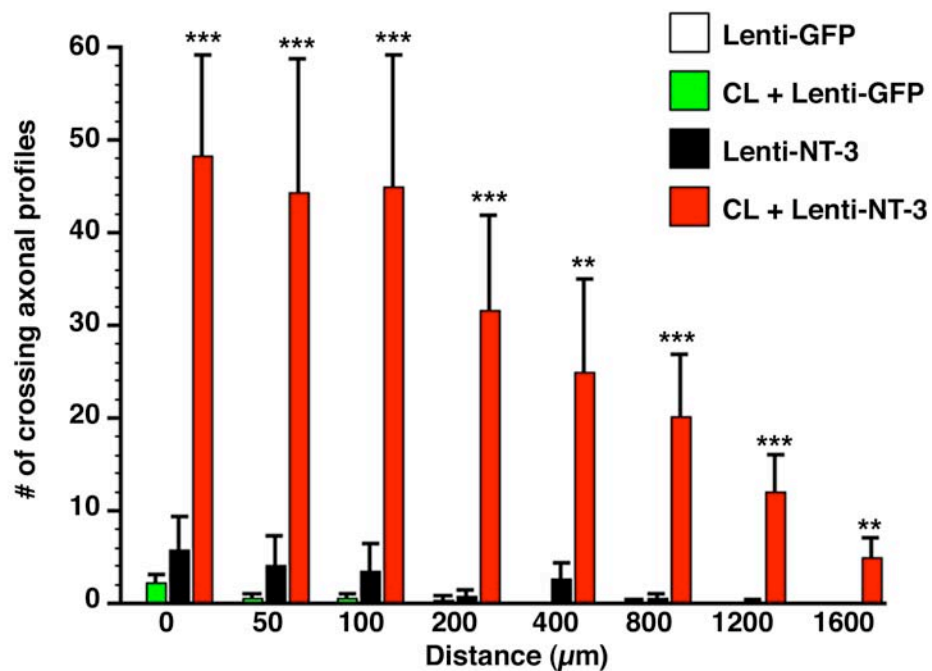
Figure 3.3. Quantification of axons bridging across the lesion site. Pre-conditioning lesions in combination with lentiviral NT-3 gene transfer increase the number of axons crossing a C3 dorsal funiculus lesion filled with MSCs. The number of axons crossing the rostral host graft interface or a virtual line 50, 100, 200, 400, 800, 1200 and 1600 μm beyond the lesion site was quantified. (ANOVA followed by Fischer's posthoc analysis *** $p<0.001$, ** $p<0.01$).

Figure 3.4. Ascending sensory axons extend beyond the graft/lesion site towards NT-3 vector-transduced cells in an animal that received conditioning lesions and lentiviral NT-3 gene transfer. A sagittaly cut spinal cord section triple immunolabeled for (**A**) CTB to label ascending sensory axons (**B**) GFAP to indicate the extent of the lesion/graft site, and (**C**) GFP to label NT-3 vector-transduced cells. (**D, E**) Higher magnification of insets in **A** shows (**D**) axons growing across the rostral host/graft (g) border (indicated by stars) into distal host tissue. (**E**) Numerous axons can be found at distances far rostral to the lesion site. (**G, H**) Double immunolabeling for CTB-labeled axons (pseudocolored blue) and GFAP (red) at the (**F**) rostral host/graft interface (indicated by stars) and (**G**) in the host spinal cord. Arrowheads indicate (**F**) axons oriented along GFAP-labeled processes and (**G**) axons extending along blood vessels. Rostral is to the left, dorsal to the top. Scale bars: A-C, 424 μm; D and E, 170 μm; F and G, 85 μm.
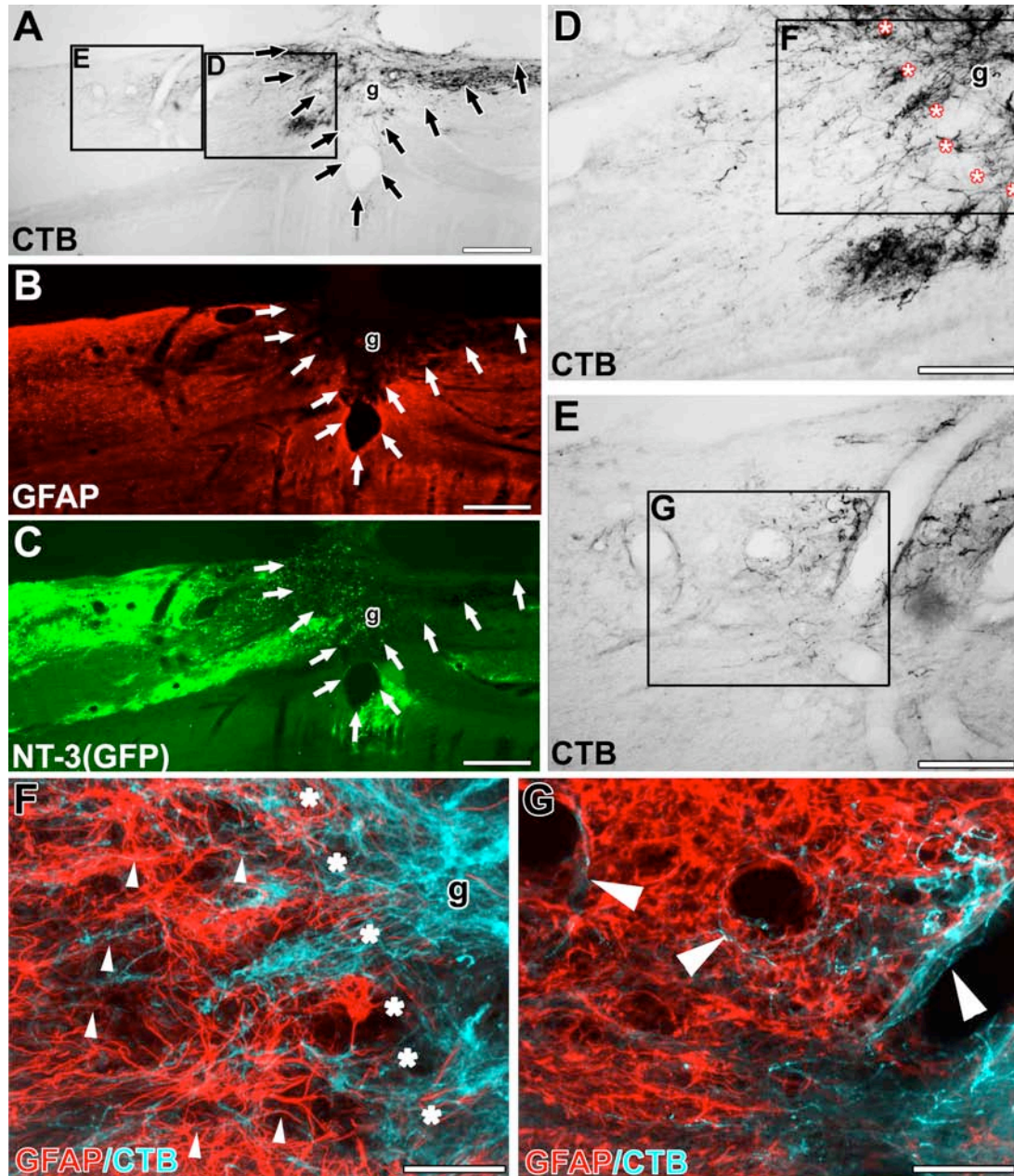
CHAPTER 4

Neurotrophin Delivery and Conditioning Lesions Promote Reinnervation of a Denervated Target and May Lead to Synapse Formation after Spinal Cord Injury

ABSTRACT

Neurotrophin gradients established beyond sites of spinal cord injury (SCI) combined with conditioning lesions promote growth of ascending sensory axons into and beyond lesion sites. We tested whether combinatorial treatments could promote growth of ascending sensory axons originating from hindlimb dorsal root ganglia (DRG) into the nucleus gracilis, the brainstem termination site for these axons. We further examined the ability of regenerating axons to establish synaptic contacts with target neurons, and to express their appropriate pre-injury excitatory phenotype on host dendrites. Adult rats underwent C1 dorsal column lesions to transect ascending sensory axons, followed by grafts of autologous bone marrow stromal cells into the lesion site. Concurrently, lentiviral vectors expressing NT-3 were injected into the nucleus gracilis to promote axon growth beyond the lesion site and toward denervated target neurons. Some subjects also received peripheral nerve conditioning lesions one week prior to spinal cord lesion surgery, cell grafting and virus injection. Four weeks after treatment, a combination of anterograde and retrograde axonal tracing revealed regenerating sensory axons in the nucleus gracilis in animals that received lentiviral NT-3 delivery with or without conditioning lesions; in contrast, axons were not

observed in the target after injections of control, GFP-expressing lentiviral vectors, in the nucleus gracilis. In animals treated with NT-3 delivery and conditioning lesions, axon density in the nucleus gracilis was significantly greater than in any other group. Ultrastructural examination of tissue from animals that received combinatorial treatment indicated the presence of possible synaptic contacts formed by regenerated axons within the nucleus gracilis. These synapses formed on dendritic arborizations rather than cell somata of gracilis neurons, similar to synapses commonly observed in the intact nucleus gracilis. These findings reveal that neurotrophic factor expression in denervated target regions combined with augmentation of neuronal growth programs at the cell soma can induce regeneration of lesioned axons into target regions, with possible formation of new synapses within denervated neuronal targets.

INTRODUCTION

Functional deficits after spinal cord injury (SCI) are caused primarily by destruction of axonal connections between neurons. Because CNS axons fail to regenerate spontaneously, lost connections cannot be restored, resulting in permanent disability. Regeneration failure has been attributed to factors intrinsic to injured neurons, such as the inability to activate genetic programs for growth (Teng and Tang, 2006), and to extrinsic factors, such as an inhibitory cellular environment (Yiu and He, 2006). Despite these barriers, a number of experimental strategies have been used to promote growth of injured axons beyond lesion sites after SCI (Thuret et al., 2006). While the observed increase in axonal growth is an important step forward, functional

recovery will also require the formation of new synaptic connections beyond the lesion site. Almost 20 years ago, studies of retinal ganglion cells (RGCs) demonstrated that synaptogenesis can occur after regeneration of adult CNS axons into an original termination site (Aguayo et al., 1990). The presence of new synapses was confirmed using ultrastructural examination (Carter et al., 1989), electrophysiology (Keirstead et al., 1989), and functional testing (Thanos, 1992). Additional studies used similar criteria to establish that regeneration of sensory axons across the dorsal root entry zone and into the spinal cord resulted in synapse formation and recovery of postsynaptic potentials or behavior (Ramer et al., 2000; Ramer et al., 2002). However, similar evidence for synapse formation after regeneration of injured axons in the spinal cord is lacking. To date, it remains unknown whether injured axons in the spinal cord retain the capacity to form functional connections upon reaching potential postsynaptic partners.

An important first step toward achieving functional regeneration is to promote axon growth into an original termination site. One means of promoting growth of axons, is delivery of neurotrophins to injured axon tips (Blesch et al., 2002). In addition to providing a positive growth stimulus for axons, neurotrophins may act chemotropically. Neurotrophins exhibit chemotropic effects on growth cones in vitro (Letourneau, 1978; Gundersen and Barrett, 1979) and are thought to play an integral role during developmental target innervation, possibly as axonal guidance molecules (Ringstedt et al., 1997; Tucker et al., 2001; Ma et al., 2002; Genc et al., 2004; Tessarollo et al., 2004). In adulthood, peripheral nerve injury causes upregulation of

neurotrophins and other neurotrophic proteins in the distal nerve stump (Frostick et al., 1998; Terenghi, 1999), suggesting a possible role for neurotrophic factor guidance in regeneration.

We previously reported that delivery of NT-3 rostral to dorsal column injuries, either with or without conditioning lesions, promoted sensory axon bridging of cell-filled lesion sites (Taylor et al., 2006). Importantly, NT-3 delivery resulted in axon growth specifically into portions of tissue beyond the lesion site where NT-3 was expressed. These studies reveal a chemotropic role for NT-3 in eliciting axonal growth in the injured spinal cord. Moreover, axons appeared to sprout locally in regions of high NT-3 expression, suggesting that NT-3 can promote local axonal arborization (Schnell et al., 1994; Cohen-Cory and Fraser, 1995). Thus, delivery of neurotrophins to denervated target regions might provide an effective means for directing axons toward target cells and promoting formation of functional synapses.

To test this possibility, we used lentiviral vectors to deliver NT-3 to the denervated nucleus gracilis after a C1 dorsal column lesion. We examined whether transected ascending sensory axons originating from hindlimb dorsal root ganglia (DRG) would extend across cellular grafts into the nucleus gracilis, the normal brainstem termination site for these axons, in response to NT-3 delivery and/or conditioning lesions. Our results indicate that axons extending across the lesion site in response to combined treatment with NT-3 and conditioning lesions can reinnervate the nucleus gracilis and may form synapses in somatotopically appropriate regions of target neurons.

MATERIALS AND METHODS

Experimental Design:

To determine whether NT-3 expression in the nucleus gracilis and/or

conditioning lesions could promote growth of transected ascending sensory axons into

a denervated target, animals received C1 dorsal column lesions and injections of NT-

3-expressing or GFP-expressing (control) lentiviral vectors into the nucleus gracilis, in

combination with sciatic nerve conditioning lesions or no peripheral lesions (Table 1).

The following groups were examined histologically: No conditioning lesion

combined with GFP virus injection (NoCL + Lenti-GFP, n=9), conditioning lesion

combined with GFP virus injection (CL + Lenti-GFP, n=8), no conditioning lesion

combined with NT-3 virus injection (NoCL + Lenti-NT-3, n=6), conditioning lesion

combined with NT-3 virus injection (CL + Lenti-NT-3, n=7). An additional group

was examined in which conditioning lesions were combined with injection of an NT-3

virus preparation with a two-fold higher vector titer than the other groups (200 μg/ml

v. 100 μg/ml; CL + Lenti-NT-3 high, n=7). Finally, additional subjects received CL +

Lenti-NT-3 at a titer of 200 μg/ml, and were examined for electron microscopic (n=2)

and electrophysiological (n=5) evidence of synapse formation in the nucleus gracilis.

Production of lentiviral vectors:

NT-3 and control (GFP-expressing) vectors were constructed as previously

described (Taylor et al., 2006). Briefly, vectors were produced by transfection of 293

cells with third generation lentiviral vector plasmids utilizing a split genome

packaging system, as previously described (Naldini, 1996, Blesch, 2004-need to insert

real refs). NT-3 vector preparations contained 100 or 200 μg/ml p24 (3.4 x 10^8 or 6.8

x 10^8 IU/ml). Control vector preparations contained 100 or 200 μg/ml p24 (8.3 x 10^7

or 1.7 x 10^8 IU/ml). Vector titers were assayed by infection of 293 cells using serial

dilutions and quantification of p24 levels using ELISA as previously described

(Naldini 1996, Taylor, 2006).

Isolation of marrow stromal cells (MSCs) to be used as graft cells:

Rat primary marrow stromal cells were isolated according to the method of

Azizi et al. (Azizi et al., 1998). Briefly, Fischer 344 adult female rats were

anesthetized with a combination (2 ml/kg) of ketamine (25 mg/ml), xylazine (1.3

mg/ml) and acepromazine (0.25 mg/ml), decapitated, and tibias and femurs were

dissected. After removing the end of each bone, 5 ml alpha-MEM medium

(Gibco/BRL) medium was injected into the central canal of the bone to extrude

marrow. Cells were cultured in alpha-MEM medium (Gibco/BRL) supplemented with

20% fetal bovine serum (FBS) and antibiotics. Non-adherent cells were removed after

24 hours. Cells were passaged twice and frozen.

Surgical procedures:

Adult female Fischer 344 rats (n = 65) weighing 150-200 gm were used.

Institutional, NIH, and Society for Neuroscience guidelines on animal care were

followed. Animals were divided into groups based on: 1) whether or not they received

sciatic nerve conditioning lesions prior to cervical spinal cord injury, 2) the type of

lentiviral vector injected into the N. gracilis, and 3) vector titer and number of dorso-

ventral sites at which vectors were injected (Table 1). Lentiviral vectors expressing

NT-3 also expressed the reporter gene GFP from an internal ribosome entry site

(IRES) (Taylor et al. 2006). Vectors with titers of 100 μg/ml were injected at 0.25,

0.5 and 1.0 mm below the dorsal brainstem surface, and subsequent experiments using

vectors with titers of 200 μg/ml p24 were injected 0.25 and 0.5 mm below the

brainstem surface (to most accurately target the nucleus gracilis). Additional intact

animals served as controls for immunolabeling or electrophysiology.

Retrograde labeling of the Nucleus Gracilis:

To allow unequivocal identification of dorsal column sensory axonal

regeneration into their normal targets in the nucleus gracilis, we performed retrograde

labeling of nucleus gracilis neurons by injections of Fluorogold into the

ventroposterolateral (VPL) nucleus of the thalamus. Neurons in the gracile and

cuneate nuclei were retrogradely labeled by injection of fluorogold into the thalamus

(VPL) 1 week before spinal cord lesion surgery (Fig. 2). Briefly, after fixation in a

stereotaxic unit, the brain surface was exposed bilaterally. A solution of 4%

fluorogold in 0.9% sterile saline was delivered stereotactically to 12 sites within the

VPL region (6 per hemisphere) using a pulled glass pipette with a 20 μm inner

diameter. Injections were made at the following coordinates in each hemisphere

(measurements were made from Bregma): coordinates #1-3: –0.23 rostro-caudal (R/C), 0.30 mediolateral (M/L), and 0.49, 0.56, 0.62 dorso-ventral (D/V); coordinate #4: -0.30 (R/C), 0.30 (M/L), 0.61 (D/V); coordinate #5: -0.30 (R/C), 0.34 (M/L), 0.53 (D/V); coordinate #6: -0.36 (R/C), 0.34 (M/L), 0.58 (D/V). Approximately 80 nl solution was delivered by pressure injection at each of the 12 sites.

Conditioning lesion:

Immediately following the retrograde labeling procedure some animals received bilateral conditioning lesions, in which the sciatic nerve was crushed with jeweler's forceps for 15 seconds.

C1 lesion surgery and vector injections:

To assess whether axonal regeneration can be induced into a normal brain target of spinal cord projections and result in synapse formation following SCI, dorsal column ascending sensory axons were transected 1.5 mm caudal to the obex. The nucleus gracilis consists of two roughly defined regions, including a larger more compact rostral region (within approximately 300 μm of the obex), and a caudal region (extending 1.8-2.8 mm caudal to the obex), in which fewer neurons are present and are intermingled with the incoming fasciculus gracilis (Paxinos 1999)(Gulley, 1973; Bermejo et al., 2003). Thus the larger compact region, containing the majority of nucleus gracilis neurons, was located rostral to our lesion site while a relatively small proportion of neurons trail caudally from this level (Lund and Webster, 1967).

For lesion surgeries, animals were fixed in a spinal stereotaxic unit with the head

flexed at a 45-degree angle to facilitate exposure of the upper cervical region of the

spinal cord and the lower brainstem. A partial laminectomy was performed to remove

the rostral half of the C1 vertebrae, and dorsal column lesions were made 1.5 mm

caudal to the obex using a Kopf microwire device (Kopf Instruments, Tujunga, CA).

A small dural incision was made and the wire knife was lowered 0.5 mm into the

spinal cord at a position 1.25 mm to the left of the midline. The tip of the wireknife

was extruded, forming a 2.5 mm-wide arc that was raised to the dorsal surface of the

cord, transecting the ascending (sensory) axon tracts located at the dorsal surface of

the cord. To ensure complete axotomy of the ascending sensory tract, spinal tissue

was compressed against the microwire knife surface using a microaspiration pipette

until all visible white matter was transected. The knife was passed through the lesion

site a minimum of 2 times before withdrawal to ensure complete transection of axons.

Immediately following the lesion, 2 μl (75,000 cells/μl) of marrow stromal

cells (MSC) were injected through a small hole in the dura mater into the lesion space.

Cells were injected using a pulled glass micropipette attached to a PicoSpritzer II

(General Valve, Fairfield, NJ). The lesion site was then covered with a square of

agarose film to prevent extrusion of injected MSCs from the lesion cavity. Next,

lentiviral vectors were injected through pulled glass capillaries 1.7 mm rostral to the

C1 lesion site, and approximately 0.2 mm rostral to the obex into the rostral portion of

the N. gracilis. A volume of 1.25 μl of vector solution was injected 0.25 mm lateral to

the midline on both the left and right sides of the brainstem, resulting in bilateral

delivery of a total volume of 2.5 μl of vector solution to the N. gracilis. The 1.25μl

volume delivered to each side was distributed equally between either three depths of

0.2 mm, 0.5 mm and 1.0 mm, or two depths of 0.2 mm and 0.5 mm below the dorsal

surface of the brainstem (see Table 1). Pipettes were left in place for 1 min after the

injection, and were then slowly withdrawn. Overlying muscle layers were sutured and

the skin was stapled.

Transganglionic labeling of ascending sensory projections:

Animals received injections of Cholera toxin B (CTB, 1%; 2 μl, List

Biologicals; Campbell, CA) into the right and left sciatic nerves three days before

perfusion to label ascending sensory projections transganglionically. Animals were

sacrificed 4 weeks after spinal cord lesions were made by perfusion with either 4%

paraformaldehyde (for histology and immunocytochemistry) or 4% paraformaldehyde

plus 2.5% glutaraldehyde (for electron microscopy).

Immunohistochemical analysis of brainstem sections:

Animals used for immunolabeling were transcardially perfused with 100 ml

cold PBS followed by 300 ml of 4% paraformaldehyde in phosphate buffer. Spinal

cords and brainstems were removed, post fixed overnight in 4% paraformaldehyde,

and cryoprotected in 0.1 M phosphate buffer containing 30% sucrose at 4˚C. For

spinal cords, tissue was blocked in TBS Tissue Freezing Media (Triangle Biomedical

Sciences) on dry ice and sagittal sections were cut on a cryostat set at 35 μm intervals.

For immunohistochemical analysis of injured animals, every other section was triple-labeled for CTB using streptavidin-HRP light level immunohistochemistry followed by GFP and GFAP fluorescent immunolabeling. The following immunocytochemical markers were used: cholera toxin beta-subunit (goat anti-CTB 1:80,000; List, Campbell, CA), to detect ascending sensory axons; glial fibrillary acidic protein (GFAP, monoclonal 1:1000; Chemicon, Temecula, CA) to label astrocytes; and green fluorescent protein (rabbit anti-GFP 1:750; Molecular Probes, Eugene, OR) to label vector-transduced cells. Immunocytochemical labeling for CTB was performed first with streptavidin-HRP-based light level immunohistochemistry using free floating sections in the following protocol: 1) 3 day incubation in primary antibodies at in a cold room, 2) incubation for one hour with biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA) at room temperature, 3) one hour incubation with avidin-biotinylated peroxidase complex (1:150; Vector Elite Kit, Vector Laboratories) at room temperature, and 4) treatment for 4 minutes with 0.05% solution of 3.3' diaminobenzidine, 0.01% H_2O_2 and 0.04% nickel chloride at room temperature. Fluorescence immunohistochemistry for GFP and GFAP was then performed on the same sections according to the following protocol: 1) overnight incubation in primary antibody, then 2) 2.5 hour incubation with fluorescent-conjugated secondary antibodies (donkey anti-rabbit Alexa-488 at 1:150; donkey anti-mouse Alexa 594 at 1:200; Molecular Probes). Labeled sections were mounted and coverslipped with Cytoseal 60 mounting media (Richard Allen Scientific, Kalamazoo, MI).

For analysis of fluorogold retrograde labeling and CTB anterograde labeling in intact animals, all sections were labeled in serial using streptavidin-HRP light level immunohistochemistry for CTB to identify ascending sensory axons, and fluorescent immunolabeling for fluorogold to identify retrogradely labeled neurons according to the procedure described above. After CTB labeling, sections were incubated with fluorogold primary antibodies (rabbit anti-fluorogold 1:2000, Chemicon) for 2 days at 4ºC followed by incubation with donkey anti-rabbit Alexa 594 (Molecular Probes).

Animal exclusion criteria:

Animals were excluded from analysis by an observer blinded to group identity if: 1) lesions were incomplete (using criteria described below), 2) vector-transduced cells were not within 100 μm of the rostral graft/host border, because previous studies have revealed that axons fail to bridge a mid-cervical lesion site when NT-3-GFP virus injections fail to reach the host/graft interface (Taylor et al., 2007), or 3) the rostral portion of the nucleus gracilis was substantially damaged or no fluorogold-labeled neurons were present within the 3 most medial sections of the brainstem. Lesions were considered incomplete based on very conservative criteria: an animal was excluded if examination of every 6th 35 μm-thick section immunolabeled for GFAP indicated an incomplete interruption of GFAP-labeled astrocytes at the lesion site, in particular at the most dorsal aspect, or if tissue architecture anywhere within the lesion site resembled host tissue rather than grafted cells based on GFAP immunolabeling and morphology using phase contrast microscopic optics. These analyses were made

by two experienced observers blinded to group identity. Based on these criteria, animals in the following groups were excluded: n=3, no conditioning lesion + Lenti-NT-3; n=1, conditioning lesion + Lenti-NT-3. One animal was also excluded based on insufficient virus spread caudally to the graft/lesion site (conditioning lesion + NT-3-GFP virus). Four animals were excluded due to nucleus gracilis damage or limited fluorogold labeling (n=2, no conditioning lesion + Lenti-NT-3; n=1 conditioning lesion + Lenti-GFP; n=1, conditioning lesion + high titer Lenti-NT-3).

Quantification of axonal density in the nucleus gracilis:

In a series of sagittal sections consisting of every 6th 35 μm-thick section triple-labeled for CTB, GFAP and GFP, and exhibiting fluorogold (FG) fluorescence in the nucleus gracilis, the three most medial sections were selected for analysis by an observer blinded to group identity. For each section, images of: 1) FG-labeled neurons, 2) CTB-labeled axons, and 3) GFP-labeled cells were digitally acquired at 200x magnification using PictureFrame 2.0 Imaging software (Optronics, Goleta, CA) and a Olympus Microfire camera. Using the FeatureJ plugin (www.imagescience.org/meijerling/software/featurej/index.html) for NIH ImageJ software (http://rsb.info.nih.gove/ij/), Hessian filtering was applied to light-level images of CTB-labeled axons to eliminate nonspecific background labeling and images were converted to black and white binary images using threshold settings such that axons appeared black and background appeared white. Nonspecific labeling of objects other than axons was edited from images as described previously (Lu 2005,

Taylor 2006). Area-matched fluorescent photographs of FG labeling were used to outline the area of the nucleus gracilis, outlines were superimposed onto binary-converted light level images, and ImageJ software was used to measure the number of black pixels per total pixels in the outlined area. The mean pixel density (pixels/area) from the three analyzed sections from each animal was compared between groups.

Quantification of GFP-labeled area within the nucleus gracilis:

In the same sections used for axon density quantification, ImageJ software was used to outline regions of GFP expression in fluorescent photographs of GFP labeling. GFP labeling outlines were superimposed onto outlines of FG labeling in the same section and the percent overlap between fluorogold and GFP labeling was calculated for the three most medial brainstem sections.

Electron microscopic analysis of brainstem sections:

Four animals that received combined treatment with conditioning lesions and lenti-NT-3 vectors rostral to the lesion site were used for electron microscopy. Subjects were transcardially perfused with 100 ml cold PBS followed by 250 ml of 4% paraformaldehyde and 2.5% gluteraldehyde in cacodylate buffer. Brainstems and spinal cords were postfixed for 8 hours in perfusion solution at and then stored in TBS + 0.01% sodium azide at 4°C. The next day tissue was sectioned at 45 μm intervals on a vibratome and sections were collected in serial. Every 4th section was labeled for CTB using streptavidin-HRP light level immunohistochemistry followed by GFAP

fluorescent immunolabeling as described above. These sections were examined for

evidence of CTB-labeled axons that had extended into the nucleus gracilis and for

lesion completeness as described above. Lesions in all 4 animals were judged

complete and brainstem sections from the 2 animals with the most CTB-labeling in the

nucleus gracilis were identified. Then, sections adjacent to these immunolabeled

sections in serially collected tissue were processed for immunoelectron microscopy by

labeling with CTB and GFAP as described above, with the addition of a 30 min.

incubation in 50% ethanol and a 30 min. incubation in 1% sodium borohydride prior

to 4 day incubation in primary antibody solution at 4°C. No detergents were used for

immunohistochemical processing of sections for electron microscopy. Spinal cord

sections containing labeled axon terminals were osmicated in 1% osmium tetroxide,

dehydrated in graded ethanols (50%, 7 min; 75%, 7 min, 95% 7 min, 100% 15 min),

and embedded in Durcopan® ACM (Electron Microscopy Sciences, Fort Washington,

PA), sandwiched between Aclar embedding films. Ultrathin sectioning of tissues was

performed using an RMC Products PowerTome X ultramicrotome (Boeckeler

Instruments, Tucson, AZ). The ultrathin sections (60-70 nm thickness) were serially

collected on formvar-coated copper one-hole grids, counterstained with uranyl acetate

and lead citrate, and examined in a JEOL 100 CX electron microscope.

Electrophysiology:

Electrophysiological recordings were collected from the nucleus gracilis after

sciatic nerve stimulation in 4 groups of animals (Table 1). Animals in groups 8 and 9

did not receive dorsal column lesions and were used to generate characteristic

recordings from the intact nucleus gracilis. Animals in groups 10 and 11, respectively,

received conditioning lesions followed 1 week later by C1 dorsal column lesions, cell

grafts, and Lenti-NT-3 (n=5) or Lenti-GFP (n=3) virus injections; these subjects were

used to test for electrophysiological activity in the denervated nucleus gracilis.

Data were obtained 6 weeks after conditioning lesion (groups 8 and 9) or 6

weeks after C1 lesion (groups 10,11) from urethane-anesthetized rats (1.2-1.6 g/kg,

i.p.). Throughout the procedure, body temperature was maintained at 37°C. The

spinal cord was exposed by a T2 laminectomy to provide access to the dorsal columns.

Wire electrodes (125 μm Teflon coated stainless steel with ~5 mm of the insulation

stripped off) to be used for stimulation were hooked around sciatic nerves bilaterally

and sutured to surrounding muscles. Animals were then secured in a precision

stereotax with heads flexed at approximately 45° and the upper cervical/brainstem

region was exposed. Glass microelectrodes (1-3 M ohm impedance) filled with 3M

NaCl were used to obtain extracellular recordings from the ipsilateral nucleus gracilis.

Recordings were made at regular intervals beginning 200 μm rostral through 1400 μm

caudal to the obex; 500 μm mediolateral of the midline; and through a range of 100-

500 μm below the dorsal surface of the brainstem. An average of 29 recording sites

were sampled in C1-injured animals.

Stimulation consisted of a single 100 μsec square monophasic pulse generated

by a constant current stimulus isolator (AMPI Isoflex, Jerusalem, Israel) under the

control of a programmable stimulator (AMPI Master-8). Responses were passed

through a high impedance headstage, band pass filtered (10Hz – 3 kHz) and amplified 1000X using a single channel differential amplifier (A-M Systems). A reference electrode consisting of a stainless steel screw was placed in the skull above the cerebellum. Amplified signals were digitized (Axon Digidata) and collected in a 500 msec sampling window using Clampfit Software (Axon Instruments). Stored traces were further filtered offline using a 100 Hz high pass filter. The magnitude of nucleus gracilis unit responses were quantified over a 10 msec window, beginning 6.0 msec after the stimulus onset, by calculating the root mean square of the waveform. Each post stimulus value was normalized by subtracting the RMS values calculated from a 10 msec window ending 6 msec before the stimulus onset. This method of analysis yielded a single numerical value for the response that was normalized to the pre-stimulus baseline gracilis activity at each recording site. The maximum RMS response observed in lesioned animals lacking regenerated axons (the conditioning lesion + GFP vector group) was used as a minimum threshold to categorize responses. Responses below the control max response were considered negative and those above it would be considered positive.

To verify that evoked responses were due to synaptic activation, the response to pharmacological blockade of glutamatergic transmission using kynurenic acid was determined. With the electrode maintained at a single location, baseline responses to sciatic nerve stimulation were obtained every 3 minutes for a period of 12-15 minutes. Then, 25 μl of a solution containing 50 μM kynurenic acid was applied topically over the nucleus gracilis while continuing to monitor evoked responses every 2 minutes.

After 10-12 minutes, the kynurenic acid solution was removed and the brainstem was bathed with repeated applications of ACSF for 30-60 minutes to wash out the kynurenic acid. Responses were continually monitored every 3 minutes throughout the washout phase. Additional verification that evoked responses were mediated by axons in the dorsal columns was obtained by comparing responses before and after a transection of the dorsal columns at spinal cord level T2.

RESULTS

Retrograde Labeling Identifies Nucleus Gracilis Neurons and Defines the Target Region for Regenerative Growth of Dorsal Column Sensory Axons.

In order to determine whether NT-3 gene delivery promotes sensory axon growth into the nucleus gracilis, we first established a reliable method to identify the extent of the nucleus and the pattern of sensory axon terminals originating from L4-6 DRG neurons. Since a large percentage of nucleus gracilis neurons project to the ventro-posterior-lateral thalamus (VPL), we injected fluorogold (FG) into the VPL to *retrogradely* label nucleus gracilis neurons, and cholera toxin B subunit (CTB) into the sciatic nerve to *anterogradely* label sensory axon terminals in the nucleus gracilis of intact rats (Table 1, group 1). An examination of serially mounted sagittal brainstem sections double-labeled for CTB and FG indicated that FG-labeled neurons were found in the same regions as CTB-labeled axons (Fig. 1). CTB-labeled axons and FG-positive neurons were found in close association throughout the medial brainstem in the region classically described as the nucleus gracilis (Paxinos

1999)((Lund and Webster, 1967; Gulley, 1973; Leong and Tan, 1987; Smith and

Bennett, 1987; Bermejo et al., 2003). FG injections to VPL also labeled neurons of

the more laterally located nucleus cuneatus, which was not innervated by CTB-labeled

axons, but very few neurons outside these nuclei were labeled. FG labeling of the

cuneate nucleus could be distinguished from nucleus gracilis labeling based on the

mediolateral position of the section. We therefore restricted our analysis in

subsequent experiments to the three most medially located sections of the series

examined.

Lentiviral NT-3 Delivery to the Nucleus Gracilis Supports Growth of Transected

Ascending Sensory Axons into the Denervated Nucleus Gracilis.

To assess regeneration of transected dorsal column sensory axons into the

denervated nucleus gracilis after lentiviral NT-3 delivery and peripheral conditioning

lesions (Fig. 2), 5 different groups of animals were investigated (Table 1). Four

experimental groups received dorsal column lesions and injections of "standard" titer

Lenti-NT-3 or Lenti-GFP vectors (100pg/ml), either with or without peripheral nerve

conditioning lesions (Table 1, groups 2-5). In addition, a fifth group of animals

received more superficial NT-3 vector injections with two-fold higher virus titer

(200pg/ml) combined with peripheral conditioning lesions (Table 1, group 6), to

maximize delivery of a trophic stimulus to the target region.

CTB-labeled axons were observed rostral to the lesion site and within the

retrogradely labeled nucleus gracilis only in animals that received lentiviral vectors

expressing NT-3 (Fig. 3). Similar to our previous observations indicating that axons

extending beyond spinal cord lesion sites associate topographically with regions of

NT-3 expression (Taylor et al. 2006), axons in the present experiment that regenerated

beyond the host/graft interface and into the nucleus gracilis were exclusively

associated with regions of NT-3 expression in host tissue. Regenerating CTB-labeled

sensory axons were present within the nucleus gracilis target region, as identified by

co-localization of CTB labeling with retrograde FG labeling (Fig, 3). Axons were

observed in the denervated target in 50% of animals (3 of 6) after NT-3 delivery

without peripheral nerve conditioning lesions (Fig. 4A). Notably, when NT-3 delivery

was combined with conditioning lesions, the proportion of animals in which axons

were found within the nucleus gracilis increased to 100% (7 of 7 animals, Fig. 4B).

High titer NT-3 vector delivery combined with preconditioning lesions also resulted in

axon growth into the nucleus gracilis in 100% of animals (7 of 7 animals, Fig. 4C). In

contrast to animals that received NT-3 lentivirus, control GFP virus injections did not

promote axon growth into the nucleus gracilis in any animal. No axons were observed

beyond the lesion site or within the nucleus gracilis in any subject treated with control

GFP virus without conditioning lesions (Fig. 4D), and only a few axons were observed

immediately beyond the rostral lesion interface, but not within the nucleus gracilis, in

38% of animals (3 of 8 animals) that received control virus combined with

conditioning lesions (Fig. 4E).

Accurate Targeting of NT-3 is Required for Axonal Regeneration into the Denervated Nucleus Gracilis.

We measured axon density within the fluorogold-labeled nucleus gracilis to determine whether axon growth into the denervated target region differed between groups. (Fig. 5A). Animals that received conditioning lesions and NT-3 delivery (low and high titer) showed a significantly higher axon density within the target nucleus compared to GFP control groups. High titer superficial NT-3 vector delivery combined with conditioning lesions resulted in the highest axon density. Density in this group was more than three times greater than the density observed in animals that received standard titer NT-3 vectors combined with conditioning lesions. In contrast, NT-3 delivery without conditioning lesions resulted in limited axon growth within the target region that did not significantly differ from control groups that received GFP vectors.

As noted above, axons regenerating beyond the lesion site and into the denervated nucleus gracilis were associated with regions of NT-3 expression (Fig. 3). Further, axon densities within the nucleus gracilis were highest among animals that received high titer NT-3 vectors, suggesting that axonal growth was directly influenced by the amount or area of NT-3 in the target. Indeed, among animals that received high titer NT-3 virus, 79% of the target area contained vector-transduced cells compared to 54% in subjects that received conditioning lesions plus low-titer NT-3, and 52% in animals that received low-titer NT-3 alone (Fig. 5). Thus, the extent

of NT-3 expression in the target was generally associated with an increase in axonal growth.

Another factor potentially influencing axonal growth into the nucleus gracilis was the distance over which axons were required to regenerate to reach the denervated target; a shorter lesion-to-target distance could favorably bias success of axonal growth. We measured the shortest distance between the rostral host/graft interface (identified by GFAP labeling) and the target (indicated by the presence of FG-labeled cells, Fig. 5C). The mean distance from lesion to target significantly differed only between animals that received conditioning lesions plus standard titer lenti-NT-3 virus and those that received conditioning lesions plus high titer lenti-NT-3 vectors (p = 0.02; Fig. 5). However, the group with the longest mean lesion-to-target distance, the high-titer lenti-NT-3 group, also exhibited the highest density of axons within the nucleus gracilis. Thus, distance from the lesion to target did not bias the outcome of the present findings.

Ectopic Axonal Regeneration Occurs Into Regions of Mistargeted NT-3 Expression.

NT-3 expression outside the nucleus gracilis promoted axon growth to ectopic locations. In some subjects, virus spread occurred into regions that were ventral and rostral to the nucleus gracilis, as well as within the target (Fig. 6). In these cases, some axons extended beyond the lesion and into these ectopic regions of NT-3 expression outside the nucleus gracilis, while other axons followed properly targeted

NT-3 expression and extended into the target. Ectopic axons extended for long distances, in some cases up to 900 μm rostral to the nucleus gracilis. Axons were never observed in these ectopic locations in uninjured, naive animals. These findings further confirm the trophic influence of NT-3 on axonal growth in the lesioned adult CNS.

Lesion Completeness

One important question is whether axonal profiles within the nucleus gracilis represented spared or regenerated axons (Steward et al., 2003). Several observations suggest that axons observed in our lesioned animals were truly regenerating. First, we employed conservative criteria in eliminating subjects wherein lesion extent was uncertain, based on observations of two experienced, blinded observers (authors LT and MT). These criteria included confirmation that GFAP labeling in all subjects was disrupted at all dorso-ventral levels of the lesion site, and that the tissue architecture throughout the lesion/graft was distinct from intact tissue, indicating a lack of spared tissue within the lesion. Second, axons were observed emerging from all levels of the rostral host/graft interface, rather than just at the most dorsal or ventral parts where spared axons might be expected. Third, we observed axons in ectopic locations beyond the lesion site, where axons are not found in intact animals. Finally, no axons were observed within the target region in any animals that received control (GFP) lentiviral vector injections (i.e. by our criteria, lesions were complete in all control

animals). Taken together, these observations support the presence of truly regenerated rather than spared axons in the nucleus gracilis.

Regenerating Axons Appear to Form Synapses in the Nucleus Gracilis.

Transected dorsal column sensory axons that extended into the nucleus gracilis in response to NT-3 delivery were in a potential location to form synapses with appropriate target neurons. To test whether synapses were formed after regenerative axon growth into a denervated target, medial brainstem sections of animals with high titer NT-3 virus injections and conditioning lesions were examined by immuno-electron microscopy for evidence of synaptic contacts between CTB-labeled axons and neurons in the nucleus gracilis (Fig. 7). Grafted marrow stromal cells were easily identified during ultrastructural examination and were well integrated with host tissue at the rostral host/graft interface (Fig. 7). Distal to this interface, several CTB-labeled synaptic boutons were identified in one of two subjects, indicating synapse formation by ascending sensory axons in the nucleus gracilis. Synaptic boutons were distinguished by a patchy distribution of electron dense particles in axons, indicating the presence of DAB reaction product introduced by CTB immunolabeling. The observed CTB-labeled boutons contained round (rather than ovoid) synaptic vesicles suggesting an excitatory phenotype (Peters and Palay, 1996). CTB-labeled regenerated axons in the nucleus gracilis also exhibited features atypical of intact axons, including multiple synaptic contacts with a single dendritic process, the presence of dendritic spines from gracilis neurons protruding through the CTB-labeled axoplasm, and

unusually high quantities of synaptic vesicles in the regenerated axon terminal. These features may constitute markers of regenerated axons.

Electrophysiological Evaluation of Synapse Formation in the Nucleus Gracilis after NT-3 Delivery.

To assess whether synaptic activity could be detected in association with regenerated axons, electrophysiological studies were conducted. Extracellular recordings were performed in 20-50 sites sampling the rostrocaudal extent of the nucleus gracilis following electrical stimulation of the sciatic nerve in 5 combinatorially-treated subjects (Fig. 7). Initial evoked responses were recorded from naïve animals (n=3), and from animals without spinal cord lesions that received conditioning lesions six weeks prior to recording (n=3). The number of sites wherein responses could be evoked, and the overall magnitude of the evoked responses (Fig. 7C), were comparable in intact and sciatic nerve conditioned (but not centrally lesioned) animals, indicating that the conditioning lesions did not affect the ability to elicit an evoked response via sciatic nerve stimulation (data not shown). Evoked responses from animals with no spinal cord lesions were synaptically mediated, because responses were nearly completely abolished following local application of the glutamate antagonist kynurenic acid (Fig. 7A and 7B). After washout of kynurenic acid with artificial CSF, responses returned to near baseline levels in these non-lesioned subejcts. In addition, transection of the dorsal columns at T3 completely

abolished evoked responses within the nucleus gracilis, indicating that recorded potentials were transmitted through the dorsal columns (Fig. 7A, B).

Having established a paradigm for recording postsynaptic potentials in the nucleus gracilis, we next compared responses in intact animals to responses in C1-injured animals that underwent full treatment with cellular grafts, conditioning lesions and either high titer NT-3 or GFP vector delivery. Fig. 7C shows the range and mean of the root mean square (RMS) response magnitude for all recording sites in individual animals. Because our histological analysis showed that GFP vector delivery did not support axonal growth into the nucleus gracilis, the maximum RMS response recorded in this control group was used as a threshold for analyzing responses in intact and NT-3-treated animals (Fig. 7C, green line). This criterion allowed us to determine whether responses at individual recordings sites exceeded the background response. Postsynaptic potentials in intact animals exceeded the threshold response in 67% of recording sites sampled. In contrast, among animals treated with exogenous NT-3, only two responses, representing 1.4% of recording sites, exceeded those of GFP control animals 6 weeks following lesions. Closer investigation of response curves associated with these two sites indicated that they did not qualitatively differ from those of GFP control animals, nor did they resemble postsynaptic potentials observed in intact animals. These findings should be considered in light of the fact that only a small proportion of the target nucleus was sampled (far less than 1%).

DISCUSSION

In order for axon growth to promote functional recovery after SCI, regenerating axons must form active synaptic contacts with neurons beyond the injury site. We show here that lentivirus-mediated NT-3 delivery to the denervated nucleus gracilis, combined with conditioning lesions, can promote significant growth of transected dorsal column sensory axons across C1 spinal cord lesion sites and into the nucleus gracilis. Furthermore, ultrastructural examination reveals synapse formation between labeled axons and dendritic processes in the nucleus gracilis. These findings demonstrate that combinatorial treatments can be used to promote and direct axon growth and provide the first anatomical evidence for formation of synaptic contacts after regenerative growth into a denervated target of spinal cord projections.

Factors Contributing to Axonal Regeneration into the Nucleus Gracilis

In the current study, we evaluated the ability of NT-3 delivery, conditioning lesions or both treatments combined, to promote axonal growth into the nucleus gracilis. Importantly, NT-3 expression appeared to be the key determinant in directing topographical axon growth into the denervated target. This conclusion is supported by the fact that NT-3 expression beyond the lesion was necessary to promote axon growth into distal host tissue. Axons extended into regions of NT-3 expression in the target (Taylor et al., 2006) and when NT-3 expression within the target was increased, axon growth into this region was enhanced. Further, when NT-3 was expressed in ectopic locations outside the nucleus gracilis, axon growth was directed toward these ectopic

sites rather than toward the appropriate target. These findings are reminiscent of neural development, in which ectopic expression of neurotrophins guides growing axons into abnormal termination sites (Tucker et al., 2001). It appears that the location of NT-3 expression, rather than cues expressed by denervated target cells, is responsible for directing chemotropic axon growth. Taken together, these data indicate that neurotrophin delivery to specific sites can provide guidance cues for new axonal projections in and beyond the injured spinal cord.

In addition to a role in directing axon growth toward the target, NT-3 may also have promoted synapse formation directly. Developmental studies have suggested that neurotrophin expression at target sites is necessary to promote innervation and synapse formation in a number of contexts (Ma et al., 2002; Patel et al., 2003; Genc et al., 2004). Thus, NT-3 delivery may provide a particular synaptogenic stimulus in development that is also effective after regenerative axon growth. For example, neurotrophin expression in the target region may have promoted local axon sprouting (Schnell et al., 1994; Cohen-Cory and Fraser, 1995) to facilitate identification of postsynaptic cells leading to synapse formation. Indeed, we have previously observed axon sprouting in response to NT-3 delivery beyond lesion sites in white matter (Taylor et al., 2006); and similar types of growth and terminal specification were observed in the present study when NT-3 was expressed in the target nucleus (Figure 3,4,6).

The application of a conditioning lesion increased the axonal growth response to NT-3. These findings confirm previous results from our laboratory that suggested

the importance of combined neurotrophin stimulation at the axon tip and somal stimulation by a conditioning lesion to enhancing the overall axonal growth response after CNS injury (Chapter 3)(Lu et al., 2004). Combinatorial stimulation may enhance growth into the nucleus gracilis in several ways. Two positive stimuli to the injured neuron (a trophic factor and a conditioning lesion) may elicit simple additive responses through identical intracellular mechanisms focused on axonal events, such as actin assembly, that increase overall axonal growth. However, it appears more likely that NT-3 delivery and conditioning lesions support axon growth through distinct mechanisms. For example, NT-3 might provide a short distance growth signal to the growth cone (Ma et al., 2002; Patel et al., 2003; Genc et al., 2004), but may be insufficient for promoting axonal elongation and extension over inhibitory substrates. Conditioning lesions may initiate an axonal extension (or long distance growth) program. The combination of enhancement of axonal extension mechanisms and growth-cone growth mechanisms may together elicit long distance and target-specific growth of the nature observed in this experiment. Further and necessarily complex studies will more fully elucidate mechanisms underlying the effects of combinatorial treatments after SCI.

A key component of this experimental design was the use of fluorogold (FG) retrograde labeling to identify neurons comprising the nucleus gracilis. Importantly, tracing studies in intact animals established that in the medial medulla, FG-labeled cells were found almost exclusively in regions in which ascending sensory axons terminated. In animals that received NT-3 delivery, we identified axons in close

proximity to FG-labeled cells, providing strong evidence that these axons regenerated into original target fields. The fact that axons were clearly in position to form synaptic contacts strengthens ultrastructural findings indicating synaptogenesis.

Synapse formation in the nucleus gracilis.

In the present study, we identified anterogradely labeled synaptic boutons in the nucleus gracilis after treatment with NT-3 delivery and conditioning lesions. To our knowledge, this is the first ultrastructural evidence for synapse formation after regeneration of spinal cord axons into a denervated target region. These studies provide proof of principle that regenerating axons in the spinal cord have the capacity to form synaptic structures upon reaching host tissue distal to lesion sites.

Several factors support the notion that synapses observed at the ultrastructural level were derived from regenerated axons. First, structural features characteristic of the labeled boutons, including multiple synaptic contacts formed with a single dendritic process, and dendrites of medullary neurons protruding through the axoplasm of regenerated axons, are not typical of synaptic contacts observed in the pre-injured, intact nucleus gracilis. In addition, labeled boutons of regenerated axons were unusually large compared with normal synaptic structures in this nucleus (Rustioni and Sotelo, 1974). These features suggest that the observed synapses were newly formed. To provide greater confidence that no spared axons were present in sections subjected to ultrastructural or light microscopic analysis, we carefully examined sections from all of these spinal cords: any subject was eliminated from

consideration if lesion completeness was uncertain. Thus, CTB-labeled synaptic boutons observed in the nucleus gracilis are likely to represent regenerated structures.

Ultrastructural evidence for synapse formation suggested the possibility that regenerated axons might be functional. The observed synapses contained numerous round synaptic vesicles, consistent with an excitatory transmitter phenotype (Peters and Palay, 1996). Only axo-dendritic synapses were observed; no axo-somatic synapses in CTB-labeled axons were identified, replicating the most common pattern of normal dorsal column somatotopy within the nucleus gracilis (Rustioni and Sotelo, 1974).

Given the preceding findings, electrophysiological studies were conducted to determine whether target reinnervation supported synaptic transmission. Sampling a relatively small proportion of the target nucleus and using conservative criteria for electrophysiological recovery, we did not detect synaptic potentials in the nucleus gracilis following sciatic nerve stimulation. This outcome occurred either because sampling was inadequate, or because regenerated axons and confirmed synaptic contacts were non-functional. The latter possibility appears unlikely, given the presence of a full set of cellular mechanisms at the ultrastructural level to support synaptic transmission. However, it is possible that portions of the regenerated axons were unmyelinated, leading to increased latency and decreased duration and strength of evoked responses (Yezierski et al., 1992) and an accordingly reduced opportunity to detect relatively weak responses. These possibilities serve to highlight the

complexities inherent in paradigms that aim to restore function in the lesioned adult CNS.

Improving the model system

Despite our inability to detect activity of regenerated axons in the present experiment, the present studies have established a paradigm that can be used to measure functional recovery in a model of target reinnervation. A number of factors that may have hindered synapse formation could potentially be minimized in future studies to enhance axon growth and increase synapse formation to levels that could potentially be detectable by electrophysiology. For example, axons may have been unable to contact denervated target cells due to upregulation of inhibitory extracellular matrix molecules surrounding potential synaptic partners (Massey et al., 2007). Such strategies as administration of chondroitinase to degrade inhibitory extracellular matrix, in combination with and NT-3 and a conditioning lesion, might further increase formation of synaptic structures (Massey et al., 2006; Massey et al., 2007). Second, expression of NT-3 by glial cells throughout the nucleus gracilis may have interfered with the ability of axons to detect potential neuronal postsynaptic targets, thereby limiting synapse formation. Expression of NT-3 specifically by target neurons might allow more axons to correctly identify target neurons.

Third, synapse formation may have been attenuated due to conditioning lesions, which may downregulate synaptic proteins that might be necessary for synapse formation (Hughes et al., 2004). Identification of specific genetic pathways

that mimic the conditioning lesion effect might provide a means to augment axon growth without potentially deleterious side effects. Fourth, axons may have been mistargeted away from the nucleus gracilis by ectopic NT-3 delivery, reducing the total number of axons regenerating into the target. To address this problem, the target could be defined more strictly by delivering repulsive proteins, such as semaphorins, to regions ventral and rostral to the nucleus gracilis, and delivering NT-3 into the target (Pasterkamp et al., 2001; Tang et al., 2007). Additional manipulations such as these might increase the number of synapses in the nucleus gracilis to levels that could be detected by electrophysiological or functional testing.

The present work illustrates that in vivo neurotrophic factor gene delivery combined with conditioning lesions can be used to guide injured axons toward appropriate target regions, and provides anatomical evidence for synapse formation after regenerative axon growth into a denervated target. Thus, we have taken the first steps toward demonstrating that axons retain the capacity to form functional synapses after injury. Modifications to this model system might allow further steps, such as demonstration of functional connectivity, to be taken. In addition, the model system developed here could be used to address a number of salient mechanistic questions surrounding formation of synapses by regenerating axons.

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Table 4.1. Experimental groups. Eleven groups of animals were used for histology, electron microscopy (EM) or electrophysiology. Intact animals were used to define the nucleus gracilis histologically (Group 1) and electrophysiologically (Group 8 and 9). All other animals received C1 dorsal column (DC) lesions and were divided into groups based on three treatments: 1) conditioning lesions, 2) lenti-NT-3 vs. lenti-GFP virus injection rostral to the lesion site, or 3) vector injection sites and concentration (100 vs. 200 μg/ml p24).

| Analysis	Groups	Conditioning lesion	DC lesion	Lentiviral vector	Vector Titer	Injection depths (mm)	Animal #	Included animals
Histology	1	No	None	None	N/A	N/A	N=2	N=2
	2	No	C1	GFP	100μg/ml	0.25, 0.5, 1.0	N=9	N=9
	3	Yes	C1	GFP	100μg/ml	0.25, 0.5, 1.0	N=9	N=8
	4	No	C1	NT-3	100μg/ml	0.25, 0.5, 1.0	N=11	N=6
	5	Yes	C1	NT-3	100μg/ml	0.25, 0.5, 1.0	N=9	N=7
	6	Yes	C1	NT-3	200μg/ml	0.25, 0.5	N=8	N=7
EM	7	Yes	C1	NT-3	200μg/ml	0.25, 0.5	N=4	N=4
Electrophysiogy	8	No	None	None	N/A	N/A	N=3	N=3
	9	Yes	None	None	N/A	N/A	N=3	N=3
	10	Yes	C1	GFP	high	0.25, 0.5	N=3	N=3
	11	Yes	C1	NT-3	high	0.25, 0.5	N=5	N=5
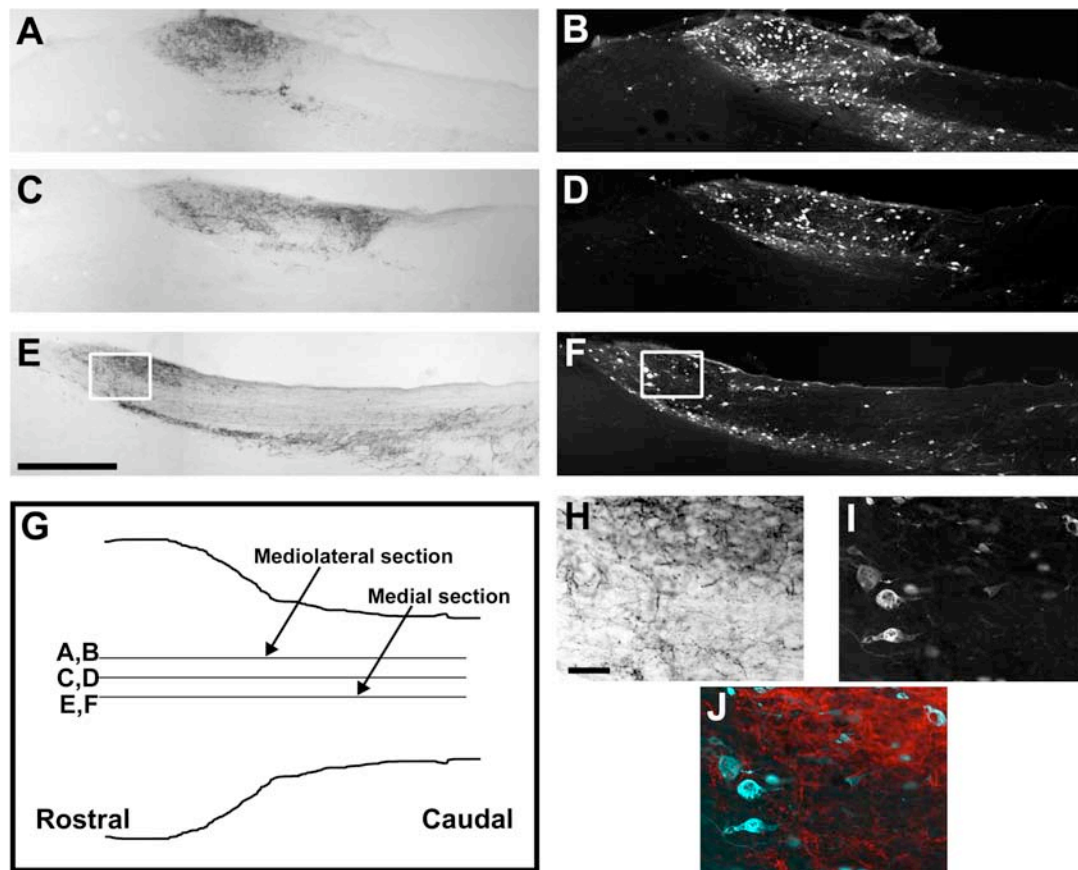
Figure 4.1. Fluorogold retrograde and CTB anterograde labeling of the nucleus gracilis and ascending sensory axons in intact animals: normal anatomy. (A, C, E) Ascending sensory axons anterogradely labeled with CTB normally terminate in the nucleus gracilis through a range of medial-to-lateral sections, (B,D,F) in the same sites wherein FG-labeled neurons are observed following retrograde transport from the VPL nucleus of the thalamus. (G) The schematic illustrates the medio-lateral positions of sections A-F. A high magnification view of the boxed region in the most medial section illustrates the close association between (H) terminating CTB-labeled axons and (I) FG-labeled nucleus gracilis neurons. (J) Merged panels H,I. Scale bars: A-F, 500 μm; I-K, 100 μm.
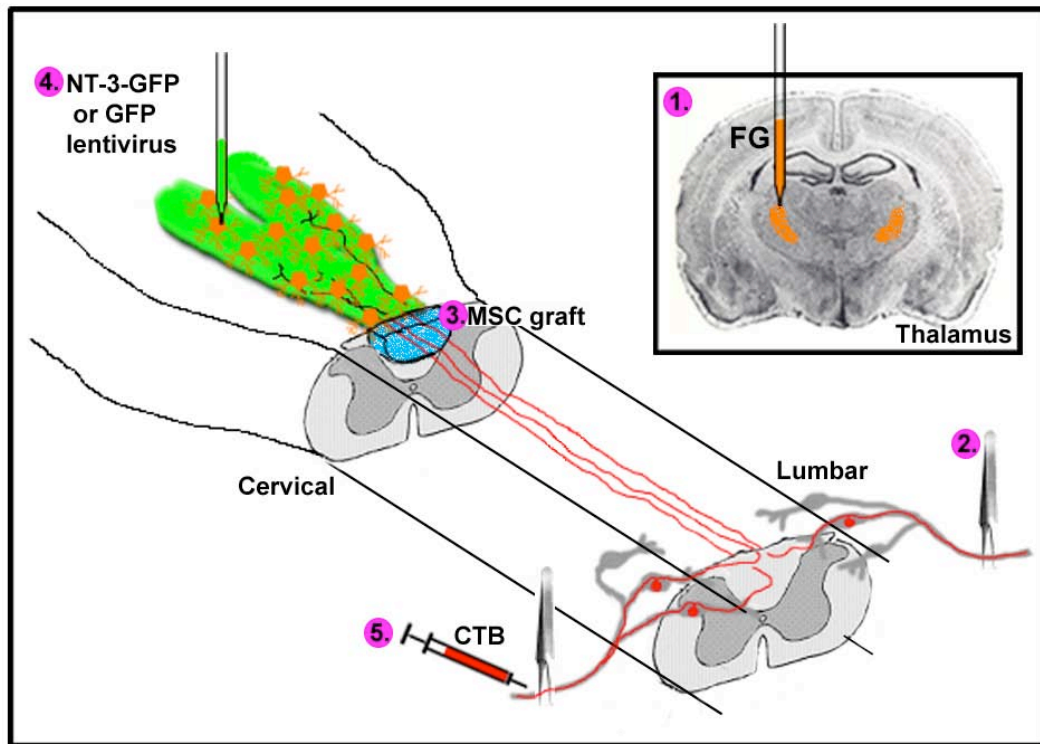
Figure 4.2. Experimental paradigm. 1) Nucleus gracilis neurons were retrogradely labeled using bilateral injection of fluorogold (FG, orange) into the ventro-posteror-lateral (VPL) thalamus one week prior to dorsal column lesions. 2) Some animals received bilateral sciatic nerve conditioning lesions, while controls received no sciatic nerve lesions. 3) 7 days later a wire knife was used to transect the dorsal columns approximately 1.5 mm caudal to the obex, and marrow stromal cells (MSCs) were grafted into the lesion site. 4) Immediately after grafting, lentiviral vectors expressing both NT-3 and GFP ("Lenti-NT-3") or GFP alone ("Lenti-GFP") were injected into the nucleus gracilis bilaterally, slightly rostral and lateral to the obex. 5) 4 weeks later, ascending sensory tracts were labeled by injecting CTB into both sciatic nerves; 3 days later, animals were perfused.
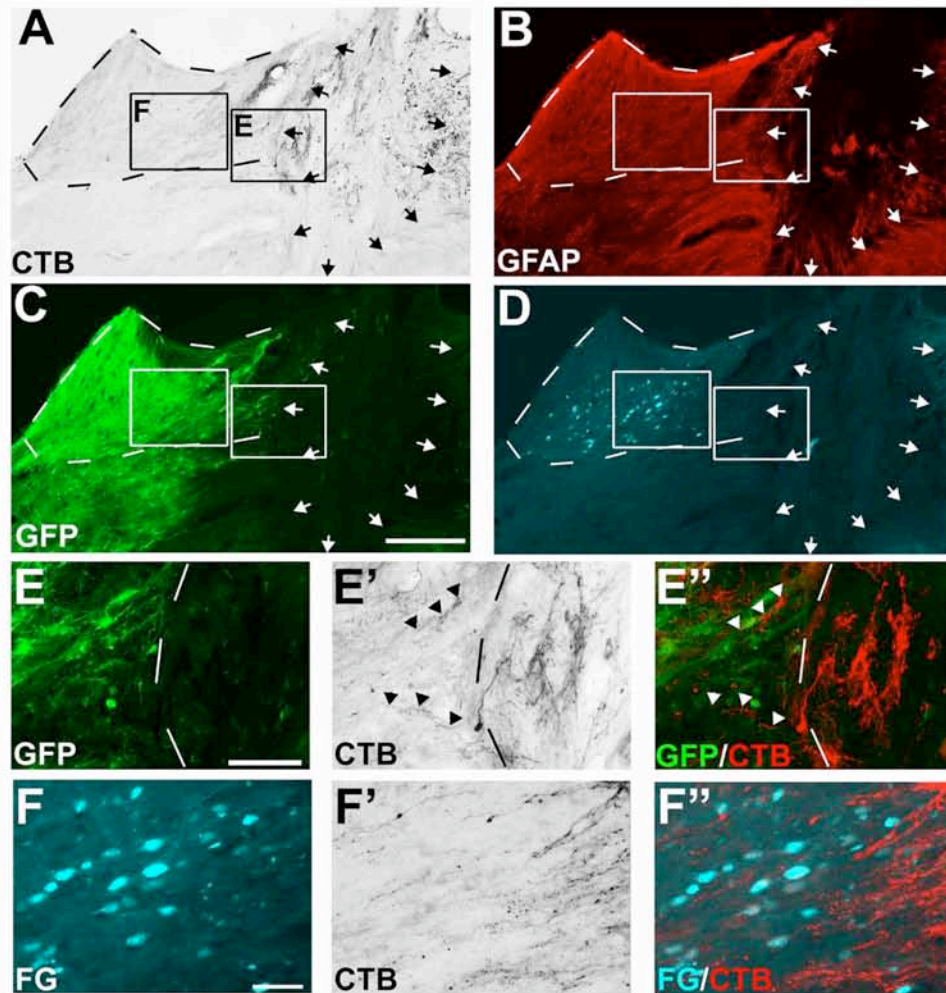
Figure 4.3. Transected ascending sensory axons extend toward Lenti-NT-3 -transduced cells in the denervated nucleus gracilis. Triple labeling for either: (A) CTB to identify ascending sensory axons, (B) GFAP to indicate the lesion/graft borders (arrows), (C) GFP to identify NT-3-GFP vector-transduced cells, and (D) fluorogold (FG) fluorescence to identify nucleus gracilis neurons, in a sagittal brainstem section 4 weeks after dorsal column lesion, cell grafting and lenti-NT-3 gene delivery (200pg/ml) to the nucleus gracilis. Dashed lines encircle the nucleus gracilis. (E) High magnification view of the graft/host border (box E) shows CTB-labeled axons crossing into rostral host tissue in a region containing (E') NT-3-GFP-expression. (E") Merge of panels E and E' with CTB-labeled axons pseudocolored red. (F) Regions of NT-3-GFP expression overlap with (F') regions containing FG-labeled nucleus gracilis neurons, and (F") CTB-labeled axons are present within the denervated nucleus. F" is a merge of panels F and F' with axons pseudocolored red. Scale bars: A-D, 250 μm; E, 100 μm; F, 50 μm.

Figure 4.4. Comparison of axon growth into the nucleus gracilis in five experimental groups. (A,B,C) Axons were observed distal to the rostral lesion border (arrows) and within the nucleus gracilis (dashed lines) in animals that received: (A) NT-3 lentivirus delivery, (B) conditioning lesions plus NT-3 lentivirus delivery, and (C) conditioning lesions plus high titer NT-3 lentivirus delivery. The greatest number of axons were observed in animals with full combination treatments. No axons were observed within the nucleus gracilis in animals that received (D) GFP lentivirus delivery, or (E) GFP lentivirus delivery with conditioning lesions. (A'-E') High magnification of boxes in A-E. Axons are indicated by arrowheads. Scale bars: A-E, 250 μm; A'-E', 100 μm.
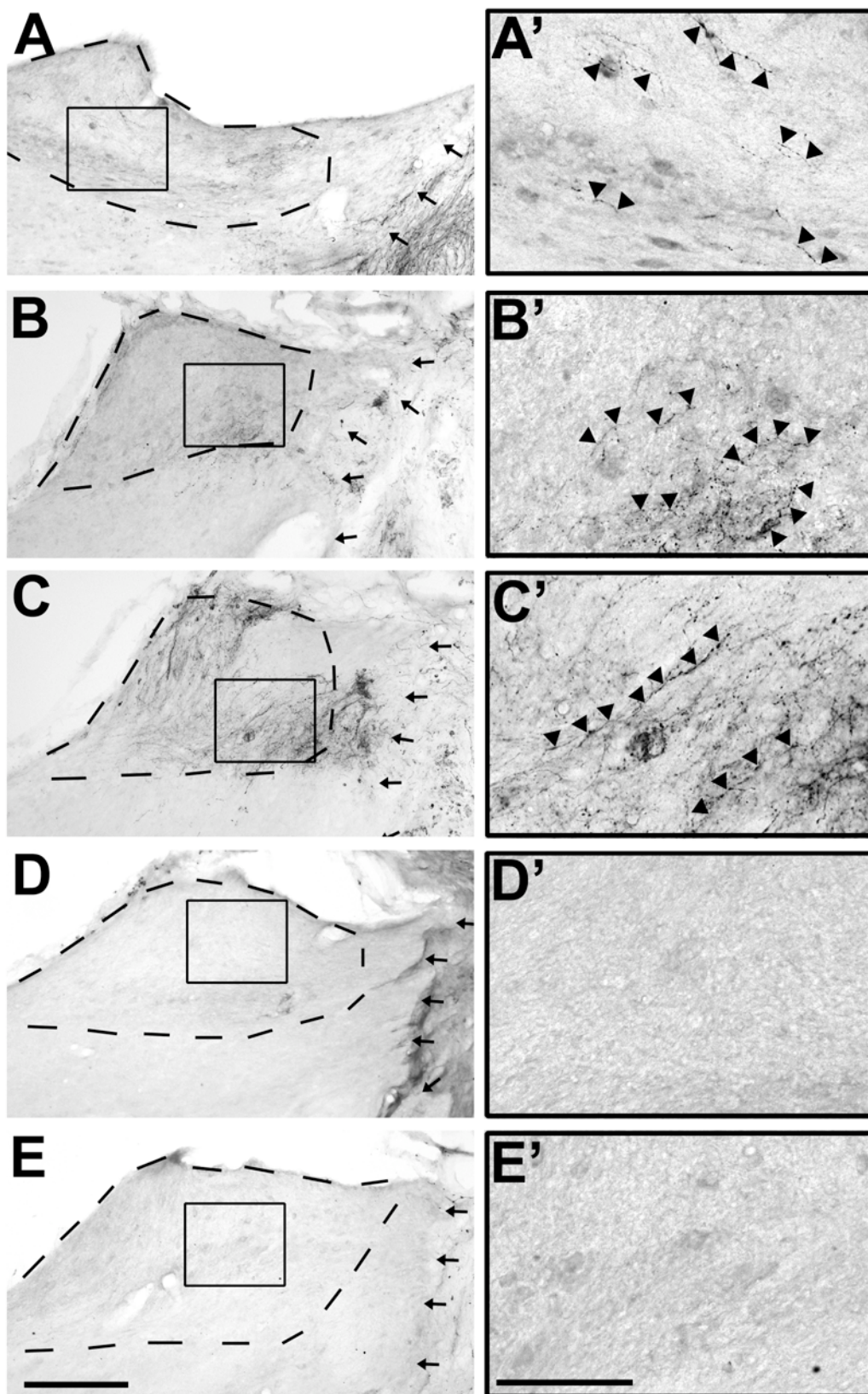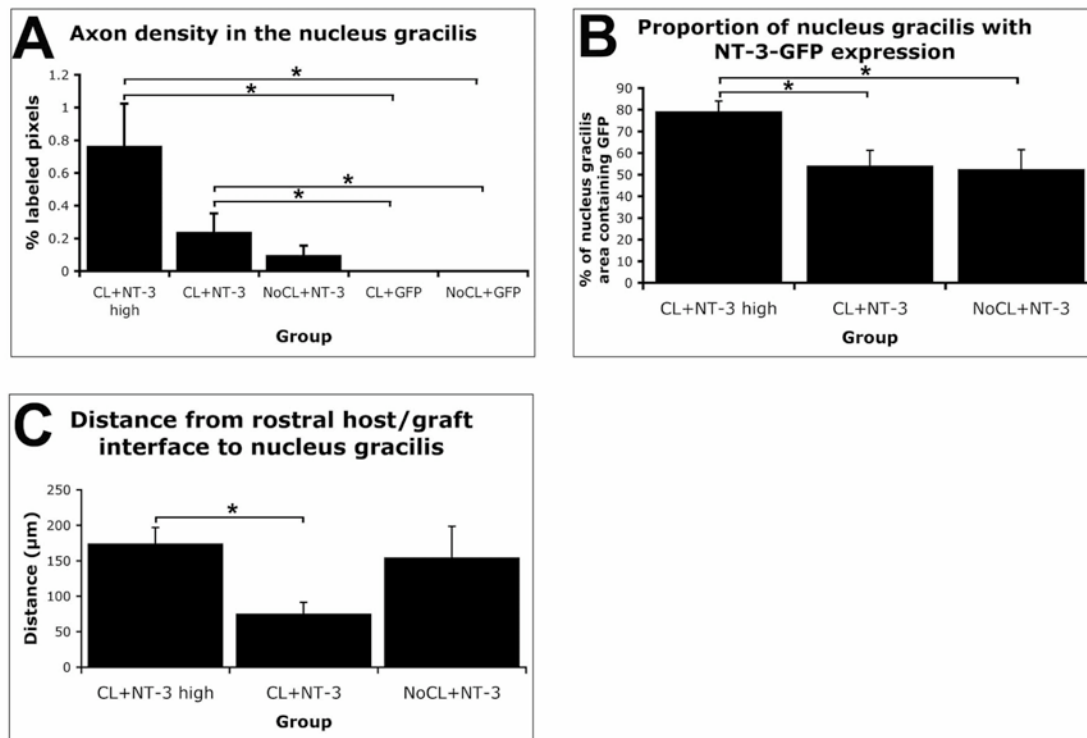
Figure 4.5. Quantification of CTB-labeled axons and NT-3 expression in the nucleus gracilis. (A) The density of CTB-labeled axonal profiles within the nucleus gracilis was significantly higher in groups that received lenti-NT-3 injections combined with peripheral nerve preconditioning lesions, compared to control groups that received lenti-GFP with or without preconditioning lesions. (B) The proportion of the nucleus gracilis displaying GFP expression was significantly higher in animals that received superficially injected, high titer lenti-NT-3 virus compared to the two other NT-3 virus-injected groups. (C) The distance between the rostral host/graft interface and the most caudally located FG-labeled cell was longest in the CL+NT-3 high treatment group. Despite the fact that axons were required to grow farther to reach the nucleus gracilis in this group compared to the CL+NT-3 group, axon regeneration into the target was greater (graph A). A, Kruskall Wallis test, chi square <0.0001; Mann-Whitney U post-hoc tests between all groups with Bonferroni adjusted significance criterion ($p<0.005$), *$p<0.001$. B, ANOVA, $p=0.027$, Fisher's PLSD, *$p<0.05$. C, ANOVA, $p=0.05$, Fisher's PLSD, *$p<0.05$. Values are mean ± S.E.M. CL, conditioning lesion; NT-3 high, $200\mu g/ml$ lenti-NT-3 vector.
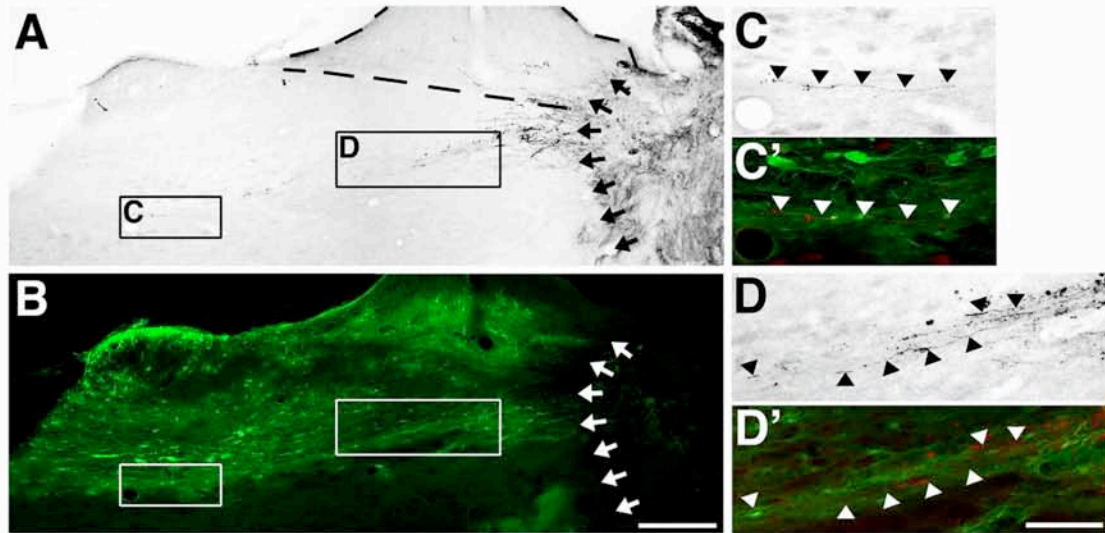
Figure 4.6. Lesioned axons exhibit ectopic regeneration when NT-3 expression is not restricted to nucleus gracilis. (A) CTB-labeled axons are found beyond the C1 lesion site (arrows), but outside the nucleus gracilis in regions where (B) GFP labeling identifies cells transduced by Lenti-NT-3 virus in an animal that received Lenti-NT-3 delivery and conditioning lesions. These findings indicate that axons exhibit chemotropic responses to NT-3, and will regenerate to ectopic locations when growth factor gradients exist outside the target nucleus. Dashed lines encircle the nucleus gracilis. (C, D) Axons in ectopic locations are found in regions where (C', D') GFP labeling indicates the presence of NT-3-GFP-virus transduced cells. Scale bars: A,B, 250 μm; C,D, 100 μm.

Figure 4.7. Axons regenerating into the nucleus gracilis may form new synapses in the denervated target region. (A) Marrow stromal cells within the lesion site, identified by large nuclei (asterisks), adjacent to host tissue beyond the lesion site, exhibiting excellent graft integration into the host/lesion interface. Dashed line represents host (h)/graft (g) border. Rostral is left. (B) Beyond the interface, CTB-labeled synaptic boutons are observed. The large bouton pictured exhibits multiple possible synaptic specializations (arrows) on medullary neuronal dendritic spines (d) that protrude through the cytoplasm. (C) High magnification view of the boxed area in B. Arrows indicate a synapse.
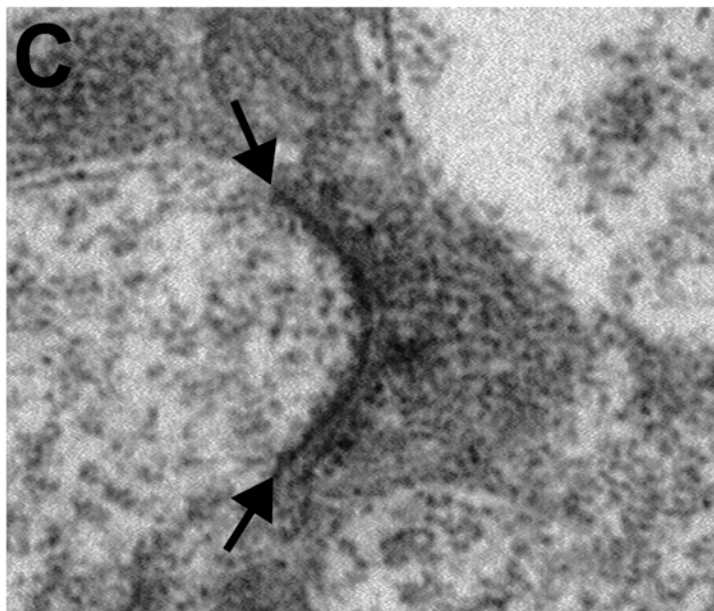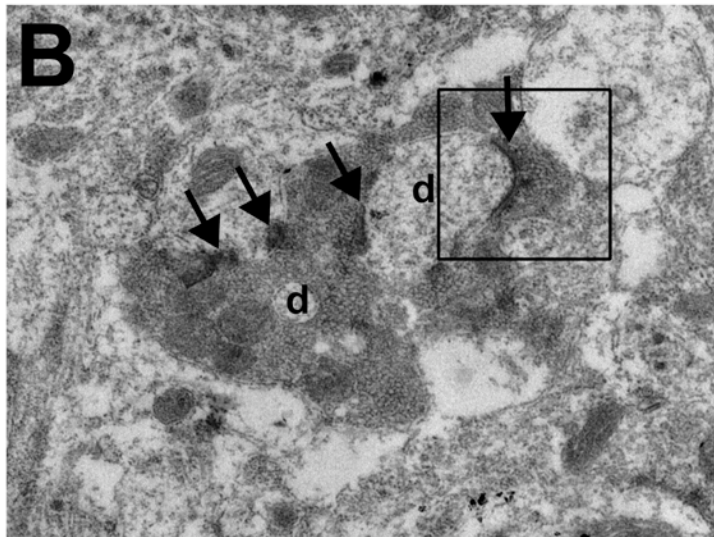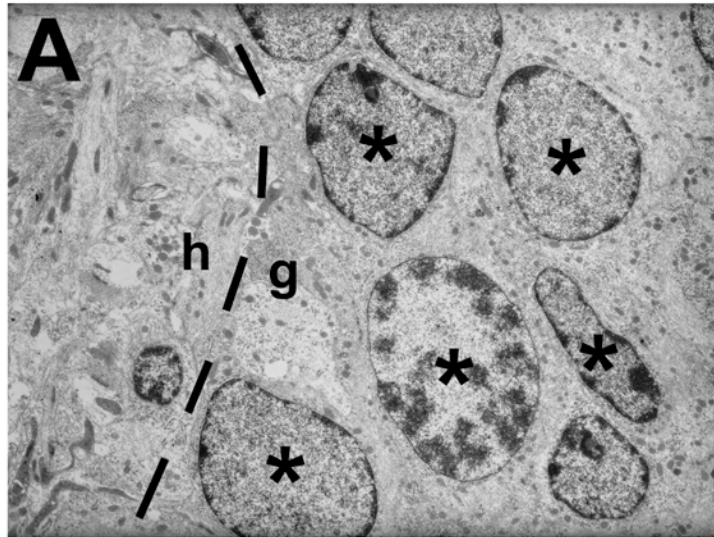
Figure 4.8. **Electrophysiological responses in the nucleus gracilis evoked by sciatic nerve stimulation in intact and C1 injured animals.**
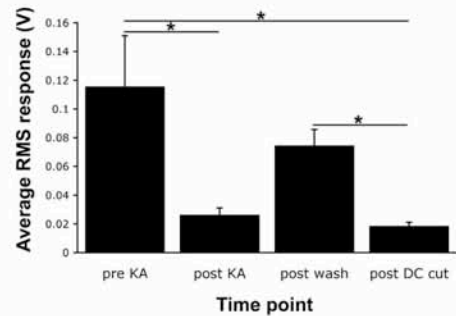(A) Representative traces from a single nucleus gracilis recording site in an intact animal. Baseline recordings obtained from a naïve (intact) animal (pre KA), after addition of the glutamatergic antagonist kynurenic acid [50 μM] (post KA), following washout of kynurenic acid (post wash) and after a dorsal column transection at T3 (post DC cut) are shown. Arrows indicate the pre- and post-stimulus windows used to calculate the root mean square (RMS) power spectrum response in each case (see materials and methods).
(B) Statistical analysis of evoked responses shows significant attenuation of pre-KA responses following addition of kynurenic acid (post-KA). Washout with ACSF partially reversed the effects of kynurenic acid (post wash). Transection of the dorsal columns at spinal level T3 completely abolished evoked responses. Naïve animals and animals with sciatic nerve lesions but uninjured spinal cords showed similar results and were therefore combined for this analysis (n=3 per group). Repeated measures ANOVA p=0.006. Fischer's least square difference posthoc test, *p<0.05. (C) A comparison of responses to sciatic nerve stimulation recorded in the nucleus gracilis in i) intact animals (INT, n=3), ii) intact animals with conditioning lesions (CL INT, n=3), iii) C1 lesioned animals that received GFP-expressing vectors and cell grafts plus conditioning lesions (CL+GFP, n=3) and iv) C1 lesioned animals that received NT-3-expressing vectors and cell grafts plus conditioning lesions (CL+NT-3, n=5). Vertical lines indicate the maximum, minimum and mean RMS response values obtained from all recording sites for individual animals. The horizontal dotted line (green) indicates the maximum RMS response level recorded in control CL+GFP animals. Recorded responses exceeded the maximum control response at 2 sites in a single animal that received conditioning lesions and NT-3 vectors (red arrow). (D) Further examination of individual traces did not indicate the presence of evoked activity in NT-3 treated animals. In the 2 instances wherein RMS response values exceeded the maximum level obtained in GFP-treated C1-lesioned animals, individual traces were further investigated (top 2 traces in D). In neither case did the post-stimulus response resemble activity found in intact or conditioned-lesioned animals; traces were more comparable to those found in C1-lesioned + GFP-treated animals (bottom trace) or in animals with acute transection of the dorsal columns at the T2 level (Figure 7A, post cut).

A Effect of kynurenic acid and dorsal column transection on evoked potentials in an intact control animal

prestim   poststim

pre KA

post KA

post wash

post DC cut

0.25 mV

20 ms
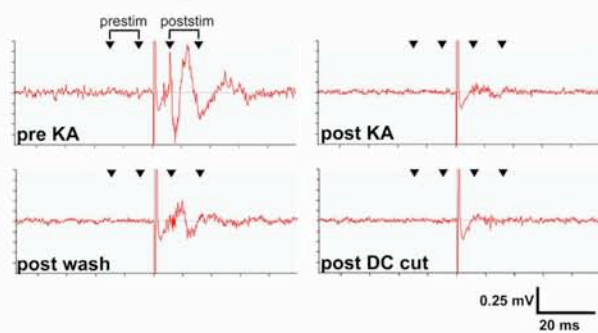
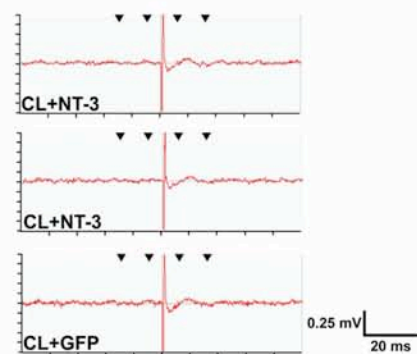**B** Average effect of kynurenic acid and dorsal column transection in all intact animals

C Range of recorded potentials in individual animals

Max

Mean

Min

INT   CL INT   CL+GFP   CL+NT-3

Animal subject

D

CL+NT-3

CL+NT-3

CL+GFP

0.25 mV

20 ms
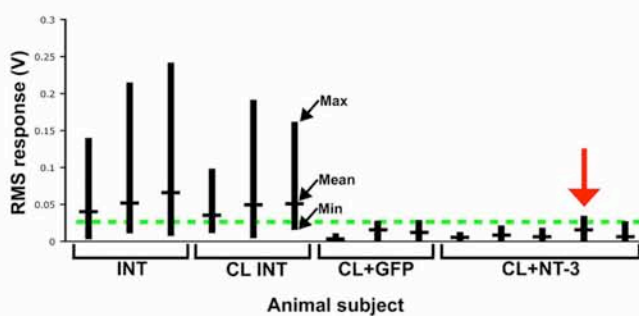
CHAPTER 5

General discussion

Neurotrophins can promote robust axon growth into lesion sites after spinal cord injury and a large body of literature demonstrates a role for these proteins in supporting and guiding axon growth. Based on these characteristics, we proposed that delivery of neurotrophins beyond spinal cord lesion sites using in vivo gene delivery could create chemotropic gradients that could promote growth of axons into distal host tissue beyond the lesion. This thesis work has demonstrated that neurotrophin gradients can indeed promote axon growth beyond spinal cord lesions, but that these gradients are insufficient to promote long distance axon regeneration. However, when neurotrophin gradients are combined with stimulation of growth programs at the level of the cell body, long distance axon growth can be achieved. Further, these combined stimuli can be used to promote and guide axon growth toward denervated targets where regenerating axons can form new synaptic connections. Below, the key findings of this thesis work will be discussed and future directions for the project will be outlined.

In vivo gene delivery can be used to establish neurotrophin gradients in the injured spinal cord.

The present work began with the observation that while ex vivo gene delivery of neurotrophins to spinal cord lesion sites was a powerful means for promoting

growth of injured axon into grafts, this delivery method did not result in axonal egress from the graft into distal host tissue (i.e. Lu 2003). The present work demonstrated that in vivo gene delivery rostral to lesion sites expands neurotrophin gradients such that the apex of the gradient is located beyond the lesion rather than within it. These studies demonstrated that in vivo gene delivery can be used to establish desirable patterns of neurotrophin expression beyond lesion sites after SCI.

In vivo gene delivery provides several important advantages over traditional delivery methods, including intrathecal delivery and protein injections. Viral delivery allows long-term gene expression in a spatially restricted and appropriately targeted manner. The ability to deliver therapeutic proteins continuously using in vivo gene delivery may be advantageous in promoting axon growth. NT-3 delivery by lentiviral vectors in the present study substantially increased the number of axons extending into distal host tissue compared to delivery of NT-3 protein to the same location (Lu et al., 2004). Possible disadvantages of in vivo gene delivery for clinical applications include potential immune responses to vectors, inability to discontinue gene delivery in case of adverse affects, and the possibility that integration of vectors into the host genome could affect normal gene expression. However, lentiviral vectors induce a minimal immune response, development of regulatable gene delivery systems is ongoing, and obvious deleterious effects caused by these vectors have not been reported in clinical trials (Wong et al., 2006). AAV vectors might also be used as an alternative to lentiviral vectors if side effects are discovered. AAV gene transfer is currently being used in several ongoing clinical trials (Lillicrap et al., 2006;

Warrington and Herzog, 2006) and AAV serotypes with infectious properties similar to those of lentiviruses have recently become available (Wu et al., 2006). Thus, in vivo gene delivery of therapeutic proteins remains a promising approach for treatment of SCI clinically.

NT-3 has a chemotropic effect on regenerating axons.

A number of observations support the conclusion that NT-3 acts tropically on regenerating axons after injury. First, we observed axon growth beyond spinal cord lesion sites specifically into regions where NT-3 was expressed and axons were found in close proximity to NT-3 expressing cells. In addition, axon growth beyond lesion sites was dependent on local availability of growth factors, such that no growth occurred unless NT-3 was expressed directly adjacent to the host/graft interface. In experiments involving delivery of neurotrophins to the nucleus gracilis, wherein axons had a clearly defined target, expression of NT-3 in ectopic locations promoted axon growth away from the target and toward the region of NT-3 delivery.

Several other studies lend support to the conclusion that NT-3 can act chemotropically to guide sensory axon growth. For example, when NT-3 was delivered to spinal cord lesion sites via ex vivo gene delivery, sensory axons penetrated cellular grafts much more extensively than when graft cells did not express NT-3 (Lu et al., 2003; Tobias et al., 2003). In these experiments axons grew directly into grafts where NT-3 was expressed, suggesting a possible chemotropic effect. Delivery of NT-3 to the spinal cord using adenoviral vectors promoted growth of TrkC

expressing sensory axons across the dorsal root entry zone and into the spinal cord into regions where NT-3 was expressed (Zhang et al., 1998). In addition, Lu et al. (Lu et al., 2004) demonstrated growth of sensory axons toward sites of NT-3 protein injection distal to spinal cord lesion sites and Bamber et al. (Bamber et al., 2001) noted that a variety of axon types were associated with regions where NT-3 was delivered to the injured spinal cord intrathecally. These studies also suggest a chemotropic effect of NT-3 on adult axons after injury.

These findings are challenged in one study by Ramer et al. (Ramer et al., 2002). This group reported that intrathecal delivery of NT-3 to the spinal cord promoted growth of large diameter proprioceptive axons across the dorsal root entry zone (DREZ) and into the spinal cord after dorsal root axotomy. NT-3 appeared not to exert chemotropic effects on these axons because axons grew away from the source of NT-3 and deep into spinal cord tissue of the dorsal horn. Nevertheless, NT-3 did promote growth from the dorsal roots toward the intrathecal injection site initially, and it was noted that a number of axons grew along the pia mater, where NT-3 concentrations were likely to be high after intrathecal delivery. Also, regeneration promoting effects of NT-3 in this study may have been mediated not only on regenerating axons but also directly at the level of the cell body via diffusion of NT-3 to DRGs.

Interestingly, it was suggested by Ramer et al. that endogenous chemotropic cues expressed by dorsal horn neurons attracted regenerating axons. We did not observe a similar phenomenon in studies of NT-3 delivery to the nucleus gracilis. In

our study, axons grew into the nucleus gracilis only if NT-3 was expressed there. In addition, axons grew away from neuronal targets toward NT-3-expressing cells when NT-3 was delivered to ectopic locations outside the target nucleus. These findings suggest that NT-3 delivery was a more potent axon attractant than cues that may have been expressed by target cells. Based on patterns of axon growth that we observed after lentiviral NT-3 delivery, the observations of several other groups that have delivered NT-3 to the injured spinal cord, and a number of studies suggesting that NT-3 can act as a target-derived growth factor during development (Gillespie, 2003; Genc et al., 2004), it is likely that NT-3 is exhibiting chemotropic effects on axons when delivered to axon tips after SCI.

Long distance axon growth beyond lesion sites requires a combination of neurotrophin delivery and activation of neuronal growth programs (conditioning lesions).

NT-3 gradients promote sensory axon growth through the permissive substrate of a cellular graft, but upon encountering the inhibitory environment of the distal spinal cord an NT-3 stimulus cannot continue to propel growth. Similarly, conditioning lesions alone failed to promote axon growth beyond lesion sites. However, when these two treatments were combined, axon growth over inhibitory substrates was greatly enhanced. These findings highlight the importance of combinatorial strategies that foster axonal growth using multiple stimuli.

Axon growth may be dependent on a balance between growth and inhibition, such that removing inhibitory factors or adding stimulatory factors can be used to tip the balance in favor of growth. For example, stimulation of the neuronal cell body by conditioning lesions can promote axon growth beyond the lesion site when combined with degradation of inhibitors associated with the glial scar (Tan et al., 2006). In this case, a positive stimulus (conditioning lesions) combined with removal of an inhibitory signal resulted in axon growth. In addition, multiple pro-growth stimuli together have a greater impact than a single positive stimulus. Neurotrophin delivery combined with cAMP delivery to cell bodies increases axon growth beyond lesion sites compared to either treatment alone (Lu et al., 2004). Moreover, Zhou et al. (Zhou and Shine, 2003) demonstrated that delivery of neurotrophins to both the cell bodies of corticospinal neurons and to their axons in the lumbar spinal cord increased sprouting of intact fibers compared to lumbar delivery alone.

Combined stimulation at the axon tip and cell soma might increase axon growth additively through the same mechanism or by contributing to distinct mechanisms that are necessary for axon growth. Studies using dual stimulation paradigms suggest that signaling events at the neuronal cell body *and* the axon tip are essential for promoting a maximum growth response after SCI. These results are in fact not surprising given how developmental and peripheral nerve regeneration occur. During development, the neuron expresses genes that keep it in a continual "growth state" until it reaches a final target. After peripheral nerve injury, neurons are capable of initiating a similar, but probably distinct (Liu and Snider, 2001), growth state due to

specific changes in gene expression that occur in response to injury. Despite the fact that neurons operate with the growth state "on" in these situations, they may still respond to environmental cues encountered by the growth cone. For example, axons clearly respond to repulsive and attractive guidance cues encountered at the growth cone during development.

The "growth state on" status of developing neurons or regenerating peripheral neurons might be fully realized only when the extending growth encounters pro-growth signals at the axon tip. During developmental growth toward peripheral targets, neurotrophic support is necessary during the entire axon pathfinding process (Farinas et al., 1996). Encountering positive growth stimuli at the axon tip might therefore be essential for utilizing an overall long distance growth program initiated at the level of the cell body. This conclusion is supported by studies described in this thesis, which show that initiation of a genetic program for axonal growth (conditioning lesion) is insufficient for long distance extension of axons, and allows for axonal bridging over extended distances only when combined with stimulation of the axon tip. Possible mechanisms for how combined stimuli might support long distance axon growth are outlined under the "Future Directions" section of this discussion.

NT-3 delivery combined with conditioning lesions can promote growth of axons into denervated target regions and may support formation of new synaptic connections.

Functional recovery after SCI can theoretically occur by at least three mechanisms: 1) Reorganization of spared neuronal circuitry, 2) compensatory sprouting of uninjured axons resulting in connections with denervated targets or 3) regeneration of injured axons resulting in reconnection with novel or appropriate denervated targets. Considerable evidence exists that enhanced or naturally occurring compensatory sprouting and reorganization of existing connections can result in functional recovery after partial spinal cord injuries (Weidner et al., 2001; Raineteau et al., 2002; Massey et al., 2006). However, these mechanisms may be limited, especially in cases of severe spinal cord lesions in which little tissue is spared.

Perhaps the most exciting finding of the present work is that we demonstrate for the first time that regeneration of injured axons may result in establishment of synaptic connections in a denervated target region after SCI. Several previous studies have suggested the possibility of synapse formation after regenerative axon growth in the injured spinal cord. However, these findings were based on observation of "bouton-like" structures in immunohistochemically labeled axons that had bridged a spinal cord lesion site (Bamber et al., 2001; Houle et al., 2006). We have extended these findings to include ultrastructural evidence for regeneration of synapses in an original target zone. Importantly, we also demonstrate that in vivo neurotrophic factor delivery can be used to successfully direct axons specifically toward regions containing appropriate target neurons. The ability to promote axon growth toward a denervated region will become increasingly important as mechanisms for promoting axon regeneration continue to improve.

Future directions

The present work has raised a number of interesting questions that need to be addressed in future studies. Perhaps the most important of these is a mechanistic one: How are genetic changes associated with conditioning lesions integrated with neurotrophin signaling at the growth cone to promote superior axon growth? Currently, a major effort is being made to determine the genetic mechanisms behind the conditioning lesion response. These studies aim to elucidate specific cellular signaling pathways that can be used to "switch on" an overall axonal growth program.

One of the best-characterized components of the conditioning lesion response is cAMP. Conditioning lesions cause cAMP levels to rise resulting in the ability of axons to overcome myelin-associated inhibition by mechanisms that are still being investigated. Some studies suggest that the conditioning lesion effect can be recapitulated completely by modulation of cAMP (Qiu et al., 2002). Pharmaceutical agents that increase cAMP levels, such as rolipram, have been tested clinically and could be used to modulate cAMP therapeutically to promote an active growth state in CNS neurons (Nikulina et al., 2004). However, a number of additional signaling events that contribute to the conditioning lesion response, besides those associated with cAMP, have now been identified. Based on these findings, it seems likely that growth responses initiated by conditioning lesions involve more complex mechanisms that could also be exploited to enhance axon regeneration.

In addition to activation of CREB by elevated cAMP, several other transcription factors, including ATF3 and STAT3, have been implicated in mediating the conditioning lesion response (Qiu et al., 2005; Seijffers et al., 2006). It is likely that modulation of cAMP levels may be responsible for promoting growth over myelin-associated inhibitors, while other pathways promote growth in other ways. For example, activation of ATF3 augments neurite outgrowth, but does not promote growth of axons over inhibitory myelin substrates (Seijffers et al., 2007). In addition to these transcription factors, a vast number of proteins that are differentially regulated in response to peripheral and central lesions have been identified using gene array studies and need to be investigated (Costigan et al., 2002).

Understanding the growth-promoting pathways associated with the conditioning lesion response represents an important avenue for development of future therapies for SCI. However, the experiments presented in this thesis demonstrate that the axonal growth program initiated by conditioning lesions is much more effective at promoting long distance axon growth over inhibitory substrates when combined with a positive stimulus at the axon tip (NT-3). Understanding the role of neurotrophin signaling in boosting the conditioned growth response, or conversely, determining how conditioning lesions enable axons to better respond to NT-3 delivery, could provide essential insight into intracellular signaling pathways that optimize long distance axon growth in an inhibitory environment.

Neurotrophins are known to signal locally at the growth cone to affect cytoskeletal dynamics and can also be retrogradely transported to affect transcription.

Thus, signaling via these proteins could interact in several ways with growth programs supported by conditioning lesions. First, neurotrophin signaling might interfere with local inhibitory signaling at the growth cone. Interference by neurotrophins could happen in several ways. For example, it is thought that the small G-proteins RhoA and Rac1 have opposing roles at the growth cone, such that RhoA activity blocks growth cone extension while Rac1 activity promotes extension. Many inhibitors probably cause growth cone collapse via activation of RhoA (Sandvig et al., 2004). However, neurotrophin signaling through Trk receptors can activate Rac (Reichardt, 2006). Thus, increasing neurotrophin signaling at the growth cone might increase pro-extension activity to counteract the affects of inhibitory signaling. Alternatively, neurotrophins might competitively bind to receptors involved in inhibitory signaling. The p75 receptor, a component of the signaling complex mediating myelin-based inhibition, is also a low affinity receptor for neurotrophins (Patapoutian and Reichardt, 2001; Yiu and He, 2006). In addition to acting at the axon tip, it is also possible that retrograde neurotrophin signaling could act to enhance growth programs at the level of the cell body resulting in a more potent growth response than that supported by conditioning lesions alone.

The examples above illustrate how NT-3 signaling might contribute to an active growth state initiated by conditioning lesions. A second possibility is that conditioning lesions augment the ability of NT-3 to function as a growth stimulant. For example, conditioning lesions might allow axons to grow in the presence of NT-3 by affecting the availability of cellular machinery needed to extend the growth cone.

In support of this idea, cytoskeletal proteins are upregulated by conditioning lesions (Costigan et al., 2002). In addition, Verma et al. recently demonstrated that regeneration of a new growth cone after axotomy requires high levels of protein synthesis machinery within axons (Verma et al., 2005). Interestingly, these studies further showed that conditioning lesions increase levels of this machinery in sensory axons. Conditioning lesions might also enhance NT-3 signaling by upregulating expression of neurotrophin receptors or downregulating expression of inhibitory receptors. Modulation of receptor availability might also occur posttranscriptionally, such that signaling associated with conditioning lesions could affect availability of receptors in axonal membranes. Notably, it has been demonstrated that Trk receptors, which can be sequestered in intracellular vesicles, can be recruited to the plasma membrane by cAMP elevation {Meyer-Franke, 1998 #627}.

Finally, conditioning lesions and NT-3 delivery may act together to elevate cAMP levels in DRG neurons to enhance long distance axon growth. It is well-established that conditioning lesions raise cAMP levels in DRG neurons allowing axons to regenerate over inhibitory myelin substrates (Spencer and Filbin, 2004). Neurotrophins can also promote growth over inhibitory substrates through elevation of cAMP levels via Erk-mediated inhibition of phosphodiesterase 4 (Cai et al., 1999). Combined conditioning lesions and NT-3 delivery may increase cAMP levels more significantly or for longer times than either treatment alone. Enhanced cAMP signaling could lead to increased or sustained expression of regeneration-associated genes that could account for the improved axonal regeneration observed in animals

treated with combined NT-3/conditioning lesion treatment. The concept that long term NT-3 delivery might act to *sustain* the effects of the conditioning lesion is particularly compelling since previous work has indicated that transient delivery of NT-3 (Lu et al., 2004) is less effective than sustained NT-3 delivery (present study) in promoting axon growth beyond lesion sites when combined with cell body stimulation.   Each of the possible mechanisms described here could be investigated in future studies to determine how NT-3 delivery and conditioning lesions act synergistically to promote axon growth beyond lesion sites.

Another fascinating line of investigation would be to determine how changes in the NT-3 delivery paradigm might increase the distance of axon growth when combined with conditioning lesions. Our original aim was to use NT-3 gradients to promote long distance axon growth from C3 lesion sites to the nucleus gracilis. However, single lentiviral injections could not produce a gradient that extended over such a long distance, due to insufficient diffusion of the virus. Therefore the lesion site was moved closer to the nucleus gracilis target (to C1) for reinnervation studies. To provide a more extended gradient, NT-3-expressing viruses could be injected into the target nucleus while NT-3 protein or lentiviruses with regulatable NT-3 expression (Blesch et al., 2000; Blesch et al., 2001), could be injected at one or more intermediate sites along the path toward the final target. Once axons reached an intermediate injection site, degradation of NT-3 protein or switching off NT-3 gene expression would limit local NT-3 availability, establishing a novel gradient towards target neurons. Axons might then be able to respond to this new NT-3 gradient to allow

growth over longer distances. These experiments would determine how changing local availability of a growth factor might affect axon growth.

Perhaps the most exciting possibilities for future experiments involve the paradigm that has been developed for promoting growth of sensory axons into an original termination site. We observed evidence that regenerated axons formed synapses upon reaching the denervated target. Thus, an important next step is to determine whether newly formed synapses can participate in recovery of function. Such a finding would demonstrate unequivocally that regenerative axon growth can result in restoration of original connectivity after SCI. Our first attempt to determine whether new synapses were functional using electrophysiology failed to uncover activity by regenerated axons. However, this was likely due to the fact that only a small number of synapses were formed. Increasing the number of axons that regenerate into the nucleus gracilis and the number of synaptic contacts made will be important in efforts to demonstrate functionality of new synapses.

A number of manipulations have the potential to increase the number of new synapses formed (see Chapter 4 discussion). A particularly interesting possibility would be to express NT-3 only in appropriate post-synaptic neurons. This could be accomplished using neuron-specific gene expression or retrogradely transported viral vectors, such as AAV. These vectors could be injected into thalamic sites, similar to fluorogold in the present experiments, leading to NT-3 expression via retrograde transport of virus specifically in target neurons. Preliminary experiments using this approach resulted in few retrogradely transduced neurons, but with improved

techniques this approach might become a viable option. Another possibility would be

to inject viruses into the nucleus gracilis that are designed specifically to infect

neurons or to allow gene expression only in neurons. Direct expression of NT-3 by

neuronal targets might increase the ability of axons to detect these neurons as post-

synaptic partners.

In addition to increasing the number of synaptic contacts in the nucleus

gracilis, demonstration of functional recovery might also require manipulations

designed to improve transmission of impulses by regenerated axons. For example,

including treatment strategies capable of remyelinating axons might strengthen

electrical transmission across synapses to improve function. Schwann cells and

oligodendrocyte precursors derived from stem cells have been used successfully to

promote remyelination in models of SCI (Keirstead, 2005; Nistor et al., 2005; Pearse

and Bunge, 2006). Including these cells as part of cellular grafts, or including

expression of neurotrophic factors known to attract Schwann cells (such as GDNF)

(Blesch and Tuszynski, 2003), might promote remyelination of regenerated axons.

Finally, in addition to electrophysiological testing and testing for behavioral recovery,

transsynaptic tracers, such as WGA, that require synaptic activity in order to be

transported across a synapse could be useful in providing evidence that regenerated

synapses are functional.

The current model, especially if improved to support even more synapse

formation, could be used to answer questions about the mechanisms underlying

synapse formation in the context of regeneration. Studies designed to examine

whether regenerative synapse formation is similar or different from developmental synaptogenesis would be of high interest. Our data provide some evidence that newly formed synapses may look quite different from synapses in intact animals. Whether these synapses eventually will resemble intact synapses or if they remain different remains to be determined. Another compelling line of investigation would be to determine the specificity of synapse formation in the target. Ascending sensory axons normally terminate in the ipsilateral nucleus gracilis. It would be of interest to determine whether regenerating axons have a preference for the correct side of the target or if growth is random. Also, if axons could be misdirected exclusively to the contralateral nucleus, behavioral consequences of aberrant synapse formation and potential compensation over time could be examined.

Finally, this work should be extended to include different axonal pathways and cell types and treatment at chronic time points. The conditioning lesion provides an important way to identify regeneration-associated genes involved in the growth state of neurons. Once these genes have been identified and characterized, manipulation of their expression patterns might be used to initiate a growth state in other neuronal populations. Then, neurotrophin delivery to target sites or expression of neurotrophins by target neurons might be used to direct other populations of axons to appropriate targets. For example, viral vector expression of BDNF in ventral motor neurons combined with treatments to the cell soma of descending neuronal populations, such as corticospinal neurons, could promote target finding and synapse formation. Current studies in our laboratory are examining whether approaches similar to the ones

developed in this thesis, can induce axonal bridging and reinnervation of neurons beyond a spinal cord lesion site in other neuronal populations.

Conclusions

Treatment strategies that promote growth of axons beyond lesion sites are essential to achieving functional recovery after SCI. The present work demonstrates that in vivo gene delivery of neurotrophic factors may be used as part of an overall growth-promoting strategy to support regeneration of injured axons over inhibitory substrates beyond lesion sites. Because neurotrophins can direct axon growth toward particular regions, delivery of these factors could be used in combination with a number of different regeneration strategies to designate target sites for regeneration. For these reasons, neurotrophin delivery to target sites may be an important component of future therapeutic treatment of SCI.

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