RUBROSPINAL NEURONS AFTER
CHRONIC CERVICAL SPINAL CORD INJURY

By

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ABSTRACT

Many experimental therapies have been developed that appear to have encouraging therapeutic potential in animal models of acute spinal cord injury. It has become increasingly evident however, that their effectiveness is reduced when applied *chronically* after the injury. This loss of effectiveness over time is an issue of obvious and critical relevance for the many individuals with chronic spinal cord injury. *Thus, the overall objective of this thesis was to evaluate some of the challenges that impede axonal regeneration in a chronic spinal cord injury setting, and develop therapeutic strategies for this condition. My hypothesis was that that axonal regeneration can be achieved by chronically injured CNS neurons with the appropriate administration of neurotrophic factors*

The findings can be summarized as follows: Two months after cervical axotomy, rubrospinal neurons undergo significant atrophy and exhibit limited expression of GAP-43 and Tα1 tubulin, genes thought to be important for axonal regeneration. They appear to maintain full length TrkB receptors on their cell bodies, and while their uninjured axons within the cervical spinal cord also contain TrkB receptors, the injured axons at the level of the spinal cord axotomy do not. Consistent with this, BDNF applied to the spinal cord injury site at three exponentially increasing concentrations did not reverse rubrospinal cell atrophy, did not stimulate GAP-43 and Tα1 tubulin expression, and did not promote axonal regeneration of rubrospinal axons into the permissive environment of a peripheral nerve transplant.

At 12 months after cervical axotomy, a stereologic evaluation of rubrospinal neurons demonstrates that rubrospinal neurons are in fact alive, but very atrophic. Similar to the findings at 2 months post-injury, the rubrospinal neurons 12 months post-injury display limited expression of GAP-43 and Tα1 tubulin but do maintain full length TrkB receptors on their cell

bodies. At this chronic time point, the administration of BDNF to the injured cell bodies reversed neuronal atrophy, stimulated GAP-43 and $T\alpha 1$ tubuin expression, and promoted axonal regeneration into peripheral nerve transplants.

These findings suggest that axonal regeneration is possible in the chronic spinal cord injury setting, but that the administration of neurotrophic factors to promote this growth response must be targetted appropriately. It is hoped that further study in the obstacles that impede axonal regeneration after chronic spinal cord injury will give rise to therapies for this devastating condition.

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LIST OF ABBREVIATIONS

BDNF – brain derived neurotrophic factor

CAP-23 – cortical cytoskeleton-associated protein-23

CGRP - calcitonin gene related peptide

CNS – central nervous system

GAP-43 – growth associated protein-43

GDNF – glial derived neurotrophid factor

cpm – counts per minute

ISH – in situ hybridization

MAG - myelin associated glycoprotein

OMgp - oligodendrocyte-myelin glycoprotein

NT-3 – neurotrophin-3

PNS – peripheral nervous system

RAG – regeneration associated gene

SEM – standard error of the mean

SSC – sodium chloride / sodium citrate

TrkB – tropomyosin receptor kinase B

Statement of Original Contributions

This thesis work contains material that has been published in the following:

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Kwon BK, Liu J, Oschipok L, Tetzlaff W (2002) Reaxotomy of chronically injured rubrospinal neurons results in only modest cell loss. Experimental Neurology, 177: 332-337.

Kwon BK, Liu J, Oschipok L, Teh J, Liu ZW, Tetzlaff W (2004) Rubrospinal neurons fail to respond to brain-derived neurotrophic factor applied to the spinal cord injury site 2 months after cervical axotomy. Experimental Neurololgy, epub Jul 3, 2004.

The thesis author, Brian Kwon, was the primary researcher for all results presented in this thesis. Assistance with surgical procedures was provided by J Liu. Pilot studies on the 1 year chronically injured rubrospinal experiments were performed by NR Kobayashi. Initial development and validation of the physical disector technique for cell counting was performed by C Messerer. Expertise with in situ hybridization studies and immunohistochemistry was provided by L Oschipok and J McGraw, respectively. Technical assistance was provided by J Teh and ZW Liu.

The above statements and assessment of work done by the thesis author and collaborators are justified by the senior author (supervisor of the thesis author), Dr. W Tetzlaff.

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W. Tetzlaff, MD, PhD

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This Thesis is dedicated to Mr. Rick Hansen, Man in Motion, and to the thousands of individuals with chronic spinal cord paralysis whose cause he champions with unimaginable courage, dedication, and commitment.   Over the last four years, he and my many patients who suffer this unfortunate injury have taught me innumerous lessons that are not found in the books.   You were right Rick, the end is just the beginning....

- CHAPTER 1 -

BACKGROUND

1.1. OVERVIEW

Each year, over 10,000 North Americans sustain a traumatic spinal cord injury (Nobunaga et al., 1999) and are left with one of the most physically and psychologically devastating impairments known to mankind. The majority of such paralyzed individuals are under the age of 30 and are otherwise healthy at the time of their injury (Sekhon and Fehlings, 2001). With advances in contemporary medical and surgical care, the survival rate of such individuals has improved dramatically, resulting in over 250,000 North Americans currently living with chronic spinal cord paralysis. Beyond the incalculable losses to the individual, the societal costs of the medical, surgical, and rehabilitative care for patients with acute and chronic spinal cord injuries are enormous - estimated over a decade ago at four billion dollars per annum (Stripling, 1990). The compelling need for therapies for individuals with spinal cord injuries has sparked great interest in the neuroscience community, where it is widely believed that the cumulative research efforts of the basic science and clinical communities will produce effective therapies in the imminent future.

The failure of paralyzed individuals to regain neurologic function reflects the failure of disrupted axons to regenerate and re-innervate their distal targets after spinal cord injury. A tremendous amount has been learned about what occurs within the spinal cord after injury, the obstacles that are established to impede axonal regeneration and repair, and potential therapeutic interventions to overcome them (reviewed in section 1.3). Conceptually, the obstacles to

regeneration can be divided into the limited intrinsic regenerative response on behalf of CNS neurons, and the extrinsic elements within the injured CNS that inhibit axonal regeneration (reviewed by Kwon and Tetzlaff, 2001 and Steeves and Tetzlaff, 1998). Following spinal cord injury, neurons within the brain or brainstem whose axons are disrupted in the spinal cord often undergo severe atrophy and may possibly die (reviewed in section 1.4). The molecular mechanisms that are triggered within CNS neurons to promote axonal growth after injury may not be qualitatively and/or quantitatively sufficient to promote long-distance axonal regeneration (reviewed in section 1.5). Many neurotrophic factors exist and have important roles in (amongst many things), neuronal survival and axonal growth, and have thus been utilized as experimental therapies to enhance the regenerative competence of injured CNS neurons. Neurotrophic factors and their receptors are reviewed in sections 1.6 and 1.7. The study of the neurobiology of spinal cord and the development of such strategies to promote recovery are dependent upon animal models of injury (reviewed in section 1.8). In this regard, I have chosen to use the rubrospinal system in adult rats (reviewed in section 1.9).

The past two decades have witnessed the emergence of a number of experimental therapeutic strategies for spinal cord injury; some of which have demonstrated modest potential in animal models of acute spinal cord injury. What has become increasingly evident over the past decade is that while many experimental therapies appear to be very promising when applied *acutely* at or near the time of injury, their effectiveness is reduced when applied *chronically* after the injury. This loss of effectiveness over time is an issue of obvious and critical relevance for the many individuals with chronic spinal cord injury. **Thus, the overall objective of this thesis is to evaluate some of the challenges that impede axonal regeneration in a chronic spinal cord injury setting, and develop therapeutic strategies for this condition.**

1.2. SUMMARY OF HYPOTHESES AND OBJECTIVES

The overall hypothesis of this thesis is that axonal regeneration can be achieved by chronically injured CNS neurons with the appropriate administration of neurotrophic factors.

The overall objective of these experiments was to investigate the cell body response of chronically injured CNS neurons to determine the obstacles imposed by this condition on axonal regeneration. The specific objectives were as follows. Using a cervical injury model of the rat rubrospinal system I attempted to:

1. Test the hypothesis that providing trophic support in the form of BDNF to chronically injured rubrospinal neurons at the site of spinal cord injury could reverse neuronal atrophy if applied in the correct dose. (Chapter 3)

2. Test the hypothesis that providing trophic support in the form of BDNF to chronically injured rubrospinal neurons directly to their cell bodies could reverse neuronal atrophy. (Chapter 3)

3. Test the hypothesis that chronically injured rubrospinal neurons are not dying after cervical spinal cord injury. (Chapter 3)

4. Test the hypothesis that regeneration associated gene expression can be stimulated in chronically injured rubrospinal neurons provided with trophic support. (chapter 4)

5. Test the hypothesis that axonal regeneration of chronically injured rubrospinal neurons can be elicited by providing them with trophic support (chapter 5)

6. Test the hypothesis that responsiveness to of chronically injured rubrospinal neurons to exogenously provided neurotrophic support is related to the expression of the appropriate receptors (chapter 6)

In this Background chapter, I will attempt to briefly review some pertinent considerations to the testing of these hypotheses. Although the scope of these topics is impossible to address comprehensively within the pages of this chapter, their relevance to the experiments and their interpretation warrants a brief – albeit somewhat superficial - overview here. I will begin by providing a general overview of the obstacles to axonal regeneration in the injured CNS (Section 1.3). Then, as a number of experiments evaluated the response of rubrospinal neurons to cervical axotomy, I will discuss issues of neuronal atrophy and death after axotomy, and methods by which death/survival are measured (Section 1.4). Further to this discussion of the rubrospinal response to axotomy, I will provide an overview of the "intrinsic determinants" of axonal growth; specifically, the cell body response to axotomy and some of the genetic components of this response, including the expression of GAP-43 and Tα1 tubulin (Section 1.5).

The primary intervention that was tested in these experiments was the administration of the neurotrophic factor, brain-derived neurotrophic factor (BDNF). I therefore will briefly discuss neurotrophic factors and provide some background on BDNF specifically (Section 1.6). As the tropomyosin receptor kinase (Trk) family of receptors are important for mediating the biologic response of the classic neurotrophins, I will then discuss Trk receptors and TrkB receptors specifically (Section 1.7).

The experiments performed to test the aforementioned hypothesis were largely in vivo experiments, and as such, I will provide an overview of how spinal cord injury is modeled in the animals, and how axonal regeneration is evaluated in such models (Section 1.8). The neuronal system that I focused on was the rubrospinal system, and therefore I will review some aspects of rubrospinal anatomy and function (Section 1.9). Finally, I will summarize the rationale for the experimental models applied in this thesis (Section 1.10).

1.3. PROMOTING AXONAL REGENERATION IN THE CNS

1.3.1. Obstacles to Axonal Regeneration and Therapies to Overcome Them

The fact that surgeons suture together peripheral nerves in the hand that have been completely transected, but yet offer a poor prognosis to the completely paralyzed individual whose spinal cord has been only bluntly contused, reflects very different expectations about the regenerative capacities of neurons within the peripheral and central nervous systems. *So why does axonal regeneration fail in the CNS while it occurs fairly readily in the PNS?* One could postulate that this failure is related either to the fact that CNS neurons simply lack the ability to regenerate axons after injury, or alternatively that there exists something in the spinal cord environment that inhibits CNS neurons from doing so. It has become apparent that, to some extent, both of these are true (Fawcett, 1998). I will speak more of the intrinsic growth propensity of neurons within the CNS (section 1.4). Suffice to say now, that while the ability of PNS neurons to regenerate is accompanied by a host of changes in gene expression (and in the extrinsic environment after axotomy), such a gene response in CNS neurons is transient and generally insufficient for long-term axonal growth (Fernandes and Tetzlaff, 2000). Enhancing this limited intrinsic growth propensity of CNS neurons therefore has a strong rationale for reparative therapies, which would include such strategies as delivering growth factors to the neurons (Kobayashi et al., 1997, Kwon et al., 2002b) and the modulation of intracellular cAMP (Spencer and Filbin, 2004).

Recent years have produced an enormous amount of insight into some of the inhibitory molecules within the CNS that make the cord less permissive to axonal regeneration than peripheral nerves (Reviewed by Kwon et al., 2002a). The two major impediments to axonal regeneration in the injured spinal cord are CNS myelin and the glial scar/cyst that forms at the

injury site. Within CNS myelin, a number of molecules that inhibit axonal growth have been identified and characterized, including Nogo (Chen et al., 2000, GrandPre et al., 2000, Prinjha et al., 2000), myelin associated glycoprotein (MAG) (McKerracher et al., 1994), and oligodendrocyte-myelin glycoprotein (OMgp) (Wang et al., 2002). Recognizing that CNS myelin may contain many more as-yet unidentified inhibitory molecules provides the rationale for immunological disruption of myelin as a potential therapeutic strategy (Dyer et al., 1998). Within the glial scar, astrocytes form a physical barrier to axonal growth and may contribute to the expression of a number of inhibitory molecules such as chondroitin sulphate proteoglycans (Morgenstern et al., 2002). Interventions directed at attenuating the inhibitory effect of these environmental impediments appear promising in their potential to allow axonal regeneration to occur. In order to "bridge" the inhibitory spinal cord injury site, numerous cellular substrates are emerging as potential candidates to facilitate axonal regeneration (reviewed by Bunge, 2000). These would include fetal tissue transplants, embryonic stem cells, genetically altered fibroblasts, Schwann cells, and olfactory ensheathing cells. (Figure 1.1)

Figure 1.1. Schematic of the obstacles to axonal regeneration after spinal cord injury and potential therapeutic strategies.

OBSTACLES TO REGENERATION

1. POOR REGENERATIVE RESPONSE at the level of the cell body

2. ENVIRONMENTAL INHIBITORS at the level of the injured axon

Failure to sufficiently express regeneration associated genes and trophic factors
eg: GAP-43, CAP-23, Tα1-Tubulin
BDNF, FGF-2

Glial Scar
eg: chondroitan sulfate proteoglycans
semaphorins, ephrins

Atrophy/death
of neurons
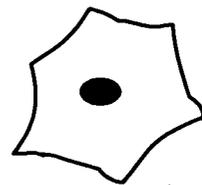
Cyst / Cavitation
- neuronal and glial death

Myelin Inhibitors
eg: NOGO, MAG, OMgp

Growth Cone Signalling
- Rho/ROCK inhibition
- cAMP, cGMP

Myelin Inhibition
- Anti-NOGO antibodies
- Immunologic disruption

Cellular Bridges
- Fetal tissue
- Schwann cells
- Stem cells
- Olfactory ensheathing cells

Glial Scar Degradation
- Chondroitinase ABC

Neurotrophic Factors
- BDNF, FGF-2, NT3

THERAPEUTIC STRATEGIES TO PROMOTE REGENERATION

1.4. NEURONAL SURVIVAL, ATROPHY, AND DEATH AFTER AXOTOMY

1.4.1. Introduction

Fundamentally, the neuropathology associated with spinal cord injury is the result of the mechanical impact (primary injury) and subsequent pathophysiologic processes (secondary injury) disrupting ascending and descending axons within the spinal cord, with the additional demise of neurons and glial cells at and adjacent to the injury site contributing to the neurologic impairment (reviewed by Kwon et al., 2004b). While much work and attention is directed to these pathologic changes that occur within the spinal cord at the site of injury, it is important to take into account what is happening to the supraspinal neurons whose axons are disrupted within the spinal cord. Herein I will discuss the issue of neuronal survival and cell size after axotomy. Apart from the issue of neuronal survival, the gene expression changes relevant to axonal regeneration that occur in response to axotomy will be reviewed elsewhere (section 1.5).

1.4.2. Neuronal Survival and Cell Size are Influenced By Neurotrophic Factors

Neuronal survival after axotomy highlights some important differences between neurons of the central and peripheral nervous systems. CNS neurons appear to receive substantially less neurotrophic support after axotomy than their PNS counterparts. Acutely axotomized PNS neurons enjoy an abundant supply of neurotrophic factors from Schwann cells and macrophages (ie. non-target tissue) (Funakoshi et al., 1993, Hoke et al., 2002) and may also upregulate their own production of both neurotrophic factors and neurotrophic factor receptors (Ernfors et al., 1993, Kobayashi et al., 1996, Piehl et al., 1994). In contrast, while neurotrophic factors are in fact produced by oligodendrocytes (Wilkins et al., 2001, Wilkins et al., 2003), it would appear that the extent to which they support neuronal survival within the CNS after axotomy is indeed

quite limited. Rubrospinal neurons, for example, undergo significant atrophy (and possibly also death – see further discussion) after cervical axotomy. This atrophy can be prevented by the intraparenchymal infusion of brain derived neurotrophic factor (BDNF) directly to the neuronal cell bodies one week after injury (Kobayashi et al., 1997, Fukuoka et al., 1997). This suggests that while sufficient trophic support may not be arriving at the cell bodies via their axons, the cell bodies themselves remain responsive to exogenously applied neurotrophins or they respond indirectly through some effect that the neurotrophins are having on surrounding tissue (discussed further in Chapter 7). Along the same lines, the exogenous intraparenchymal infusion of BDNF to the motor cortex prevents the near 50% loss of corticospinal neurons after axotomy at the level of the internal capsule (Giehl and Tetzlaff, 1996).

It is well recognized that rubrospinal neurons undergo significant atrophy after cervical axotomy (Kobayashi et al., 1997, Kwon et al., 2002b, Kwon et al., 2002c). Such atrophy after axotomy is certainly not unique to the rubrospinal system, and has been reported in other neuronal populations within the central nervous system such as corticospinal neurons (Giehl et al., 1997) and basal forebrain cholinergic neurons (van der Zee and Hagg, 2002). As stated earlier, the exogenous delivery of specific neurotrophic factors has the potential to prevent or reverse some of this atrophy, suggesting that, similar to cell survival, the aspects of cellular physiology that contribute to the maintenance of cell size are also dependent on neurotrophic factor support. For the rubrospinal system specifically, Kobayashi et al. reported that the acute administration of BDNF to the cell bodies of rubrospinal neurons can prevent their atrophy after cervical axotomy (Kobayashi et al., 1997). Furthermore, in the experiments of this thesis, I observed that the similar cell-body application of BDNF one year after injury could reverse the atrophy of rubrospinal neurons (Kwon et al., 2002b). The exact mechanism by which cell

atrophy occurs and is reversed is not entirely understood, but may relate to the expression and synthesis of cytoskeletal proteins such as tubulins and neurofilament (McKerracher et al., 1993, Bisby and Tetzlaff, 1992, Bregman et al., 1998). In support of this association, Fernandes et al. observed that rubrospinal neurons that increased their expression of GAP-43 and Tα1 tubulin and regenerated through peripheral nerve transplants were also the largest neurons within the injured red nucleus (Fernandes et al., 1999).

1.4.3. Measuring Neuronal Death After Axotomy

The measurement of neuronal death (or conversely, the counting of surviving cells remaining) after axotomy requires not only careful histologic techniques but also careful counting methods. This is by no means an exact science. As stated by Guillery and Herrup, *"counting objects in histological material is an exercise in estimation"* (Guillery and Herrup, 1997). In this regard, numerous histologic techniques are available to visualize neurons (eg. immunohistochemistry, retrograde labeling, Nissl staining), and a number of techniques have been developed to address the very practical problem that the majority of neuronal systems contain such large numbers of neurons that an absolute numerical determination is not feasible. Fundamentally, the problem is that when tissue is sectioned, cells within it may appear in more than one section, which will lead to the overestimation of the total number of cells in the population, particularly if the cells are large. To accommodate for this error, one can apply a mathematical correction that incorporates the height of the cells within the section and the thickness of the section, as originally proposed by Abercrombie (Abercrombie, 1946). An alternative "empirical method" employs a formula that incorporates an estimation of the number of sections a single cell is visualized within in order to account for the potential overestimation of cell number (Coggeshall et al., 1984, Coggeshall and Chung, 1984). In theory, the weakness

of the former method is that it requires an estimation of the height of the cells within the section (and an assumption of cell sphericity), while the latter requires an estimation of the number of sections per cell (and an assumption of cell shape).

Furthermore, one of the major considerations in the application of these mathematical corrections in studies of neuronal populations after axotomy is the well recognized atrophy that occurs after axotomy. Stereometric methods had been developed that, in theory require no *a priori* knowledge of the size or shape of the neurons being counted. In principle, stereologic counting compares the cells in one section against the cells in an adjacent section, and by avoiding the double-counting of cells one arrives at a more true account of the number of cells, irrespective of their size or shape (West, 1999). The problem that is generated by not performing stereologic counts in the setting of atrophy is schematically illustrated in Figure 1.2. There has been considerable debate over the past decade about the validity of the various counting techniques (Guillery and Herrup, 1997, Benes and Lange, 2001, West and Slomanka, 2001). With no definitive resolution of the controversy surrounding which counting technique is best, it would appear reasonable to chose the counting technique - recognizing both its strengths and limitations – after accumulating as much prior knowledge of the behavior of the neuronal system in question and after deciding upon the research question to be answered. In this thesis, I applied a stereologic counting technique (the physical disector method, described in more detail in Chapter 2) to count rubrospinal neurons after axotomy.

Figure 1.2.   Schematic illustration of the difference between standard counting and stereologic counting (eg. disector method).

This schematic demonstrates the difference between performing standard and stereologic counting and how the atrophy of neurons after axotomy might be mis-represented as death if standard counts alone are used to determine the number of neurons remaining. In this schematic, the dark grey section (A) is the index section that is counted. In the normal, uninjured state (above), a standard count would produce a cell count of 8 (all 8 cells would be visualized in the index section). However, a stereologic count of the same tissue would compare the cells in A against those visualized in B and then exclude from A those cells that were visualized in both sections.   So in this case, cells 5, 6, 7 and 8 would be seen in both sections, and the "stereologic" count of section A would be only 4.

Now, after the axotomy, the neurons atrophy.   Again, performing a standard count on section A would produce a cell count of only 5 (cells 2, 3, 4, 6, and 8).   So for the same section of tissue, the death of neurons would be estimated to be 3/8, or 37.5%, merely from atrophy alone. However, a stereologic count comparing section A against B would arrive at a cell count of 4, unchanged from the non-atrophic state.

Figure 1.2

Normal Sized Cells

	Standard Count	Stereologic Count
→	8 cells	4 cells

Axotomy

	Standard Count	Stereologic Count
→	5 cells	4 cells

Atrophic Cells
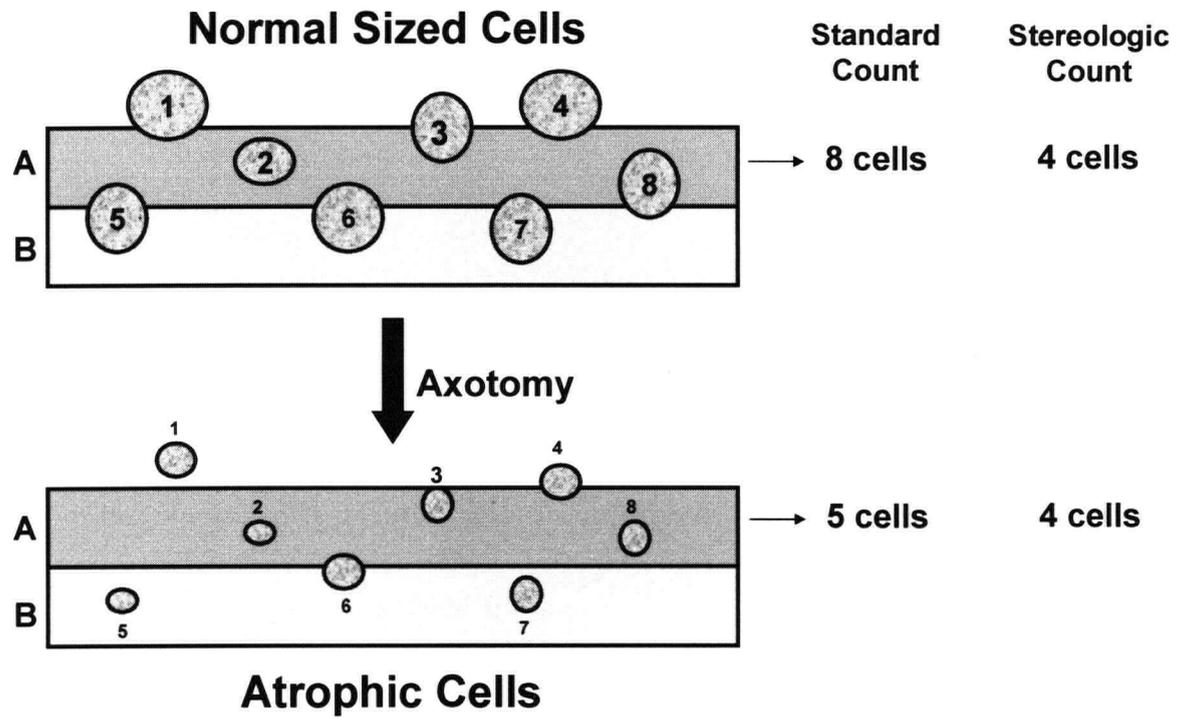
1.5. INTRINSIC DETERMINANTS OF AXONAL GROWTH AFTER CNS INJURY

1.5.1. Introduction

Rather pivotal to the issue of promoting neurologic recovery after spinal cord injury is the actual capacity of CNS neurons to regenerate their axons after injury. The ability of CNS neurons to regenerate their injured axons was first described at the turn of the century in the laboratory of the Spanish neuroscientist Ramón y Cajal (Ramon y Cajal S, 1928). Experiments in the 1980s by Aguayo, David, and Richardson (David and Aguayo, 1981, Richardson et al., 1984, Richardson et al., 1980) reiterated and extended these findings by demonstrating that when presented with permissive conditions (ie. a peripheral nerve graft), some CNS neurons are indeed able to regenerate their injured axons. It has been subsequently shown that the even in non-permissive conditions, it is possible to influence the intrinsic growth state of the neuron in such a manner as to still promote axonal regeneration (Spencer and Filbin, 2004, Fischer et al., 2004). Characterizing and harnessing this intrinsic ability to regenerate axons after injury is thus a key element in strategies to promote functional recovery after spinal cord injury.

1.5.2. The Cell Body Response to Axotomy

After axotomy, the intrinsic ability of the neuron to effect long-distance axonal regeneration is thought to be closely tied to molecular and biochemical activity within the cell body. A study by Richardson and Issa in 1984 demonstrated that the response of the neuronal cell body to axonal injury plays a pivotal role in the regenerative capacity of the neuron (Richardson and Issa, 1984). In this study, the central spinal projection of a dorsal root ganglion cell was shown to regenerate into a peripheral nerve transplant only *after* the peripheral projection had been previously transected, demonstrating that the cell body response evoked by

transection of the peripheral projection was in some manner essential for the subsequent regeneration of the central spinal projection. It was subsequently demonstrated that transection of the peripheral axon induces changes in gene expression in the parent neuron which are not seen after injury of the central process (Schreyer and Skene, 1993). These observations suggested that these neurons did in fact possess the appropriate regenerative machinery, but required the initial stimulus of the peripheral transection to activate it and thus become "regeneration-capable". Significant interest has therefore been generated to delineate these gene regulatory mechanisms that must be initiated at the cell body level, with hopes that such understanding will allow for strategies to persuade otherwise incompetent neurons to regenerate their injured axons. We now know that this regenerative competence and the response to axotomy is unequal amongst different neuronal types (Woolhead et al., 1998, Morrow et al., 1993), and also varies significantly with neuronal age (Chen et al., 1995) and distance from the site of injury (Fernandes et al., 1999, Richardson et al., 1984, Mason et al., 2003). In summary, while injured axons may intrinsically be capable of short disorganized terminal sprouting and in some cases even slow axonal elongation (Bisby et al., 1996, Andersen and Schreyer, 1999), the sustained and distant growth of axons requires the participation of the cell body, manifested by the expression of a number of regeneration associated genes (Smith and Skene, 1997, Fernandes and Tetzlaff, 2000).

1.5.3. Regeneration Associated Gene Expression

A number of genes have been shown to be up-regulated or constitutively expressed in association with axonal growth, both during development and during axonal regeneration. These have collectively been termed "regeneration associated genes" or RAGs (reviewed by Fernandes and Tetzlaff, 2000). The increases in RAG expression that occur in response to axotomy of CNS neurons are weaker and more transient (or altogether absent) than those that occur in the PNS, and the successful regeneration of the latter implicate these RAGs as important mediators of axonal regeneration (reviewed by Plunet et al., 2002). Regeneration of the central (spinal) axons of the dorsal root ganglion has been demonstrated after the combined transgenic overexpression of GAP-43 and CAP-23, but not when each of these genes was expressed alone (Bomze et al., 2001). Interestingly, the number of regenerating axons with the dual GAP-43 and CAP-23 overexpression was still only a third of that observed after a pre-conditioning peripheral nerve axotomy, which presumably initiates the "full" cell body response. The results of this study highlight two important considerations: that RAG expression is a prerequisite rather than an associative phenomenon for axonal elongation, and that a complex, coordinated expression of a battery of genes (rather than just one or two) is necessary for the optimal growth response. The pace at which the many components of this molecular response to axotomy are identified in the future will undoubtedly be facilitated by gene chip technology (Fan et al., 2001, Gris et al., 2003).

The products of these genes include transcription factors such as c-jun which mediates subsequent gene expression (Jenkins et al., 1993, Herdegen et al., 1997), cell adhesion molecules such as L1 and NCAM involved in growth cone guidance (Becker et al., 1998, Jung et al., 1997, Woolhead et al., 1998), cytoskeletal proteins involved in axonal extension such as Tα1-tubulin

(Fernandes et al., 1999, Miller et al., 1989), and growth cone proteins involved in mediating axonal guidance and synaptic plasticity such as GAP-43 and CAP-23 (Frey et al., 2000, Skene, 1989). The importance of these genes in axonal regeneration has generally been extrapolated from the correlation of their upregulation with axonal growth and the absence of their expression with regenerative failure (Anderson and Lieberman, 2000, Fernandes et al., 1999, Tetzlaff et al., 1991, Becker et al., 1998, Anderson and Lieberman, 2000, Schreyer and Skene, 1993). **In this thesis, I have evaluated the expression of Tα1-tubulin and GAP-43 as a manifestation of the regenerative response to axotomy of rubrospinal neurons.**

1.5.4. GAP-43

GAP-43 (also known as B50, neuromodulin, and F1) is a calcium-regulated phosphoprotein that is closely related to initial axonal outgrowth during differentiation and successful axonal regeneration in both the CNS and PNS. It is a major constituent of the developing growth cone, where it appears to be involved in axonal steering and the formation of new synaptic connections (reviewed by Benowitz and Routtenberg, 1997). By modulating the assembly of phosphoinositide lipid $PI(4,5)P_2$ containing rafts on the inner surface of the cell membrane and its interactions with calmodulin and G proteins, GAP-43 appears to play an important role in the regulation of the actin cytoskeleton (Caroni, 2001, Frey et al., 2000, Laux et al., 2000). GAP-43 knockout mice exhibit defects in axonal guidance (Maier et al., 1999), and growth cones of primary sensory neurons depleted of GAP-43 in vitro demonstrate poor adhesion and limited resistance to inhibitory substrates (Aigner and Caroni, 1995). Conversely, the transgenic overexpression of GAP-43 in adult mice induces significant spontaneous axonal sprouting (Aigner et al., 1995).

While PNS neurons express high levels of GAP-43 during development and regeneration (Skene, 1989), the ability of CNS neurons to increase and sustain GAP-43 expression after axotomy is generally limited (reviewed by Fernandes and Tetzlaff, 2000). Within the CNS, rubrospinal neurons were observed to increase the expression of GAP-43 after axotomy at the cervical but not at the thoracic level, and correspondingly only regenerated into peripheral nerve transplants inserted into the cervical spinal cord but not the thoracic spinal cord (Fernandes et al., 1999). Similar observations correlating regeneration into peripheral nerve transplants and GAP-43 expression have been made in other CNS neuronal systems (Vaudano et al., 1995, Woolhead et al., 1998). In interpreting these findings, however, one should not forget that the expression of a number of other RAGs such as L1 and c-jun and a host of as yet unidentified genes may also be stimulated in response to axotomy (Chaisuksunt et al., 2000). Furthermore, there are examples in which the overexpression of GAP-43 alone was not sufficient to promote regeneration (Buffo et al., 1997, Mason et al., 2000, Bomze et al., 2001). Conversely, GAP-43 knockout mice have a grossly normal nervous system with axonal growth characteristics not unlike wild-type mice, but they demonstrate abnormal axonal pathfinding (Strittmatter et al., 1995). These findings suggest that while much evidence points to GAP-43 being an indicator of neuronal growth propensity, it is neither absolutely necessary for axonal growth due to other compensatory mechanisms, nor is it by itself sufficient to promote axonal growth.

1.5.5. Tα1 Tubulin

Microtubules are one of the most important constituents of the neuronal cytoskeleton, playing an essential role in the development and maintenance of neuronal morphology, neurite outgrowth, and intracellular transport (reviewed by Laferriere et al., 1997). Microtubules are formed by the polymerization of tubulin molecules into protofilaments which then laterally associate to form a hollow tube. Most commonly, tubulin molecules are comprised of an α and a β subunit, although new members of the tubulin superfamily have been discovered more recently, including gamma (γ), delta (δ), epsilon (ε), zeta (ζ), eta (η), and iota (ι) tubulin (reviewed by Dutcher, 2003).

A number of α-tubulin and β-tubulin isoforms have been identified in neuronal tissue. The expression of Tα1 tubulin mRNA was reported to be very high in the embryonic nervous system, particularly in neurons actively undergoing neurite extension (Miller et al., 1987). This study also demonstrated that PC12 cells stimulated in vitro by NGF significantly increased their Tα1 tubulin mRNA expression concurrent with their differentiation and their extension of neurite processes. Miller and colleagues subsequently demonstrated a rapid increase in Tα1 tubulin mRNA expression in facial and sciatic motor neurons after axotomy, which remained elevated until axonal growth re-established terminal connections (Miller et al., 1989). Tetzlaff and colleagues reported that rubrospinal neurons also upregulate Tα1 tubulin expression after cervical axotomy (Tetzlaff et al., 1991), findings that were extended by Fernandes and colleagues who demonstrated that while Tα1 tubulin expression was increased after cervical axotomy of the rubrospinal tract, it was not significantly increased by thoracic axotomy (Fernandes et al., 1999). The expression (and lack thereof) of Tα1 tubulin in the latter study corresponded with the ability to regenerate into peripheral nerve transplants inserted into the

cervical spinal cord but not the thoracic cord. Combined, these studies suggest that Tα1 tubulin is an aspect of the regenerative response of axotomized neurons, with an increase in its expression correlating with a growth propensity.

1.6. NEUROTROPHIC FACTORS

1.6.1. Introduction

Neurotrophic factors are proteins that exert considerable influence on a wide spectrum of processes within the developing and mature nervous system, including neuronal survival, axonal growth, synaptic plasticity, and neurotransmission (reviewed by Tuszynski, 1999). Since the identification of the first neurotrophic factor, nerve growth factor (NGF) by Levi-Montalcini and Hamburger over 5 decades ago (Lev-Montalcini and Hamburger, 1951, Lev-Montalcini and Hamburger, 1953), dozens of such factors have been uncovered. The classical description of neurotrophic factor action on neurons is that of a target-derived, retrogradely transported signal delivered back to the neuronal soma by the axon. This concept has been expanded somewhat, as it has become apparent that neurons receive trophic support not only from their distal targets in a retrograde fashion, but also from afferent neurons in an anterograde manner, from adjacent or ensheathing glial cells in a paracrine manner, and even from themselves in an autocrine manner (reviewed by Korsching, 1993). While the term "growth factors" is used loosely to collectively describe these proteins, they are structurally very diverse, and as such, have distinct targets within the nervous system, and exert their activity through different receptors and signalling pathways. The responsiveness of a particular cell population within the nervous system to a specific neurotrophic factor is therefore partially dependent on the expression of the appropriate receptors.

Because of their wide-ranging influence on many aspects of neural biology, neurotrophic factors have been extensively studied as a potential therapeutic strategy for promoting neural repair after spinal cord injury (Jones et al., 2001). Within this context, it is important to recognize the extremely diverse nature of neurotrophic factors and their functions. However, the

exogenous delivery of a single trophic factor - while demonstrating some potential as a therapeutic strategy for spinal cord injury - is unlikely by itself to elicit a comprehensive regenerative response in all relevant neuronal and glial cell populations. The "classic" family of neurotrophic factors, otherwise referred to as neurotrophins, includes NGF, BDNF, Neurotrophin-3 (NT-3), NT-4/5, NT-6, and NT-7.

1.6.2. Brain Derived Neurotrophic Factor (BDNF)

Brain derived neurotrophic factor (BDNF) was identified by Barde and colleagues in 1982 as a 12.3 kDa protein purified from pig brain that promoted both the survival and neurite outgrowth of embryonic chick sensory neurons in an additive fashion to NGF (Barde et al., 1982). The BDNF gene was subsequently cloned and sequenced by Barde's laboratory in 1989, where it was also found that the brain and spinal cord contained much larger amounts of BDNF mRNA than NGF (Leibrock et al., 1989). The temporal pattern of BDNF mRNA expression demonstrates a higher level of expression during adulthood than during development (exactly in contrast to NT-3 expression), suggesting that BDNF may be more involved as a maturation and maintenance factor later in development, while NT-3 plays an earlier role in neuronal development (Maisonpierre et al., 1990). The exact role that BDNF (and other neurotrophic factors for that matter), plays in the developing nervous system is difficult to elucidate in knockout mice lacking the BDNF gene given the compensation and redundancy that undoubtedly exists with many other neurotrophic factors. Such BDNF null mice demonstrate substantial loss of neurons in a number of sensory ganglia, including the DRG, trigeminal, vestibular, and nodose ganglia, but no loss of neurons in the cortical and hippocampal regions of the brain and motor neurons in the spinal cord (Ernfors et al., 1994, Jones et al., 1994).

Neuronal populations known to be responsive to BDNF include sensory neurons of the DRG, nodose ganglion and geniculate ganglion, dopaminergic neurons of the substantia nigra, basal forebrain cholinergic neurons, hippocampal neurons, cerebellar granule cells, and retinal ganglion cells (Korsching, 1993, Barde et al., 1987). The widespread expression of BDNF and its many potential targets has made it an appealing neurotrophic factor to evaluate as a potential therapeutic agent for a number of neurological disorders, including stroke (Schabitz et al., 2004), amyotrophic lateral sclerosis (for which it has already undergone human clinical trials) (Ochs et al., 2000, BDNF Study Group, 1999), Alzheimer's and Parkinson's disease (reviewed by Murer et al., 2001), head injury (Blaha et al., 2000), peripheral nerve injury (Ho et al., 1998) and, as will be discussed in more detail later, spinal cord injury.

1.7. TRK NEUROTROPHIN RECEPTORS

1.7.1. Introduction

As stated earlier, the biological activity of neurotrophic factors depends on the presence of the appropriate receptors on target tissue. The neurotrophins (eg. NGF, BDNF, NT-3) act primarily through high-affinity binding to the Trk (tropomyosin receptor kinase) family of protein tyrosine kinases, but also bind to the low-affinity p75 receptor (reviewed by Barbacid, 1995). The trk oncogene, present in a human colon carcinoma, was originally described as a transforming gene containing sequences of both a non-muscle tropomyosin and a protein tyrosine kinase (Martin-Zanca et al., 1986). Activity of the tyrosine kinase was found to be stimulated by NGF, which identified the product of the trk oncogene to be a putative receptor for this neurotrophic factor (Kaplan et al., 1991a, Hempstead et al., 1991, Kaplan et al., 1991b, Klein et al., 1991). Additional studies revealed other highly related Trk receptors, including TrkB (Klein et al., 1989) and TrkC, (Lamballe et al., 1991) which also serve to bind to and effect the signal transduction of members of the neurotrophin family.

While highly related, the Trk receptors demonstrate specificity in their binding, with NGF binding to Trk (TrkA), BDNF and NT-4/5 acting through TrkB, and NT-3 acting primarily on TrkC. All neurotrophins bind with low affinity to the p75 receptor, a cell surface glycoprotein belonging to the tumor necrosis factor receptor superfamily (Chao, 1994). Structurally, the Trk receptors are characterized by an extracellular ligand binding domain consisting of cysteine clusters, a leucine-rich motif with Ig-like domains, and an intracellular domain consisting of the tyrosine kinase catalytic domain (Reviewed by Barbacid, 1994). Extracellular ligand binding facilitates a dimerization of the Trk receptor, allowing for autophosphorylation of the intracellular tyrosine residues (Jing et al., 1992). These serve as

docking sites for adaptor proteins that mediate their interaction with the phosphotyrosine residues via SH2 domains (Schlessinger and Ullrich, 1992) and then initiate downstream intracellular signaling cascades. Intracellular signalling pathways initiated in this fashion influence cell survival and neurite outgrowth through PLCγ, Akt1/2, and MAP kinase (reviewed by Kaplan and Miller, 2000).

1.7.2. TrkB Receptors

TrkB receptor mRNA expression is widely found in the brain, spinal cord, and peripheral nervous system (Klein et al., 1989, 1990, and 1993). Immunoreactivity of the TrkB extracellular domain is also widespread but is most intensely seen within the olfactory bulb, pyramidal neurons of the hippocampus, granular cells of the dentage gyrus, striatal neurons, Purkinje cells of the cerebellum, substantia nigra pars compacta, locus coeruleus, brainstem and spinal motoneurons, and rubrospinal neurons (Yan et al., 1997). TrkB knockout mice appear phenotypically normal at birth, but die typically within days from starvation which is thought to be related to sensory and motor deficiencies in systems related to feeding and gastrointestinal functions (Klein et al., 1993). Neuronal loss in the trigeminal ganglia, facial nucleus, and DRG in particular was noted in these animals. Interestingly, while TrkB knockout mice demonstrate significant loss of motorneurons (Klein et al., 1993), BDNF knockout mice have normal numbers of spinal motorneurons (Jones et al., 1994), suggesting that during the development of the latter, other neurotrophic factors can compensate for the absence of BDNF.

The TrkB locus encodes not only a full length receptor, gp145trkB (or TrkBTK+), with both the extracellular and intracellular domains described above, but also two truncated versions, TrkB.T1 and TrkB.T2 that lack the catalytic intracellular kinase domain (Middlemas et al., 1991). In the rat forebrain, the expression of the full length TrkB predominates in early

development, while the truncated forms predominate in late postnatal and adult life (Fryer et al., 1996). The function of these truncated forms of the receptor is not clear. It has been proposed that they act in a dominant-negative fashion to sequester BDNF and thereby restricts the diffusion and binding of this neurotrophin to full length receptors (Haapasalo et al., 2001, Biffo et al., 1995, Offenhauser et al., 2002). Alternatively, their truncated intracellular domains may in fact have some signal transduction function after BDNF binding (Baxter et al., 1997), possibly by activating a G protein that leads to inositol-1,4,5-trisphosphate-dependent calcium release (Rose et al., 2003) or by interacting with other cytoplasmic or membrane proteins one of which has been identified and named TrkB-T1 Interacting Protein, or TTIP (Kryl and Barker, 2000). While the physiologic function of these truncated forms of TrkB is still in question, it is interesting to note that they are significantly upregulated at the site of spinal cord injury (Frisen et al., 1993, King et al., 2000). In this regard, it is proposed that they may restrict the local bioavailability of exogenously administered or endogenously produced BDNF. The purpose of this upregulation of truncated TrkB receptors at the injury site is unknown.

1.8. ANIMAL MODELING OF SPINAL CORD INJURY

1.8.1. Introduction

The delineation of the pathology and pathophysiology of spinal cord injury and the development of strategies to overcome the paralysis associated with them depends heavily upon animal models of spinal cord injury. The interest in this line of research has led to the establishment and refinement of a number of animal models which employ a variety of animal species and a spectrum of injury paradigms, ranging from sharp transection to blunt contusion (reviewed by Kwon et al., 2002d and Rosenzweig and McDonald, 2004). Recently, to more closely reproduce the clinical situation in which the spinal cord is injured by the mechanical failure of the surrounding spinal column, an animal model of spinal cord injury via spinal dislocation rather than by impaction has been reported (Fiford et al., 2004). Currently, the rat and mouse are the most popular animals utilized in spinal cord injury research, both for cost and accessibility reasons, as well as for the latter's transgenic potential (Jakeman et al., 2000). Experimental interventions in animal spinal cord injury models are most commonly evaluated anatomically, biochemically, neurophysiologically, and/or behaviorally.

1.8.2. Anatomical Assessment of Axonal Growth

Anatomic assessment of axonal growth in animal models is highly dependent upon immunohistochemistry and on axonal tract tracers. Immunohistochemical techniques utilize antibodies targeted against proteins uniquely found in certain axonal populations, allowing for the visualization of these axons in histologic sections of the spinal cord. Examples of such proteins include calcitonin gene related peptide (CGRP), which is a marker of small diameter primary sensory axons, serotonin (5-HT), a marker of raphe-spinal axons, tyrosine hydroxylase

(TH), a marker of coerulospinal axons and sympathetic axons, and choline acetyltransferase (ChAT), a marker of cholinergic motor axons (Jones et al., 2001).

Axonal tracers are molecules that can be picked up by neurons or axons and transported in either an anterograde or retrograde fashion. Anterograde tracers are applied within the vicinity of the cell bodies of neurons and are then transported along the axons where they can be visualized. Such tracers are therefore useful for visualizing axons that are injured and/or regenerating at the injury site. Examples of such anterograde tracers include biotinylated dextran amine (BDA), the enzyme horseradish peroxidase conjugated to wheat germ agglutinin (HRP-WGA), and the cholera toxin B subunit (CTB)(Alisky and Tolbert, 1994). A tracer that is used retrogradely is applied in the vicinity of the axons and, depending on the characteristics of the tracer, is taken up by cut and/or intact axons, or by their terminal endings and transported back to the cell body. The previously mentioned anterograde tracers (HRP-WGA, CTB, and BDA) can also serve as retrograde tracers. A variety of fluorochromes such as FluoroGold, Fast-Blue, and Nuclear Yellow are commonly used retrograde tracers (Cowan, 1998). The differential absorption and emission characteristics of these fluorochromes allows them to be distinguished on histologic sections with various filters and wavelengths of light.

It is a common practice in CNS regeneration studies to apply a tracer distal to the injury, and then evaluate its presence in the neuronal bodies proximally. For example, if the corticospinal tract is cut completely at C3, and a retrograde tracer is applied at T1, the presence of tracer-labeled corticospinal neurons in the motor cortex implies that their axons successfully regenerated across the lesion at C3, picked up the tracer at T1, and transported it back (Figure 1.3). This tracing paradigm can be misleading if the tract is incompletely cut and axons have been spared, or if excessive tracer diffuses proximally beyond the lesion.

1.8.3. Sharp Versus Blunt Spinal Cord Injury Paradigms

The vast majority of spinal cord injuries that occur in humans are the result of blunt trauma which rarely causes a complete transection of the spinal cord. To this effect, a number of devices have been developed to cause reproducible blunt contusive or compressive injuries to the spinal cord (Kwon et al., 2002d). Similar to human injuries, these injury models typically create a central area of damage within the spinal cord, leaving variable amounts of spared tissue along the periphery (Hill et al., 2001). Alternatively, sharp injury models either partially or completely transect the spinal cord. It is important to recognize that while the blunt injury paradigm may be more representative of clinical reality, both sharp and blunt injury paradigms are important for spinal cord injury research. Models in which the spinal cord is fully or partially transected are useful for examining axonal regeneration because they more dependably disrupt the axons of a tract, while the sparing that results from contusion injuries is much more difficult to control and account for. Hence, the anatomical study of axonal regeneration and the cell body response to axotomy is best performed in sharp spinal cord injury models, where one can be reasonably confident that the axons were actually severed in the first place. On the other hand, models in which the spinal cord is bluntly injured are useful for examining the acute pathophysiologic responses to injury and are the setting for the development of neuroprotective agents that act to minimize secondary damage. **As this thesis is focused on the study of the cell body response to axotomy and methods for promoting axonal regeneration, the injury model I employ is that of a sharp, partial transection of the spinal cord which unilaterally disrupts the laterally placed rubrospinal tract.**

Figure 1.3. Schematic of retrograde tracing paradigm after partial cord transection

After injury/axotomy of the corticospinal tract (A), the distal axons degenerate. Axons that regenerate across the lesion site will pick up the tracer which is injected distally (B). The tracer is then retrogradely transported back to the cell body (C). Misinterpretation within this tracing paradigm can occur if the tracer diffuses proximal to the lesion site, which will occur if too much tracer is injected or if it is injected too close to the lesion site (D). Also, if the partial injury is incomplete and misses some of the axons of the tract (E), spared axons will pick up the label. In both scenarios (D and E), the neuronal cell bodies will be labeled and may be mistaken for regenerating neurons.
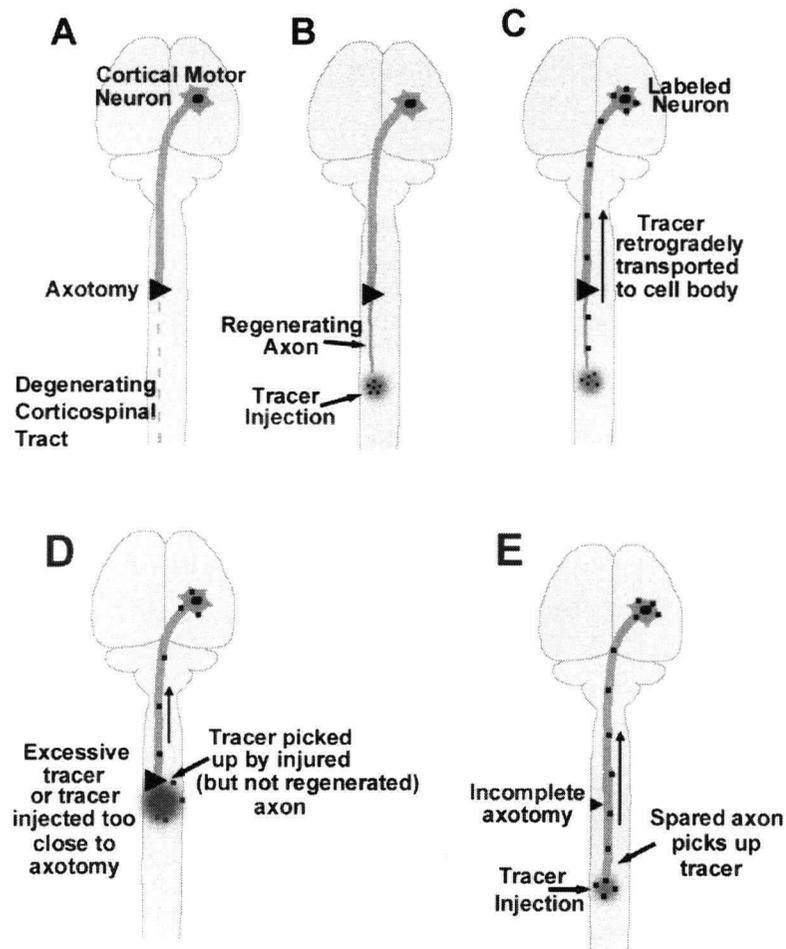
1.9. THE RUBROSPINAL SYSTEM

1.9.1. Introduction

There are many neuronal systems of interest to those who study repair strategies for spinal cord injury. In general, the study of a CNS neuronal system in this context is facilitated by access to both the neuronal cell bodies (to evaluate morphologic and molecular changes that occur at the level of the soma) and to the axonal tract in the spinal cord (to both mimic the spinal cord injury and to evaluate the axonal response to injury and its capacity to regenerate). The rubrospinal system in rats possesses both of these characteristics and as such is a useful neuronal system to study with regards to identifying regeneration obstacles within the CNS and potential therapeutic strategies.

1.9.2. Anatomy of the Red Nucleus and Rubrospinal Tract

The rubrospinal system consists of rubrospinal neurons concentrated within the red nucleus and their descending axons which almost exclusively cross midline and travel as the rubrospinal tract. In rats, the red nucleus is identified as a ovoid collection of neurons beginning approximately 2.5 mm rostral to the interaural line and extending rostrally for approximately 1 mm to a less well defined cephalad border (Huigrok and Cella, 1995). The relatively well defined boundaries of the nucleus allow for its identification histologically and facilitates stereotactic access to the rubrospinal neurons and their exiting axons in vivo (Murray and Gurule, 1979, Whishaw et al., 1990, Jeffery and Fitzgerald, 2001, Houle and Jin, 2001). Two distinct populations of rubrospinal neurons are thought to exist within the red nucleus, referred to generally as the magnocellular neurons located predominantly ventrolaterally in the caudal pole of the nucleus and the smaller, parvicellular neurons located dorsomedially in the more rostral

aspects of the nucleus (Kennedy et al., 1986). The caudal, ventromedial magnocellular neurons contribute the majority of axons to the descending rubrospinal tract, particularly to the distal lumbar spinal cord, while the smaller dorsomedial parvocellular neurons project axons to the cervical spinal cord (although the distinction between these neuronal subtypes and their projections is not absolute) (Murray and Gurule, 1979, Daniel et al., 1987, Strominger et al., 1987). It is interesting to note that while the magnocellular component of the red nucleus appears to play a predominant role in the rubrospinal tract of lower mammals such as rodents, it diminishes in importance as one moves up the evolutionary ladder and becomes almost rudimentary in man (ten Donkelaar, 1988, Nathan and Smith, 1982). Rubrospinal neurons receive their afferent input primarily from the cerebellum, but also are targets for cortical, posterior thalamic, dorsal raphe, and locus coeruleus afferents (Huigrok and Cella, 1995).

Rubrospinal axons cross the ventral tegmental decussation and descend within the dorsal part of the lateral funiculus as the rubrospinal tract (Brown, 1974). In this position, it is possible to reliably transect the rubrospinal tract unilaterally, leaving the contralateral side for comparison. The rubrospinal axons terminate mainly within laminae 5 to 7 of the dorsal horn where they synapse with excitatory and inhibitory interneurons (Antal et al., 1992), but they have also been shown to extend into the ventral horn where they directly innervate pools of motoneurons supplying the distal and intermediate forelimb muscles (Kuchler et al., 2002). The rubrospinal tract extends along the entire length of the rat spinal cord, although for the most part it appears to end within the cervical spinal cord, with only 20% extending to the lumbosacral enlargement (Huisman et al., 1982).

1.9.3. Function of the Rubrospinal System

The actual function of the rubrospinal system is subject to some uncertainty. As its neuronal anatomy appears to change as one ascends through phylogeny (ten Donkelaar, 1988, Nathan and Smith, 1982), it is quite likely that its function changes. The actual presence of the system appears to be related to the presence of limbs or limb-like structures, suggesting a role in motor control of the extremities. A rubrospinal tract has not been identified in some primitive vertebrates such as the shark and boid snakes, while rays which use their enlarged pectoral fins for locomotion do possess a rubrospinal tract (ten Donkelaar, 1988). While this association implicates the rubrospinal system in extremity motor control, in Nathan and Smith's classic report on the rubrospinal tract in humans – obviously, a species with high demands for extremity control - the rubrospinal tract was reported to be virtually non-existent below the mid-cervical spinal cord (Nathan and Smith, 1982). These findings, however, were based on degeneration studies rather than more sophisticated axonal tracing techniques.

From studies in lower mammals, Kennedy has proposed that the rubrospinal tract mediates the automation of motor skills that are first learned and established through activity of the corticospinal tract (Kennedy, 1990). While such a theory has some appeal, it is difficult to reconcile with the lack of an evident corticospinal tract in avian species such as prehensile parrots, ducks, and geese, who clearly have automated motor skills in their extremities (Webster et al., 1990, Webster and Steeves, 1988). Whishaw et al. ascribed subtle deficiencies in forelimb accuracy and grasp to lesions of the red nucleus in animals having combined rubrospinal and corticospinal lesions, although animals with isolated red nucleus lesions generally did not demonstrate forelimb impairments, possibly due to corticospinal tract compensation (Whishaw et al., 1990). However, red nucleus ablation was found to cause measurable and sustained

alterations in overground locomotion (Muir and Whishaw, 2000). Electrophysiologic studies in monkeys have suggested that the rubrospinal system preferentially activates extensor muscles in both proximal and distal joints of the extremities (Belhaj-Saif et al., 1998).

Determining the exact function of the rubrospinal tract (and many other tracts for that matter) in rodents is made difficult by neuronal plasticity and their ability to compensate over time (Raineteau et al., 2001). Increasing sophistication in the behavioral testing of animals may eventually provide a more clear picture of the relationship between the rubrospinal system and neurologic function, which will obviously be of pragmatic importance to the interpretation of therapeutic interventions in animal models of spinal cord injury. I should note that behavioral testing was not a component of this thesis work, although it is recognized that functional improvement is ultimately the goal of regeneration strategies.

1.10. RATIONALE FOR EXPERIMENTAL MODELS

The general approach taken in this thesis is to investigate the response the rubrospinal system in the state of chronic injury due to a sharp partial transection of the spinal cord at the mid-cervical level.

1.10.1. Cervically Axotomized Rubrospinal Neurons As A Model of Chronic CNS Injury

As discussed earlier, the rubrospinal neurons are contained within the red nucleus, a relatively discrete nucleus within the midbrain, allowing the neurons to be accessed in vivo using stereotactic techniques (eg. for the administration of trophic factors or the anterograde tracing of axons), and facilitating histologic (eg. immunohistochemistry) and molecular (eg. in situ hybridization) evaluation. The axons that emerge from the rubrospinal neurons cross the midbrain tegmentum and descend within the spinal cord as the rubrospinal tract in the dorsal part of the lateral funiculus. In this lateral position, the tract can be completely transected on one side of the spinal cord, leaving the contralateral side intact for comparison of both the axons within the spinal cord and the neuronal cell bodies in the red nucleus.

The acute cell body response of rubrospinal neurons to cervical and thoracic axotomy is fairly well characterized within the rubrospinal system, which provides some baseline information for the evaluation of chronic changes. For example, transection of the rubrospinal tract in the cervical spinal cord results in severe neuronal atrophy and the transient expression of the RAGs GAP-43 and Tα1 tubulin (Fernandes et al., 1999, Tetzlaff et al., 1991). The inability to sustain this elevation in RAG gene expression correlates with regenerative failure (Kobayashi et al., 1997, Fernandes et al., 1999), and in this regard, the rubrospinal system is representative of the "intrinsic" regenerative incompetence that impedes axonal regeneration within the CNS in

general. The rubrospinal axons within the spinal cord are subjected to the "extrinsic" inhibitors of regeneration such as CNS myelin and glial scarring after axotomy. Thus, the rubrospinal system allows for the evaluation of both the intrinsic and extrinsic obstacles to axonal regeneration.

1.10.2. Rat Models

In this thesis, I have exclusively used the Sprague-Dawley rat model. The animals are readily available, and the surgical protocols for such procedures as the spinal cord axotomy and stereotactic injections into the brainstem have been established. The protocols for a number of histologic and molecular techniques have been established within our lab for this species. The rats can be subjected to a contusion type injury as well, allowing for the extension of the findings from an axotomy injury paradigm to be taken forward into a more clinically relevant injury model. Finally, as stated, the rubrospinal system in rats exemplifies both the intrinsic and extrinsic obstacles to axonal regeneration; as such, the fact that the rubrospinal system's importance in humans is uncertain does not negate the valuable lessons that can be learned about these obstacles and methods for overcoming them.

- CHAPTER 2 -

MATERIALS AND METHODS

2.1 SURGICAL TECHNIQUES

2.1.1. Anaesthetic Technique

The animals used in this study were adult male Sprague-Dawley rats, weighing between 275-550 grams (the variation related to the chronicity of some of the animals). The animals were obtained from Charles River Laboratory (Quebec, Canada) or bred within the University of British Columbia animal facility. Throughout the course of experiments, they were housed in an alternating 12 hour light-dark cycle with free access to a standard diet and water. All animal experiments were performed in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the University of British Columbia Committee on Animal Care.

The anesthetics used for surgical procedures were xylazine (Rompun®, Bayer, Toronto, ON), and ketamine (Ketalean®, Bimeda-MTC, Cambridge, ON). The standard anesthetic mixture was 2 ml of xylazine (20 mg/ml) and 3 ml of ketamine (100 mg/ml) in 45 ml of distilled water (to make a solution of 0.8 mg/ml xylazine, 6 mg/ml ketamine). Each animal received approximately 1 ml of solution per 100 gm body weight via intraperitoneal injection, and then was checked regularly during the procedures to ensure adequate anesthesia and analgesia. With the length of some of the surgical procedures being quite long (over an hour), it was frequently

necessary to provide additional anesthesia with 0.5 to 1.0 ml bolus injections of anesthetic mixture.

2.1.2. Cervical Axotomy of the Rubrospinal Tract

After inducing deep anesthesia, the dorsal cervical region of the animal was shaved and the head was gently secured within a stereotactic frame, with light traction (approximately 100 gm) applied to the tail to spread the lamina and allow easier access to the spinal cord. The exposed skin was sterilized with Betadine® solution (Purdue Pharmaceuticals, Wilson, NC), a 10% providone-iodine solution that acts as a wide spectrum antimicrobial. Using a surgical operating microscope with 4.5x to 6.5x magnification, a midline longitudinal incision was made over the mid-cervical spine and the overlaying muscles split to expose the posterior elements of C3 and C4. A small laminectomy was performed with a bone rongeur and the left side of the spinal cord was visualized. At the lateral border of the dorsal horn, a 26 gauge needle was inserted to create a small hole just medial to the rubrospinal tract, and the left dorsolateral funiculus was then cut sharply by inserting one blade of a pair of fine iris scissors into this hole to a depth of approximately two thirds that of the spinal cord, and then closing the scissors. All spinal cord injuries were performed on the left side. After making the initial cut in the spinal cord, a defect of approximately half a millimeter in length along the rostro-caudal axis was created by aspiration through a tapered glass pipette. No attempt was made to suture the dura. The muscle and subcutaneous tissue were allowed to fall back towards midline and surgical clips were used to close the skin. The animal was taken out of the stereotactic frame and placed back into a cage with an electric heating blanket underneath to prevent hypothermia during convalescence.

2.1.3. Reaxotomy of Chronically Injured Rubrospinal Axons ("Refreshment Injury")

A number of time periods, from 2 months to 18 months, were utilized during these experiments to induce a state of "chronicity" in the rubrospinal system. After the period of time to establish chronicity, the rats were anesthetized and their cervical region prepared and exposed in the same fashion as described earlier. On occasion, the area of prior laminectomy occasionally fills with bone which needs to be re-resected to gain access to the spinal cord; most times, however, the previous laminectomy defect is easy to identify and the prior spinal cord injury site can be accessed by enlarging the defect slightly with a rongeur. The defect created within the spinal cord by aspiration is typically filled with scar tissue which, under magnification, is readily distinguished from the normal spinal cord tissue on either side of it. (Figure 2.1)

Reaxotomy of the rubrospinal tract (ie. "refreshment" injury) is performed by again puncturing the cord at the lateral border of the dorsal column with a 26 gauge needle and cutting it with a pair of fine iris scissors, approximately 1 mm proximal to the rostral border of the previous defect. Houle has demonstrated that retraction or "die back" of rubrospinal axons following cervical axotomy is quite modest, averaging approximately 500 μm with terminal end bulbs "rarely" visualized more than 1 mm rostral to the injury (Houle and Jin, 2001). Therefore, this second axotomy should re-injure the majority of rubrospinal axons in this area.

Figure 2.1. Two months after cervical axotomy, the scar from previous spinal cord injury is readily visible on the dorsal surface of the cord.

The incision through the dorsolateral funiculus is readily visible on the dorsal surface of the spinal cord; the original injury is performed by making an incision with a pair of fine iris scissors, and then aspirating the cord for a length of approximately 0.5 mm. The refreshment injury extends the injury site proximally about 1 mm. (rostral is to the left, caudal to the right)
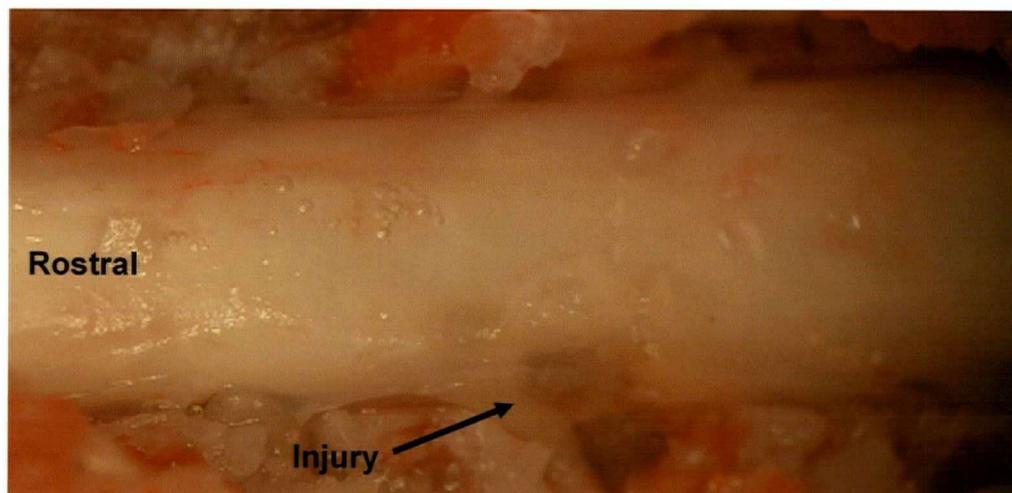
2.1.4. BDNF Application to the Spinal Cord Via Gelfoam®

After the refreshment injury was performed, small pledgets of Gelfoam® (Pharmacia & Upjohn, Peapack, NJ) were soaked with BDNF and then placed into the spinal cord defect. The concentrations of BDNF that we employed for local application to the spinal cord included a low concentration of 50 ng/µl, a medium concentration of 1,000 ng/µl, and a high concentration of 20,000 ng/µl. The BDNF-soaked Gelfoam was left in place for 5 minutes, then replaced by another pledget of fresh, BDNF-soaked Gelfoam. This procedure was repeated three more times for a total of 4 applications of freshly soaked Gelfoam over one hour. Control animals received Gelfoam soaked in sterile phosphate-buffered saline (PBS, pH 7.5). This method of applying the BDNF in Gelfoam and then replacing the BDNF-soaked Gelfoam sponge with fresh neurotrophic factor every 15 minutes over one hour, was reported to attenuate rubrospinal death after second axotomy performed 4 weeks after initial injury (Houle and Ye, 1999), and so we chose to employ this same regimen in our studies.

The BDNF used for the experiments in which direct spinal cord application of the neurotrophic factor was performed was kindly provided by Regeneron Pharmaceuticals Inc., Tarrytown, NY.

2.1.5. BDNF Application to the Red Nucleus via Osmotic Minipump

To apply BDNF directly to the rubrospinal cell bodies, an infusion system was established. An Alzet osmotic minipump (Alzet no 2001, 1 ml/hr, DURECT Corp., Cupertino, CA) was filled with BDNF (approximate total volume of 250 µl) at a concentration of 500 ng/ml within a vehicle solution of 20 mM sterile PBS, 100 U Penicillin/Streptomycin, and 0.5% rat serum albumin (Sigma-Aldrich Canada, Oakville, ON, #A-6272). Minipumps were filled with vehicle solution alone for control animals. The pumps were connected to a 28 gauge, 8 mm long

cannula (Plastic One Inc., Roanoke, VA) via a 6 to 8 cm silastic tubing (no 508-003, VWR Canlab, Mississauga, ON). This whole assembly (minipump, tubing, cannula) was preincubated for 4 to 12 hours in sterile 20 mM PBS at 37°C to initiate an even flow rate before implantation.

With the anesthetized animal held within the stereotactic frame, the Betadine-sterilized skin over the dorsal aspect of the cranium was incised in a longitudinal midline fashion. With the cranium exposed, an electric high speed burr was used to make a small hole (approximately 1 mm in diameter) in the skull 6.3 mm posterior to Bregma and 1.7 mm to the right of midline. Great care was taken to ensure that the burr did not plunge through the inner table of the cranium and injure the epidural or subdural structures. The cannula for the BDNF infusion pump was then positioned on the stereotactic frame 6.3 mm posterior to Bregma and 1.7 mm to the right of midline, in line with the burr hole. The cannula was carefully lowered through the burr hole until its tip rested on the dural surface. From this point, it was lowered 6.5 mm into the brain parenchyma, with the intention of leaving the tip of the cannula just lateral to the red nucleus. Two watchmaker screws and acrylic cement were used to secure the cannula rigidly in place and seal the burr hole. The skin was closed with skin clips and the animal placed back into a cage with an electric heating blanket underneath to prevent hypothermia during convalescence.

The BDNF used for application at the level of the rubrospinal cell bodies was kindly provided by Regeneron Pharmaceuticals Inc., Tarrytown, NY. A pegylated version of BDNF was also generously provided by Dr. Qiao Yan of AMGEN (AMGEN, Thousand Oaks, CA) under a Materials Transfer Agreement. The non-pegylated BDNF (from Regeneron Pharmaceuticals) was used for studies of the rubrospinal cell body response (ie. atrophy, cell number, and in situ hybridization) while the pegylated BDNF was used for studies of regeneration into peripheral nerve transplants.

2.1.6. Peripheral Nerve Transplantation

Because of the many inhibitory elements within myelin and the glial scar that prevent axonal regeneration within the CNS, the transplantation of peripheral nerve grafts into the spinal cord is intended to provide CNS axons with a permissive environment for growth. Approximately ten days prior to anticipated transplantation, the anesthetized animal underwent a transection of the right sciatic nerve at the level of the obturator tendon. After being cut, the nerve was left *in situ* to allow Wallerian degeneration of the distal stump, thus creating the optimal trophic environment for axonal regeneration. Ten days, later, the animal was anesthetized again and secured within the stereotactic frame. The cervical spinal cord was then re-exposed and re-axotomized as described in Section 2.1.3. The right sciatic nerve was re-exposed and a 30 to 35 mm segment was harvested. The proximal end of this pre-degenerated nerve graft was then inserted into the spinal cord defect and held in place with two 10-0 Prolene sutures (Ethicon, Somerville, NJ). The distal end of the graft was brought out to the subcutaneous tissue and marked with a suture for easy identification later. The wounds were closed in the standard fashion with skin clips. Two months later, the animal was re-anesthetized, the distal end of the graft was exposed, the distal 2 mm of the graft resected (to provide a fresh nerve ending) and a carbocyanine dye, DiI (Molecular Probes, Eugene, OR) or Biotin Dextran Amine (BDA) (Molecular Probes, Eugene, OR) was applied to this tip to retrogradely label neurons whose axons regenerated to the end of the graft.

2.1.7. Anterograde Tracing of Rubrospinal Axons

In order to evaluate TrkB receptor expression in rubrospinal axons within the spinal cord, the axons were anterogradely labeled via a stereotactic injection of BDA. The anesthetized animals were gently held within the stereotactic frame and the dorsal surface of the cranium was exposed. An electric high-speed burr was then used to create a 1 mm hole in the skull centered 6.2 mm caudal to Bregma and 0.7 mm lateral to midline. A 25% solution of BDA (10,000 MW, Molecular Probes, Eugene, OR) in 0.5% DMSO was drawn up into a glass micropipette with a Hamilton syringe. The tip of the micropipette was positioned on the dural surface 6.2 mm caudal to Bregma and 0.7 mm lateral to midline, then advanced into the brainstem to a depth of 7.2 mm. BDA was injected at a rate of 0.04 μl/min to a total volume of 0.6 μl (15 minutes), and the micropipette was left in place for an additional 5 minutes to allow diffusion of the tracer. The injection was performed on each side of midline to label both the injured and uninjured rubrospinal axons. The coordinates of this stereotactic injection were those described by Houle and Jin in their analysis of anterogradely labeled rubrospinal axons and their dieback from a cervical injury site (Houle and Jin, 2001), with a slight modification to accommodate for the larger size of our animals.

2.1.8. Retrograde and Anterograde Labeling of Rubrospinal Neurons and Axons

A number of retrograde tracers were used in these experiments, including 1% Fast Blue (Sigma-Aldrich Canada, Oakville, ON), 5% FluoroGold (Fluorochrome Inc. Eaglewood, CA), DiI (Molecular Probes, Eugene, OR) and Biotin Dextran Amine (BDA) (Molecular Probes, Eugene, OR). The Fast Blue and FluoroGold tracers were micro-injected with a Hamilton syringe into the left side of the spinal cord at the T1 level to label the lumbar-projecting population of rubrospinal neurons (predominantly magnocellular neurons, as discussed in the

Background Section 1.8.2). These tracers have the ability to be taken up by intact axons en passage, although the ability of FluoroGold to do so appeared to be better than Fast Blue, the latter being more readily picked up by intact nerve terminals (Kobbert et al., 2000). A 0.2 µl injection of 5% FluoroGold directly into the spinal cord was therefore used for the majority of experiments in which retrograde labeling of the rubrospinal tract was required. FluoroGold is known to be retrogradely transported in a robust fashion, even by chronically transected rubrospinal axons (McBride et al., 1990), and we found it to be present within the rubrospinal cell bodies even 6 months post-injection (Kwon et al., 2002c) (see Figure 3.6), although we recognize that others have not found it to be maintained within the cell bodies for quite so long (Novikova et al., 1997). The FluoroGold retrograde labeling of rubrospinal neurons was performed *prior* to cervical axotomy in these experiments to identify the lumbar projecting neurons for one of two reasons: (1) so that the boundaries of the injured aspects of the red nucleus could be identified, or (2) so that rubrospinal neurons could be identified as being chronically injured in the evaluation of axonal regeneration into peripheral nerve transplants (see further description of the double-labeling paradigm). Alternatively, in earlier stages of the animal model development, the FluoroGold was used *after* the cervical axotomy to ensure that no sparing of fibers had occurred. After some experience with the technique for unilaterally transecting the rubrospinal tract, we felt confident that our cervical axotomies were not leaving any rubrospinal axons spared (based on the absence of FluoroGold retrograde labeling of rubrospinal neurons) and thus this step was abandoned.

DiI is a lipophilic carbocyanine dye that actively can be retrogradely transported or passively diffuses laterally within the membrane as phospholipid molecules, and can thus label both live and fixed cells. In vivo, DiI can diffuse rapidly (up to 6 mm/day) (Holmqvist et al.,

1992).   DiI was employed in axonal regeneration experiments to evaluate regeneration through the peripheral nerve transplant.   Using the tip a needle, we applied a small crystal of DiI directly to the distal end of the peripheral nerve transplant to retrogradely label axons that grew to the tip of the graft.

Dextrans are hydrophilic polysaccharides that can be used as axonal tracers when biotinylated or conjugated to fluorescent molecules (Vercelli et al., 2000).   Biotin dextran amine (BDA) requires an immunohistochemical reaction for detection, but this provides a much more stable end-product for visualization than the fluorescent dextrans.   It can be used as an anterograde or retrograde tracer, and in our studies was used as both.   Anterogradely, it was injected medially to the red nucleus (coordinates described above) to label the descending axons. Retrogradely, it was injected into the distal tip of the peripheral nerve graft to label axons that had successfully regenerated through the graft.

2.2. HISTOLOGIC TECHNIQUES

2.2.1. Tissue Collection

Animals were given a lethal dose of chloral hydrate (approximately 1 g/kg). The animals were transcardially perfused with 350 ml of PBS followed immediately by 350 ml of ice cold, freshly hydrolyzed 4% paraformaldehyde (pH 7.4). Following exposure of the cranium and spinal column, the brainstem and spinal cord around the injury site was carefully extracted and placed in 4% paraformaldehyde overnight. The tissues were then sequentially cryoprotected in 12%, 16%, and 22% sucrose, before being rapidly frozen in isopentane cooled on dry ice.

2.2.2. Cryostat Cutting

The brainstem was mounted with the rostral side down for axial cryostat cutting in a caudal to rostral direction. The spinal cord was mounted either with the rostral side down for axial cutting or with the dorsal surface down for longitudinal, coronal cryostat cutting. Brain sections were cut at 20 μm thickness at temperatures ranging from -16 to -19°C. Sections were mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80°C until use. While cutting the brainstem tissue in a caudal to rostral direction, the tissue block was adjusted when the facial nucleus came into view in order to achieve the correct left-right balance before reaching the red nucleus. As the number and size of rubrospinal neurons was to be compared between injured and uninjured red nuclei, it was felt that left-right balance should be achieved so that each section that was cut represented equivalent levels of the injured and uninjured nuclei. Because the rubrospinal neurons in general become smaller as one progresses from the caudal (more magnocellular) to rostral (more parvocellular) end of the nucleus, a left-right imbalance in the cutting might produce a section with a higher percentage of smaller,

parvocellular neurons in one red nucleus compared to the other. Hence, great attention was paid to ensuring this balancing prior to reaching the red nucleus.

Brain sections used for the counting of rubrospinal neurons using the physical dissector method (described in more detail in the Background) were mounted adjacent to each other on the same slide so that they would be subjected to identical histological conditions.

For the spinal cord, the sections were taken in a ventral to dorsal fashion so that the tissue could be balanced in a left-right fashion using the ventral grey matter before arriving at the more dorsally placed rubrospinal tracts. This right-left balancing was performed so that when evaluating TrkB expression within the injured spinal cord, both the injured and uninjured rubrospinal tracts would be visualized on the same section.

2.2.3. NeuN Immunohistochemistry

NeuN immunohistochemistry was performed to enhance the identification of rubrospinal neurons, particularly after axotomy and the resultant atrophy. The NeuN monoclonal antibody was initially described by Mullen et al. (Mullen et al., 1992) who observed that the antigen recognized by this antibody was neuron-specific. It has subsequently been popularized as a specific marker of neuronal populations. The antigen to which it binds has yet to be fully characterized. Recent work by McPhail et al. has demonstrated that after cervical axotomy, rubrospinal neurons maintain their expression of NeuN for at least 28 days post-injury, while axotomized facial neurons do not (McPhail et al., 2004).

Slides containing the perfusion-fixed sections of the caudal 500 μm of the red nucleus were taken from the -80°C freezer and thawed for 10 minutes at room temperature. Following several washes in 0.01M PBS containing 0.1% Tween-20, sections were incubated overnight at 4°C in the primary antibody solution (NeuN mouse monoclonal, 1:100; Chemicon International

Inc. Temecula, CA). Following this, the slides were washed in 0.01 M PBS, blocked with 5%

normal goat serum, and incubated overnight at 4°C in a solution containing a secondary antibody

raised in goat, conjugated to Alexa 488 or Cy3 (goat anti-mouse, 1:100; Molecular Probes

Incorporated, Eugene, Oregon). The slides were then washed in 0.01 M PBS and coverslipped

with a glycerol / 0.% sodium azide mounting medium (Sigma Diagnostics Inc, St. Louis, MO).

Although the NeuN antibody has been widely used as a neuronal-specific marker, cross-

reactivity of NeuN with astrocytes and microglial cells was ruled out by performing

immunohistochemistry for both NeuN as well as an additional primary antibody either to GFAP

(Dakopatts Corp, Carpinteria, CA, Z334) or to isolectin B4 (Sigma-Aldrich Canada, Oakville,

ON, L2140) respectively. A 1:200 secondary antibody conjugated with ExtrAvidin Cy3 (Sigma-

Aldrich Canada, Oakville, ON, E-4142) was used for the GFAP and isolectin B4, and the

sections evaluated for cross-reactivity with NeuN (using a secondary antibody conjugated to

Alexa 488). These "control" slides demonstrated no cross-reactivity between NeuN and

astrocytes or microglia. (Figure 2.2)

Figure 2.2 NeuN immunostaining specifically labels neurons and does not label astrocytes or microglia.

NeuN (green) and GFAP (red) immunohistochemistry for neurons and astrocytes respectively shows no overlap of labeling (top row). NeuN (green) and isolectin B4 (red) immunohistochemistry for neurons and microglia respectively shows no overlap of labeling (bottom row). Scale bar, 50 μm.
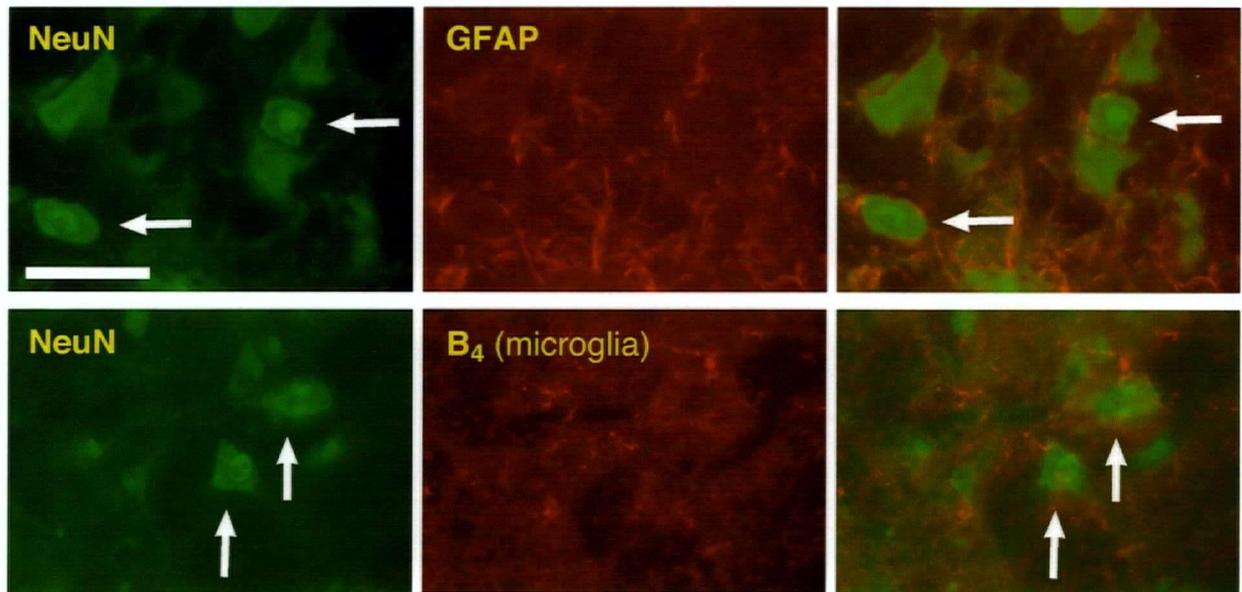
2.2.4. TrkB Immunohistochemistry

TrkB immunohistochemistry was performed on both the rubrospinal cell bodies and on the rubrospinal tracts within the spinal cord. For sections of the red nucleus, the slides were taken from the -80°C freezer and thawed for 10 minutes. After initial washes with 0.01 M PBS with 0.1% Tween-20, the slides were incubated overnight at 4°C with rabbit polyclonal TrkB antibody directed at the intracellular carboxy terminus of the full length TrkB receptor (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-12). Following blocking in 5% normal donkey serum, slides were incubated at room temperature for 2 hours in a solution containing a biotinylated donkey anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA), and subsequent development with the ABC kit (Elite ABC Kit, Vector Laboratories, Burlinghame, CA). After several brief washes in distilled water, the slides were dehydrated using a graded series of ethanol, being immersed briefly in 100% isopropanol, and cleared in toluene before being mounted in Entellan® (Electron Microscopy Sciences, Hatfield, PA) and coverslipped.

To visualize TrkB receptor expression in the spinal cord, slides were incubated overnight at 4°C with TrkB primary antibody (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-12). Slides were then blocked using 5% donkey normal serum, and incubation overnight with both an Alexa 488 conjugated anti-rabbit secondary antibody raised in donkey (1:200; Molecular Probes Incorporated, Eugene, Oregon) to visualize TrkB immunoreactivity, as well as streptavidin conjugated to Cy3 (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) to visualize the anterograde BDA labeling of the rubrospinal tract. Finally, slides were washed in 0.01 M PBS and coverslipped with an anti-fade media to reduce fluorescent bleaching

(SlowFade®, Molecular Probes, Eugene, OR), with the anticipation that bleaching of the fluorescence would be a problem with confocal imaging of this tissue.

2.3. ANALYSIS OF TISSUES

2.3.1. Disector Counting Technique of Rubrospinal Neurons

The principles of the disector method of stereologic counting have been described in the background chapter. In brief, the method involves comparing the cells identified on one section with the cells identified on the adjacent section, and counting only those that are not visualized on both in order to avoid double-counting the same cell. The caudal 500 μm of the red nucleus was sectioned into 25 sections of 20 μm thickness. After NeuN immunohistochemistry to identify the rubrospinal neurons, the image of each red nucleus was captured with a Zeiss Axioskop microscope equipped with a SPOT digital colour camera (Diagnostic Instruments Inc, Sterling Heights, MI) and Northern Eclipse image analysis software (Empix, Mississauga, ON). The application of FluoroGold into the spinal cord allowed for the retrograde labeling of the rubrospinal neurons and outlined the boundaries of the injured aspect of the red nucleus. NeuN-positive rubrospinal neurons were then outlined on each section (the "sampling" section) and compared to neurons on the rostral adjacent section (the "lookup" section). *Neurons were counted on the sampling section only if they were not present on the lookup section.* NeuN-positive neurons were outlined if their nucleus or nucleolus could be detected, or if they possessed a characteristic stellate morphology.

Neuronal counts were performed on both the injured and uninjured red nuclei. The evaluation of neuronal survival was performed in a comparative fashion, with the number of neurons in the injured red nucleus compared to the number of neurons in the contralateral uninjured red nucleus.

2.3.2. Measurement of Cross Sectional Area

Six sections spaced evenly throughout the caudal 500 μm of the red nucleus (each approximately 60-80 μm apart from one another) were used for the measurement of cross-sectional area. Again, NeuN-positive rubrospinal neurons were outlined if their nucleus or nucleolus could be detected, or if they possessed a characteristic stellate morphology. An image analysis application for Adobe PhotoShop was used to measure the cross sectional area of each outlined neuron (Image Processing Took Kit, Reindeer Graphics, Asheville, NC).

The evaluation of neuronal atrophy was performed in a comparative fashion, with the cross sectional area of neurons in the injured red nucleus compared to the cross sectional area of neurons in the contralateral uninjured red nucleus.

2.3.3. *In Situ* Hybridization (ISH)

To evaluate the expression of the regeneration associated genes GAP-43 and Tα1 tubulin, we performed *in situ* hybridization (ISH) on sections of the red nucleus. This technique allows for the cellular localization of specific mRNA sequences, from which a semi-quantitative analysis of gene expression changes can be performed. ISH was performed with radioactively labeled oligonucleotide probes complementary to GAP-43 and Tα1 tubulin. The oligonucleotide sequence for the GAP-43 probe and Tα1 tubulin probes are listed in Figure 2.3. The oligonucleotides were end-labeled with ^{35}S-dATP using deoxynucleotide terminal transferase according to standard molecular protocols (Ausubel et al., 1987). This adds a radioactive polyA tail to the 3' end of the oligonucleotide probe. The specific activity of the probes was at least 600,000 cpm/μl. Control slides were performed to confirm the sensitivity and specificity of the sense and antisense GAP-43 and Tα1 tubulin probes on animals that were acutely injured. (Figures 2.4 and 2.5)

Slides with 20 µm thick sections of the red nucleus were taken from storage in the -80°C freezer, dried for 5 minutes at room temperature, and then post-fixed for 30 minutes in 4% paraformaldehyde at 4°C before being rinsed in two washes of 1x PBS with DEPC. Sections were then permeabilized in a solution of proteinase K (20 µg/ml in 50 mM Tris and 5 mM EDTA) to improve the access of the oligonucleotide probes to their complementary mRNAs within the cells. The slides were rinsed in PBS and fixed in 4% paraformaldehyde again for 5 minutes to stabilize the proteins after permeabilization. The slides were washed twice in PBS and dehydrated in a graded series of ethanol. The slides were placed in chloroform for 5 minutes to delipidize the sections before being placed back into 100 and 95% EtOH washes for one minute each. The sections were air-dried at room temperature for 10 minutes. 100µl of hybridization cocktail containing 1.2 x 10^6 cpm of labeled oligonucleotide was applied to each slide, which was coverslipped and incubated at 43°C for 16-18 hours. Each ml of hybidization cocktail contains 1.2 x 10^7 cpm of labeled oligonucleotide, 200 mg of salmon sperm DNA, and 200 mg tRNA, in a solution of 50% deionized formamide, 10% dextran sulfate, 5x SSC, 5x Denhardt's solution, and 200 mM dithiothreitol.

After hybridization, the slides were washed in decreasing concentrations sodium chloride / saline citrate (SSC) solutions in order to remove unbound and non-specifically bound oligonucleotide probe. All SSC wash solutions contained 2-mercaptoethanol (200ml/ml) except for the final two washes in 0.25x and 0.1x SSC. After removing the coverslips in 4x SSC at room temperature, the slides were washed for 20 minutes each at 48-50°C in 2x SSC, 1x SSC, and 0.5x SSC. The two final washes were at 55°C in 0.25x and 0.1x SSC. Remaining salts were rinsed from the slides using distilled water at room temperature, and the sections were dehydrated in 60 and 95% ETOH.

For autoradiographic development, the slides were dipped in Kodak NTB2 photographic emulsion (Kodak Canada, Toronto, ON) diluted 1:1 with distilled water. For GAP-43, the slides were incubated for 5 days prior to development, while for Tα1 tubulin, slides were incubated for 2 days prior to development. Slides were developed using Kodak D-18 developer and fixed with Kodak Fixer. To visualize the rubrospinal neurons, the slides were immersed in 0.01% ethidium bromide in 1:100 PBS, washed for one hour in tap water, then coverslipped.

A semi-quantitative analysis of GAP-43 and Tα1 tubulin ISH signal was performed in order to compare RAG expression in the injured red nucleus with that of neurons in the uninjured red nucleus. Darkfield images of the ISH silver grains and fluorescent images of the ethidium bromide stained rubrospinal neurons were captured with the Zeiss Axioskop, and stored as TIFF files. Using SigmaScan Pro ImageAnalysis 5.0 Software (Systat Software, Inc, Point Richmond, CA), the ethidium bromide stained rubrospinal neurons were then outlined, using the same criteria as described above for the counting of rubrospinal neurons and measurement of neuronal cross sectional area. The darkfield image of ISH signal was then overlaid on top of the neuronal profiles and the area fraction occupied by the grains (ie. grain density) was calculated per neuron. An region of the section devoid of neuronal or glial cells was outlined (with an area approximately equal to that of a rubrospinal neuron) and the ISH signal from this area was measured as the "background" autoradiographic signal. The ISH signal per cell was then calculated as the difference between the ISH signal within the cell profile and the background signal.

Figure 2.3. In Situ Hybridization Probes

GAP-43 Probe (52mer):

5'-GCATCGGTAGTAGCAGAGCCATCTCCCTCCTTCTTCTCCACACCATCAGCAA-3'

100% complementary to bases 273-324 of rat GAP-43 mRNA {Basi, Jacobson, et al. 1987 310 /id} (accession # J02809).

Tα1 Tubulin Probe (47mer):

5'-AAACCCATCAGTGAAGTGGACGGCTCGGGTCTCTGACAAATCATTCA-3'

100 % complementary to bases 1548-1594 of rat Tα1 α-tubulin mRNA {Lemischka, Farmer, et al. 1981 952 /id} (accession# V01227).

Figure 2.4. GAP-43 anti-sense and sense probes on rubrospinal neurons 7 days after cervical axotomy

GAP-43 expression is low in adult rubrospinal neurons, but increases rapidly after cervical axotomy (unfortunately, this upregulation is not sustained, correlating with the failure of axonal regeneration). We therefore performed control ISH on the axotomized red nucleus, 7 days after injury. As expected, while the GAP-43 anti-sense probe demonstrated robust binding, there was no specific binding of the sense probe.
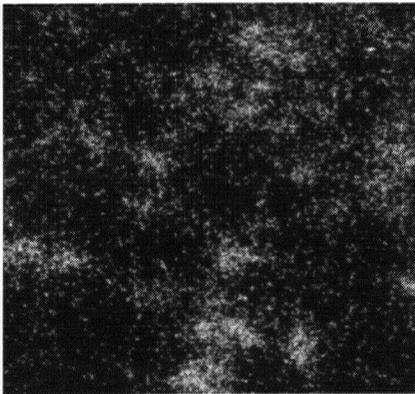
GAP-43 anti-sense probe on rubrospinal neurons 7 days after cervical axotomy.
(Note the expected rise of GAP-43 expression at this early time point after axotomy - this expression eventually falls back down to a low, baseline level of expression.)
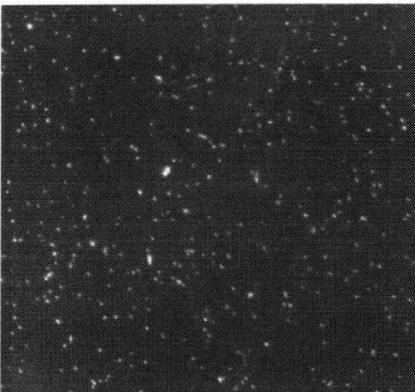
GAP-43 sense probe on rubrospinal neurons 7 days after cervical axotomy.
(Note the absence of specific binding.)

Figure 2.5. Tα1 tubulin anti-sense and sense probes on rubrospinal neurons 7 days after cervical axotomy

Baseline Tα1 tubulin expression is maintained at relatively high levels in adult rubrospinal neurons (and tends to decrease over time after axotomy). We therefore evaluated the binding of our sense and antisense probes on the uninjured red nucleus. As expected, while the Tα1 tubulin anti-sense probe demonstrated robust binding, there was no specific binding of the sense probe.
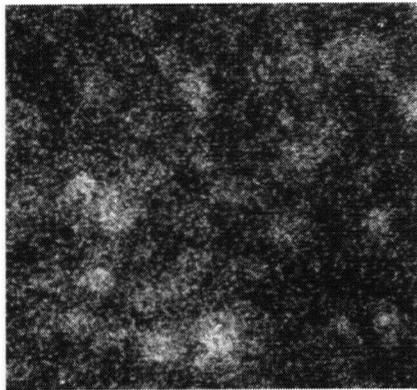
Tα1 tubulin anti-sense probe on uninjured rubrospinal neurons
(This is the expected high baseline expression in the adult)
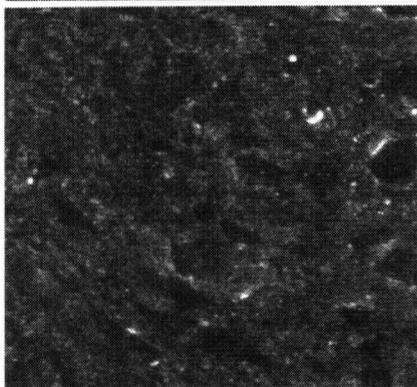
Tα1 tubulin sense probe on uninjured rubrospinal neurons
(Note the absence of specific binding)

- CHAPTER 3 -

RUBROSPINAL NEURONAL ATROPHY AND SURVIVAL

AFTER CERVICAL AXOTOMY AND

THE RESPONSE TO BDNF APPLICATION

3.1. SUMMARY

Following cervical axotomy, rubrospinal neurons undergo a well-documented atrophy and have also been described as undergoing extensive cell death within 4 to 8 weeks. The prevention, attenuation, or reversal of this atrophy has been associated with a greater regenerative propensity of rubrospinal neurons. Obviously, the prevention of cell death is of paramount importance to the possibility of promoting regeneration of this neuronal system after injury. The retrograde death of supraspinal neurons after spinal cord injury has obvious implications for the therapeutic potential of interventions to promote axonal regeneration or plasticity, as the more neurons that die after spinal cord injury, the less likely recovery will be achieved with such treatments. In this chapter, I summarize a body of work that examines rubrospinal atrophy and survival at various time points after cervical axotomy and the effect that BDNF applied either at the spinal cord injury site or to the red nucleus has on this.

I found that two months after cervical axotomy, injured rubrospinal neurons were significantly smaller than their contralateral uninjured counterparts. The application of BDNF within gelfoam pledgets directly to the spinal cord injury site, over a wide spectrum of

concentrations, did not reverse this atrophy (Kwon et al., 2004a). Compared to control axotomized rubrospinal neurons with no interventions, a slight increase in neuronal cross sectional area was collectively observed with all animals that received a repeat excision of the spinal cord injury site (a "refreshment" injury) and application of both BDNF or PBS, suggesting an effect of the refreshment injury itself. In contrast, the application of BDNF via osmotic mini-pumps directly to the injured red nucleus did reverse rubrospinal atrophy, even 12 months after cervical axotomy.

The measurement of the number of surviving rubrospinal neurons using stereologic counting techniques and NeuN immunohistochemistry (a neuron specific marker), demonstrated that rubrospinal neurons were still present 12 months after cervical axotomy, albeit in a severely atrophic state (Kwon et al., 2002b). Even with conventional histologic techniques (cresyl violet staining), equal numbers of injured and uninjured rubrospinal neurons were observed in animals treated with cell body administration of BDNF. This indicated the reversal of neuronal atrophy and the ability to detect the larger neurons within the injured red nucleus. Using the same counting and histologic techniques, I found that a second cervical injury (refreshment) caused only a modest amount of cell loss (Kwon et al., 2002c).

3.2. INTRODUCTION

3.2.1. Atrophy and Death of Rubrospinal Neurons After Axotomy and the Administration of Neurotrophic Factors

Rubrospinal neurons that are axotomized in the cervical spinal cord undergo significant atrophy within weeks of injury (Tetzlaff et al., 1991, Kobayashi et al., 1997) and reportedly undergo substantial cell death within months of injury (Goshgarian et al., 1983, Houle and Ye, 1999, Mori et al., 1997). The cause of this atrophy and cell loss is likely closely related to the interruption of target-derived neurotrophic factor support (reviewed by Goldberg and Barres, 2000). Support for this concept is derived from both developmental and adult studies of the rubrospinal system. For example, axotomy of the rubrospinal tract in developing animals prior to the establishment of axon collaterals causes severe retrograde atrophy and cell loss (Bregman and Reier, 1986). It was postulated by these authors that axon collaterals would have provided the means for attaining neurotrophic support from other targets that might have prevented such death. Such was supported by further work which demonstrated survival and axonal regeneration of rubrospinal neurons in neonatal rats that maintain axon collaterals (Bernstein-Goral and Bregman, 1997), and that the exogenous application of BDNF to the spinal cord of newborn rats acutely after hemisection prevents the loss of rubrospinal neurons (Diener and Bregman, 1994).

Of particular relevance to spinal cord injury in the mature nervous system, the exogenous administration of BDNF and NT-3 into the intrathecal space of adult rats acutely after cervical axotomy promoted neuronal survival and lead to reversal of rubrospinal atrophy (Novikova et al., 2000). These authors noted that the combined infusion of both BDNF and NT-3 effected better neuronal survival and hypertrophy than the neurotrophic factors alone. The atrophy of

adult rubrospinal neurons after thoracic axotomy was shown to be prevented by the acute transplantation of fetal tissue with or without BDNF and NT-3 into the lesion site, again with the most striking results seen in animals receiving the full combination of transplants, BDNF, and NT-3 (Bregman et al., 1998).

In summary therefore, the relationship between the interruption of target-derived neurotrophic support and the atrophy and loss of rubrospinal neurons after cervical axotomy provides a compelling rationale for the exogenous application of neurotrophic factors to the rubrospinal system after injury.

3.2.2. The Targets for Therapeutic Intervention – Cell Body Versus Axon

For patients with spinal cord injuries, the obvious site for therapeutic intervention would be their injured spinal cord, where the disrupted axons and their hostile CNS environment can be directly accessed. Indeed, most experimental therapies (including neurotrophic factors) in animal models of spinal cord injury have been initially applied to the spinal cord injury site where their potential effectiveness has been first established. Studies in which BDNF has been applied exogenously to the spinal cord in the acute injury setting have demonstrated both a neuroprotective and regenerative effects (Novikova et al., 2002, Sayer et al., 2002, Sharma et al., 2000, Jakeman et al., 1998, Lu et al., 2001, Ankeny et al., 2001, Bamber et al., 2001, Blits et al., 2003). Specifically relevant to my work in the rubrospinal system, the acute administration of BDNF to the spinal cord injury site (either by direct infusion or by genetically modified cell lines) has been reported to reduce retrograde atrophy (Liu et al., 2002) and loss (Diener and Bregman, 1994) of axotomized rubrospinal neurons, reduce oligodendrocyte apoptosis (Koda et al., 2002), promote rubrospinal axonal regeneration (Liu et al., 1999), and promote functional

recovery (Liu et al., 1999, Namiki et al., 2000, Jakeman et al., 1998, Li et al., 2003, Ikeda et al., 2002).

Alternatively, the target of intervention after spinal cord injury can be the actual cell body of the axotomized neuron. Kobayashi et al. demonstrated that the direct infusion of BDNF to the red nucleus prevented rubrospinal atrophy at cervical axotomy, promoted the expression of regeneration associated genes, and facilitated axonal regeneration into peripheral nerve transplants (Kobayashi et al., 1997). Fukuoka et al. also observed the complete prevention of rubrospinal atrophy after cervical axotomy with the direct infusion of BDNF into the vicinity of the red nucleus (Fukuoka et al., 1997). Transfection of rubrospinal neurons by adeno-associated virus vector mediated BDNF gene therapy also reversed rubrospinal atrophy after cervical axotomy (Ruitenberg et al., 2004). The cell body as a potential target of intervention extends outside the rubrospinal system as well. Berry et al. demonstrated increased regeneration of retinal ganglion cells through an optic nerve injury after the application of a peripheral nerve segment into the vitreous body of the eye, where the many neurotrophic factors being expressed by reactive Schwann cells of the peripheral nerve would be in the vicinity of the retinal ganglion cell bodies (Berry et al., 1996). Also, Toma et al. demonstrated on sympathetic neurons using compartmentalized cultures that NGF exposure to the cell bodies elicited a much higher gene expression response than NGF exposure to the distal axons (Toma et al., 1997). While the application of such interventions aimed at the cell bodies is moderately invasive (and would therefore limit their clinical translation), these studies highlight the importance of the cell body as a potential target for intervention.

3.2.3. The Timing of Therapeutic Intervention

While substantial excitement and hope has been generated over experimental therapies such as neurotrophic factors that appear to be beneficial in animal models of spinal cord injury, these models typically employ an acute injury paradigm where the intervention occurs at or just after the actual injury. The applicability of such therapies for individuals whose spinal cord injury occurred long ago and who are considered "chronic" is less clearly defined (reviewed by Houle and Tessler, 2003). Unfortunately, axonal regeneration therapies in chronic models of spinal cord injury have frequently been met with a reduced efficacy compared to that seen with acute application (discussed further in Chapter 5).

Of course, such animal studies in which a delay in therapeutic intervention is included raise the question of how to define the term "chronic" as it relates to the neurobiology of spinal cord injury. From a clinical perspective, the improvement in neurologic function that patients who sustain a complete or incomplete spinal cord injury achieve will usually plateau around 1 year post-injury (Marino et al., 1999). How this pattern of recovery is best reproduced in a small rodent model is unclear, and thus it is difficult to know how long of a delay needs to be instated in order to simulate a chronic state. A large amount of work was done on animals 12 months post-axotomy, and while this period of convalescence represents a fairly substantial part of the animals' total life span, it is a somewhat impractical length of time to propose for a model of injury chronicity. Keeping the animals alive for this period of time is expensive, and in an elderly state their ability to tolerate anesthesia and invasive surgical interventions is reduced. One subsequent experiment was carried out with a 6 month delay in intervention, but the remainder were performed two months post-axotomy, justified in part by the reports of other authors of gross morphologic changes within the spinal cord after injury (Hill et al., 2001, Houle

and Jin, 2001) and of molecular changes within the red nucleus after injury (Tetzlaff et al., 1991, Kobayashi et al., 1997, Fernandes et al., 1999). This rationalization is discussed in more detail in Chapter 7.

3.3. OVERVIEW OF EXPERIMENTAL QUESTIONS AND HYPOTHESES

In this chapter, I evaluated the cross-sectional area and the survival of chronically injured rubrospinal neurons after cervical axotomy to test the following hypotheses:

1. Investigators who have applied BDNF directly to the injured spinal cord have done so in various doses/concentrations (ie. a standard dose/concentration does not currently exist). Given that rubrospinal neurons undergo significant atrophy after cervical axotomy, and that such atrophy was not reversed by the chronic administration of BDNF to the spinal cord injury site (Storer et al., 2003), *I hypothesized that the loss of BDNF effectiveness in the "chronic" setting might be related to providing the appropriate dose/concentration of the neurotrophic factor.*

To test this hypothesis, animals underwent a cervical axotomy, then two months later, BDNF in 3 exponentially increasing concentrations (50, 1000, and 20,000 ng/μl) was applied within gelfoam to the refreshed injury site (in addition to PBS-treated controls). Cross-sectional area of NeuN immunolabeled injured and uninjured neurons was measured and compared.

2. Given that the infusion of BDNF into the vicinity of the rubrospinal cell bodies was shown to prevent their atrophy when applied acutely after cervical axotomy (Kobayashi et al., 1997), *I hypothesized that the cell-body administration of BDNF could reverse the atrophy of rubrospinal neurons when applied chronically after cervical axotomy.*

To test this hypothesis, animals underwent a cervical axotomy, then twelve months later, BDNF was infused through an osmotic minipump cannula stereotactically placed just lateral to the red nucleus. Cross-sectional area of NeuN immunolabeled injured and uninjured rubrospinal neurons was measured and compared.

3. Given that the reported rates of retrograde rubrospinal death after spinal cord axotomy have been quite variable and have employed different techniques for counting neurons (eg. different histologic techniques and counting techniques – see Table 3.1), *I hypothesized that substantial cell death was not occurring in chronically axotomized rubrospinal neurons.*

To test this hypothesis, animal underwent a cervical axotomy, then twelve months later, BDNF was infused into the vicinity of the red nucleus (as above), and the physical dissector method of stereologic counting was employed to count injured and uninjured rubrospinal neurons after both NeuN immunohistochemistry and cresyl violet staining.

4. While performing an intervention on the chronically injured spinal cord (eg. a cell transplantation), a second axotomy of already injured axons may be unavoidable (or even necessary). Given that second axotomy of chronically injured rubrospinal neurons has been reported to result in a significant acceleration of cell death using non-stereologic counting techniques (Houle and Ye, 1999), *I hypothesized that this cell death after second axotomy might be over-estimated.*

To test this hypothesis, animal underwent a cervical axotomy, then six months later, the injury site was refreshed to re-axotomize these chronically injured neurons. The physical dissector method of stereologic counting was employed to count injured and uninjured rubrospinal neurons after NeuN immunohistochemistry.

3.4. RESULTS

3.4.1. Neuronal Atrophy 2 Months Post-Axotomy and the Response to Spinal Cord Application of BDNF

Two months post-axotomy, I observed significant atrophy of the injured rubrospinal neurons compared to uninjured. In six animals sacrificed two months after injury with no other interventions, the mean cross sectional area of the axotomized rubrospinal neurons was 224.7 ± 15.9 μm^2 while the mean cross sectional area of the contralateral uninjured rubrospinal neurons was 379.6 ± 50.0 μm^2 ($p < 0.001$, paired t test). Compared to the contralateral uninjured neurons, the mean cross sectional area of the injured rubrospinal neurons was $59.7 \pm 2.3\%$. (Figure 3.1)

I tested the hypothesis that the application of various concentrations of BDNF to the spinal cord could reverse this atrophy. Two months post-axotomy, the spinal cord was re-exposed, the chronic lesion was "refreshed" by extending it rostrally by 1 mm, and either PBS or BDNF-soaked gelfoam was inserted. The mean cross sectional areas of injured rubrospinal neurons treated with BDNF-soaked gelfoam in concentrations of 50 ng/μl (low), 1000 ng/μl (medium), and 20,000 ng/μl (high) were 231.2 ± 6.8, 232.0 ± 4.7, and 219.9 ± 12.1 μm^2 respectively (n=5 animals per group). Expressed as a percent of the contralateral uninjured neurons, the mean cross sectional areas were $69.0 \pm 4.5\%$, $67.1 \pm 1.2\%$, and $66.7 \pm 4.8\%$ for the low, medium, and high dose BDNF groups. In animals treated with PBS-soaked gelfoam, the injured rubrospinal neurons had a cross sectional area of 234.7 ± 24.2 μm^2 (n=5 animals per group), and were $63.9 \pm 3.3\%$ of the contralateral uninjured neurons. (Figure 3.1)

There was no significant difference in the cross sectional area percentages between the PBS treated animals (63.9±3.3%) and the three groups of BDNF treated animals (69.0±4.5%, 67.1±1.2%, and 66.7±4.8%) (p=0.81, one way ANOVA). Given that the cross sectional area percentages were each higher for the BDNF treated groups than the PBS treated group, I pooled the BDNF treated groups to look for a neurotrophic factor effect. However, when comparing the percent cross-sectional area of the pooled BDNF-treated animals (67.6±2.1%; n=15) against the PBS treated animals (63.9±3.3%; n=5), there was again no statistically significant difference (p=0.38, unpaired t test). This suggests that BDNF at any of the three doses did not promote the reversal of neuronal atrophy when applied to the refreshed spinal cord injury site, 2 months post-axotomy.

I then compared the cross sectional area percentages of the injured rubrospinal neurons without a refreshment injury against those of the PBS and BDNF treated animals. Amongst all 5 groups (No refreshment injury, PBS, low, medium, and high dose BDNF) there was no significant difference (p=0.34, one way ANOVA). Given that the cross sectional area percentages were each higher for the groups of animals undergoing a second intervention (the PBS and BDNF treated groups) than the PBS treated group, I pooled the animals undergoing the second intervention to look for an effect of the refreshment injury. When comparing the percent cross-sectional area of the animals that did not have a refreshment injury (59.7±2.3%., n=6) against the combined PBS and BDNF treated animals (66.7±1.8%; n=20), there was a trend towards a significant difference (p=0.057, unpaired t test). The biological relevance of a mean difference of approximately 7% in cross sectional area is uncertain, but does suggest that the neurons may have reacted to the refreshment injury with a slight increase in cell size.

Figure 3.1. Rubrospinal neuronal atrophy is not reversed with any of the three doses of BDNF applied to the spinal cord injury site.

The atrophy of the rubrospinal neurons and the manner in which the FluoroGold retrograde tracer was utilized to outline the borders of the injured red nucleus is illustrated. With the neuronal atrophy (A), the borders of the red nucleus become less distinct. Therefore, the border is outlined in the FluoroGold image (B) and this is overlaid (C) on top of the NeuN image (A). Cross sectional area is then measured. Note the significant atrophy of the injured rubrospinal neurons compared to the uninjured contralateral neurons. There was no significant difference between the cross sectional areas of the groups on one way ANOVA (E). However, when pooling the PBS, Low, Medium, and High BDNF dose groups (ie. all animals that received a refreshment injury) and comparing them to the No Refreshment group, there was an increase in mean cross sectional area of approximately 7%, which approached statistical significance (p=0.057).

Figure 3.1
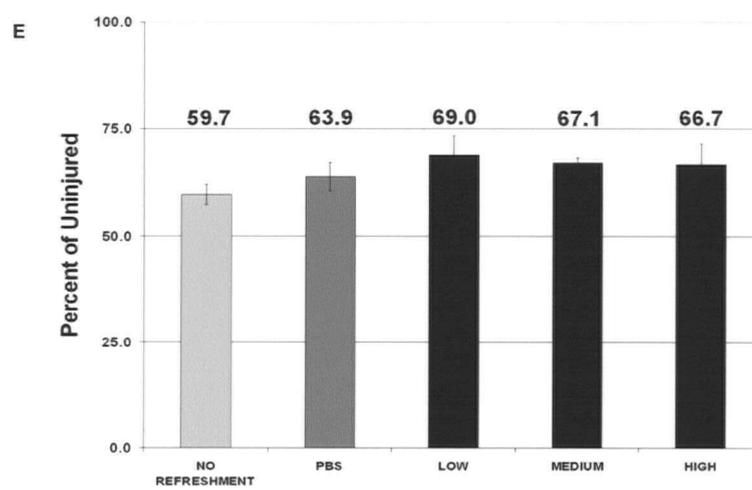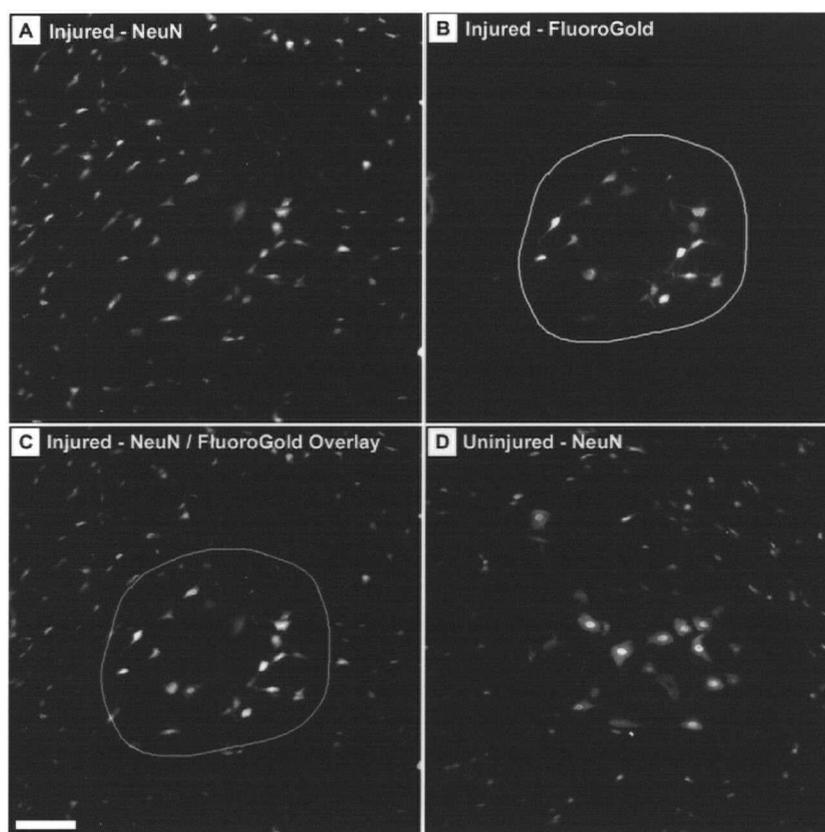
3.4.2. Neuronal Atrophy 12 Months Post-Axotomy and the Response to Cell Body Application of BDNF

Twelve months after cervical axotomy, I evaluated rubrospinal neuronal atrophy with cresyl violet staining and tested the hypothesis that the application of BDNF directly to the cell bodies rather than to the cut axons in the spinal cord could reverse this atrophy. Pilot studies of 3 animals 12 months post-axotomy were performed with the application of BDNF 500 ng/μl within osmotic minipumps directly to the spinal cord injury site. I observed no apparent reversal of neuronal atrophy (Figure 3.2) which prompted the attempts to apply the BDNF to the cell bodies. In control animals treated 12 months post-axotomy with osmotic minipumps filled with vehicle solution alone (n=8), I observed severe atrophy of injured rubrospinal neurons. The cross sectional area of these injured neurons was 178.6 ± 5.8 μm^2, which was significantly smaller than the contralateral uninjured neurons with a mean cross-sectional area of 277.0 ± 23.2 μm^2 (p<0.001, paired t test). (Figure 3.3) Represented as a percentage of the uninjured neurons, the cross sectional area of the injured rubrospinal neurons in these control animals was $66.7\pm4.1\%$. When evaluating the distribution of neuronal sizes across the injured and uninjured red nuclei, I observed a preponderance of smaller, atrophic neurons in the injured red nucleus. (Figure 3.4).

In animals treated 12 months post-axotomy with osmotic minipumps filled with BDNF (n=5), the mean cross-sectional area of the injured and contralateral uninjured rubrospinal neurons was 240.0 ± 26.8 μm^2 and 266.6 ± 30.7 μm^2 respectively. (Figure 3.3) Represented as a percentage of the uninjured neurons, the cross sectional area of the injured rubrospinal neurons in these BDNF treated animals was $90.9\pm6.2\%$. While still slightly smaller, the difference between the injured and uninjured cross sectional areas was not statistically significant (p=0.074, paired t test). Comparing the injured rubrospinal neurons between those animals treated with

vehicle solution alone against those treated with BDNF, the increase in cross sectional area observed with BDNF treated animals was statistically significant (240.0 ± 26.8 μm^2 versus 178.6 ± 5.8 μm^2, p=0.009, unpaired t test). While the control animals had a preponderance of smaller, atrophic neurons in the injured red nucleus, the distribution of neuronal sizes in the animals treated with cell body application of BDNF was very similar to that seen in the uninjured red nucleus. (Figure 3.4) Of note, there was no significant difference in size between uninjured neurons of the control and BDNF treated animals (p=0.395, unpaired t test).

Figure 3.2. BDNF applied to the spinal cord injury site 12 months after cervical axotomy does not reverse atrophy of injured rubrospinal neurons.

In pilot experiments leading to the decision to try administering BDNF directly to the cell bodies of chronically injured rubrospinal neurons, BDNF (500 ng/μl) was applied via osmotic minipumps to the spinal cord injury site, 12 months post-axotomy. This unfortunately was not associated with any noticeable reversal of atrophy in these chronically injured rubrospinal neurons, thus stimulating the interest in providing the neurotrophic factor via infusion to the brainstem. Note the significant atrophy of the injured rubrospinal neurons.
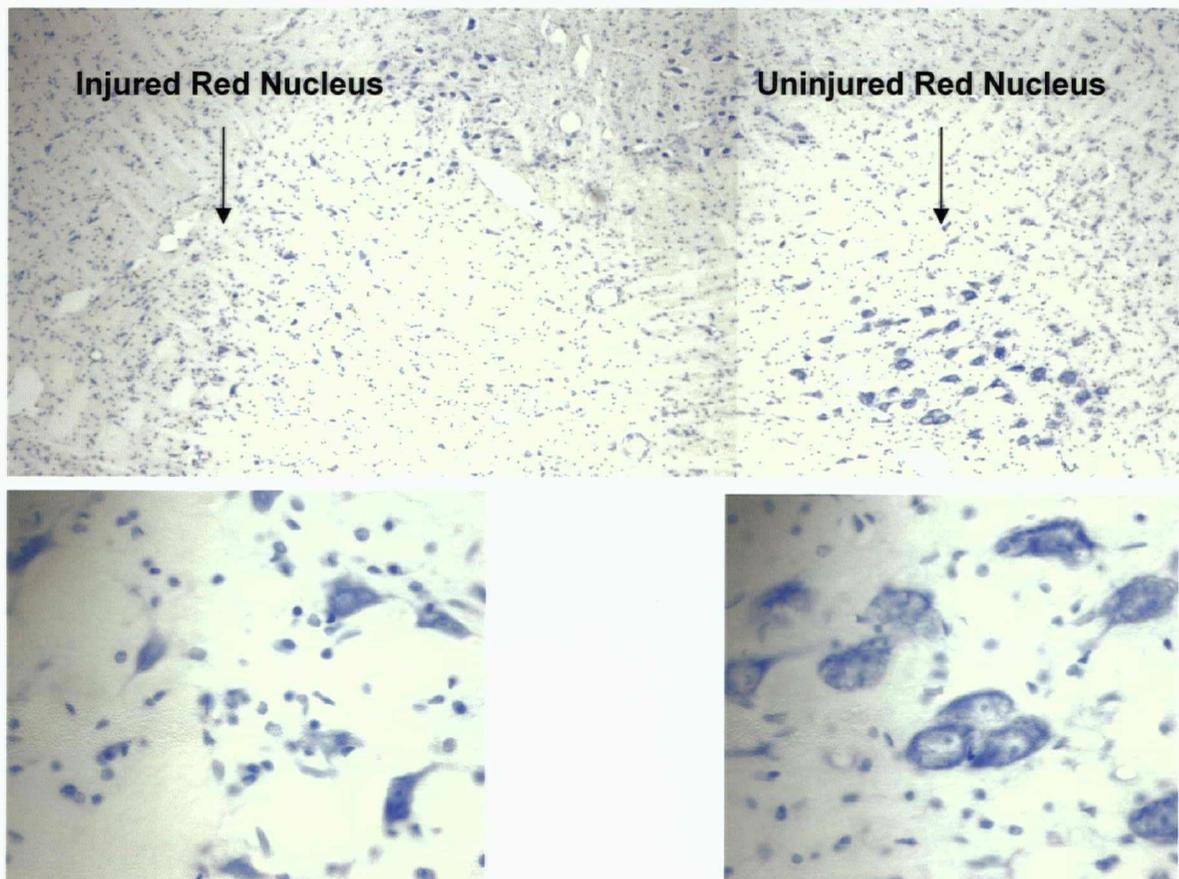
Figure 3.3. Atrophy of rubrospinal neurons can be reversed by BDNF twelve months after injury.

NeuN immunohistochemistry demonstrates numerous atrophic neurons on the injured side treated with vehicle alone. Note the recovery in cell size of the BDNF treated injured neurons to almost normal size as seen on the contralateral side. All sections are taken from comparable areas of the rubrospinal nucleus, approximately 240 μm from the caudal pole. Scale bar, 50 μm.

Neu N Immunohistochemistry
Injured   Contralateral Uninjured
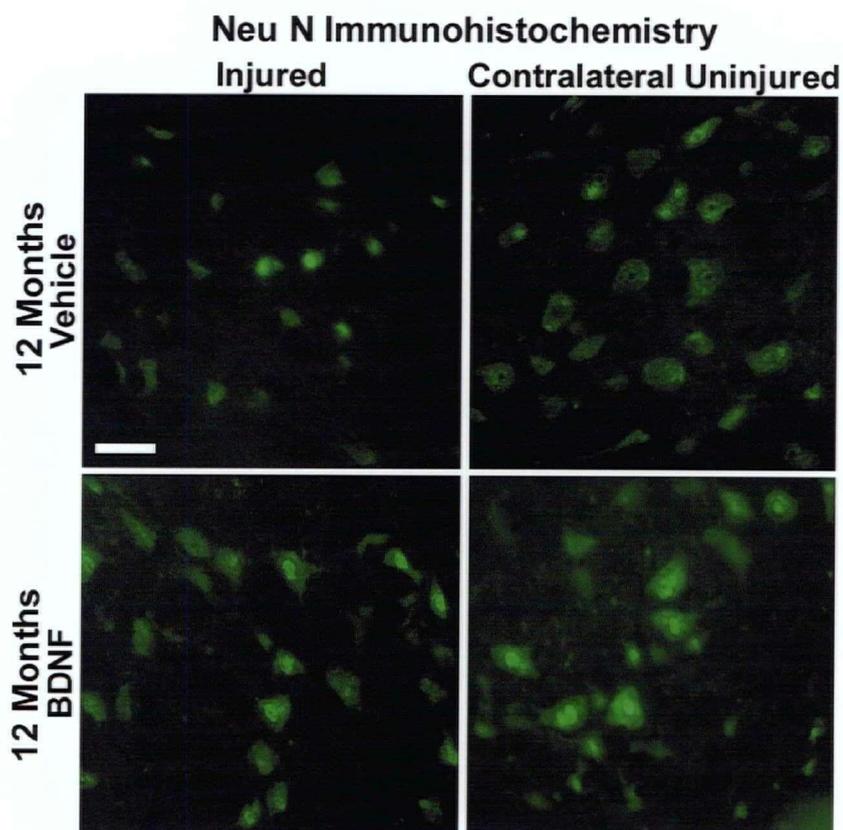
Figure 3.4. BDNF administration normalizes the distribution of cell sizes in the chronically injured red nucleus.

Histogram of cross-sectional area plotting neurons in 100 μm^2 increments demonstrates a normalization of the distribution of cell sizes with the BDNF treatment. Note the predominance of small neurons in the vehicle treated group.
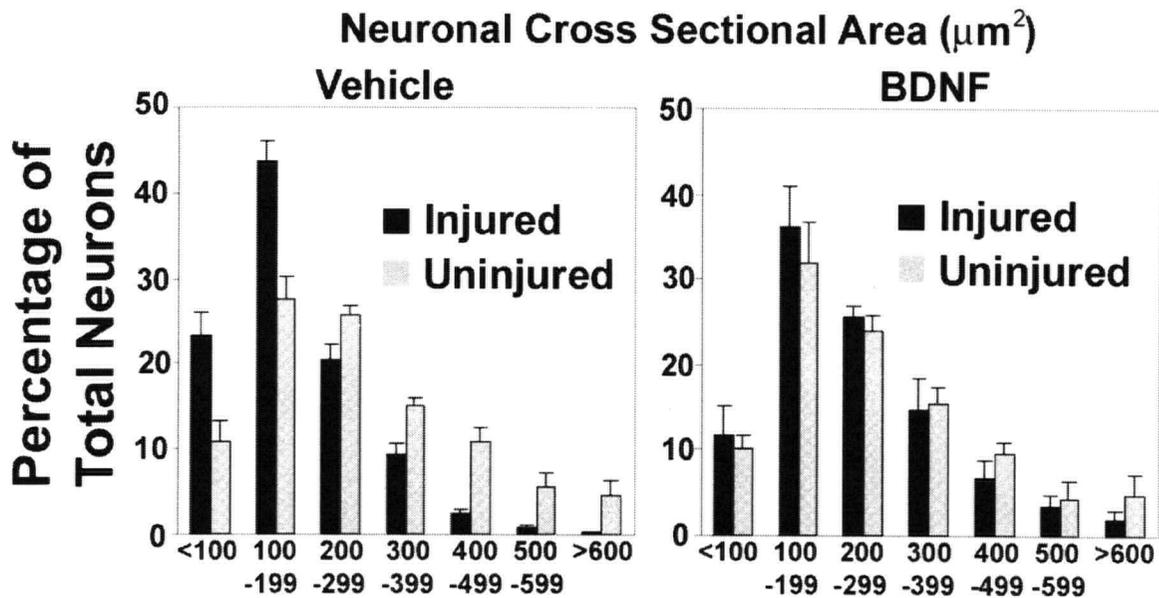
3.4.3. Rubrospinal Neuronal Counts 12 Months Post-Axotomy With Cell Body Application of BDNF

The disector method of counting was used to determine the number of rubrospinal neurons in the injured and uninjured red nucleus, to test the hypothesis that significant cell death was not occurring after cervical axotomy. As discussed earlier, this technique compares each section against its adjacent section so that neurons are counted only once. For each animal, the number of neurons on the side of the injured red nucleus was counted and compared to the number of neurons found on the contralateral uninjured red nucleus. The number of injured neurons is therefore represented as a percentage of the presumably normal number of neurons counted on the uninjured side. (Figure 3.5) In the BDNF treated animals, using NeuN immunohistochemistry I counted similar numbers of neurons (101.8±1.3%, p=0.11) in the injured red nucleus compared to uninjured. Similarly, in the vehicle treated animals, equal numbers of neurons were counted between injured and uninjured red nuclei (99.5±4.2%, p=0.47) using the NeuN marker. With cresyl violet staining, in the BDNF treated animals I again counted similar numbers of neurons in the injured red nucleus compared to uninjured (106.8±18.5%, p=0.42). For the vehicle treated animals I counted 89.0±7.6% as many neurons in the injured red nucleus compared to uninjured (p=0.10). These results using stereologic counting techniques suggest that twelve months after injury, while significantly atrophic, cervically axotomized neurons remain alive, and that using a neuronal specific marker (NeuN) aids in the visualization of atrophic neurons. That I counted on the cresyl violet sections a normal number of injured rubrospinal neurons with BDNF treatment suggests that the reversal of atrophy induced by the neurotrophic factor made it easier to visualize these neurons and thus count them as being present. Conversely, neurons that remain atrophic with the vehicle

treatment may not be visualized on cresyl violet staining, but may be better identified with NeuN immunohistochemistry. This was confirmed in further experiments at a 6 month post-axotomy time point (see below and Figure 3.6).

Figure 3.5. Stereologic counting of the injured and uninjured red nuclei demonstrates that chronically injured rubrospinal neurons remain alive long after cervical axotomy.

The number of injured neurons is represented as a percentage of the number of contralateral uninjured neurons. Note that with BDNF treatment, the number of injured neurons counted is approximately 100% of the uninjured, in both NeuN and cresyl violet staining. With cresyl violet staining only 89% of the number of uninjured neurons was detected in the vehicle treatment group. This difference is likely the result of the NeuN immunohistochemistry being a more effective method of identifying atrophic neurons than cresyl violet staining (see Figure 3.6).
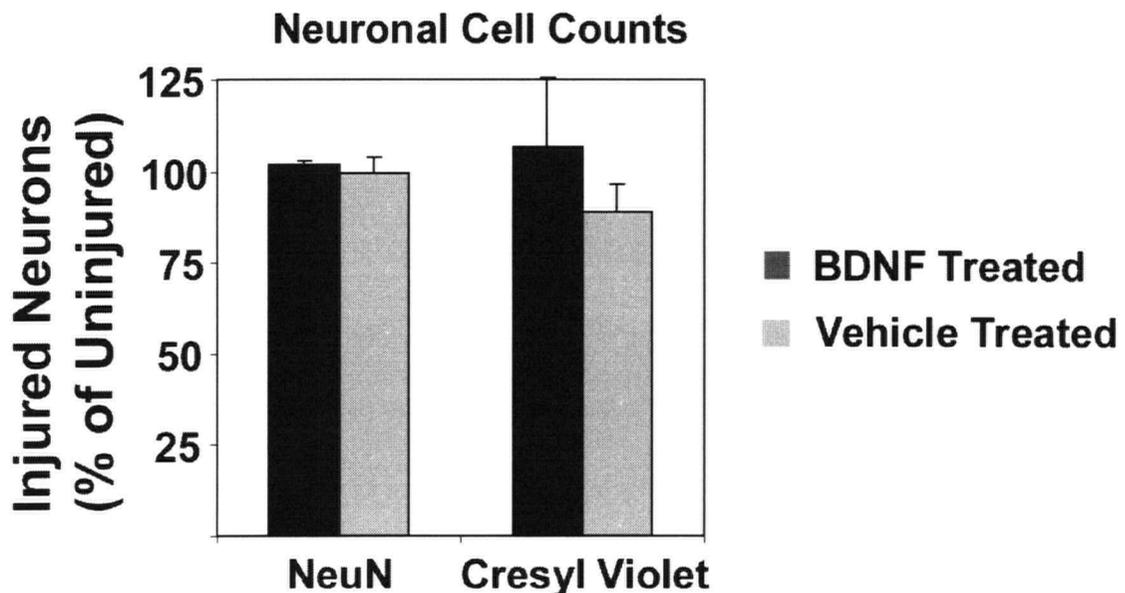
3.4.4. Neuronal Counts Following a Re-Axotomy 6 Months After Original Injury

Houle and Ye reported that a second axotomy of chronically injured rubrospinal axons (injured one month previously) caused a pronounced acceleration of cell death when compared to single-axotomized rubrospinal neurons at eight weeks after the initial lesion (Houle and Ye, 1999). In that study, the authors used retrograde labeling of rubrospinal neurons and observed approximately 70% cell loss in double-axotomized rubrospinal neurons compared to 25% cell loss in those with a single-axotomy (ie. a massive acceleration of cell death after second axotomy). In followup to my above findings of neuronal survival long after cervical axotomy with the use of a stereologic counting technique and a neuronal specific marker, I re-evaluated this issue of cell loss after second axotomy in rubrospinal neurons, six months after their original cervical injury. FluoroGold retrograde labeling of the injured rubrospinal neurons was performed to help delineate the boundaries of the injured red nucleus. (Figure 3.6, panels A-D)

One month after the second axotomy (ie. 7 months after the original axotomy), using NeuN and the disector method of counting, I counted $87.3\pm2.3\%$ of neurons in the injured red nucleus compared to the uninjured (n=4 animals). This cell loss of 13% represented a statistically significant decrease in the number of injured rubrospinal neurons ($p<0.05$, paired t test). Measurement of cross sectional area again demonstrated that axotomized rubrospinal neurons undergo marked atrophy. Injured rubrospinal neurons measured $182.\pm5.4 \ \mu m^2$ versus $290\pm6.0 \ \mu m^2$ for the contralateral uninjured neurons. This reduction in size of the injured rubrospinal neurons to $62.9\pm2.1\%$ of the cross sectional area of uninjured contralateral neurons was statistically significant ($p< 0.01$, paired t test).

Analysis of sections on which NeuN immunohistochemistry was performed initially, the digital images captured, and then cresyl violet staining was performed demonstrated a number of

atrophic NeuN positive neurons in the injured red nucleus that did not stain effectively with cresyl-violet. (Figure 3.6, panels E-J) This finding is consistent with the differences in the cell counts between NeuN and cresyl violet staining that I recorded in my evaluation of rubrospinal neurons 12 months post-axotomy. Note that in the counts of the atrophic, vehicle-treated neurons, with NeuN immunohistochemistry I detected 99.5±4.2% of the number of neurons in the uninjured red nucleus, while with cresyl violet staining I detected 89.0±7.6%. The reversal of atrophy with cell body application of BDNF facilitated the identification of the injured rubrospinal neurons and/or their distinction from surrounding glial cells with the standard cresyl violet staining.

Figure 3.6. FluoroGold retrograde labeling helps to identify the boundaries of the injured red nucleus, and NeuN immunohistochemistry better identifies atrophic neurons than cresyl violet staining.

NeuN immunohistochemistry and FluoroGold (FG) retrograde labeling of rubrospinal neurons approximately 160 μm from the caudal pole of the red nucleus (magnocellular portion). NeuN immunohistochemistry is shown in the green channel (A and D) while FluoroGold labeling of the injured rubrospinal neurons is represented in false colour in the red channel (B). For the injured red nucleus, the NeuN (A) and FluoroGold (B) images of are combined (C) so that the FluoroGold labeling facilitates identification of the rubrospinal neurons projecting down to the lumbar spinal cord (ie. those that are axotomized at the C3/4 level). Note the atrophic neurons in the ventrolateral portion of the injured red nucleus that stain poorly with FluoroGold but are seen well with the NeuN staining (arrow). The FluoroGold staining is, however, particularly useful for identifying the borders of the axotomized population of neurons in the red nucleus. In the NeuN immunostaining of the injured red nucleus (A), note the large neurons in the dorsomedial part of the nucleus (arrowhead) that are located in an area devoid of FluoroGold staining (B). Such neurons would be considered to be outside the area of the axotomized population of rubrospinal neurons and would therefore not be included in the evaluation of neuronal counts or cross sectional area. In E and F, the sections were stained with cresyl violet. Note on the injured side (E), the difficulty in identifying neurons and the borders of the red nucleus after the significant atrophy. Higher magnification of injured rubrospinal neurons (G versus H and I versus J) the demonstrates the poor visualization of these atrophic neurons with cresyl violet staining compared to NeuN immunohistochemistry. Scale bar: 50 μm.

A Injured (NeuN)

B Injured (FG)

C Injured (NeuN + FG)

D Uninjured (NeuN)

E Injured (Cresyl Violet)

F Uninjured (Cresyl Violet)

G

H

I

J
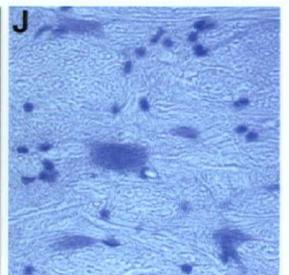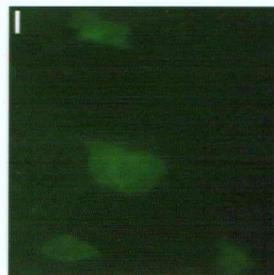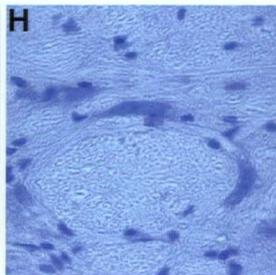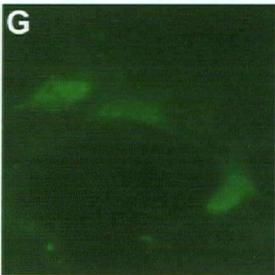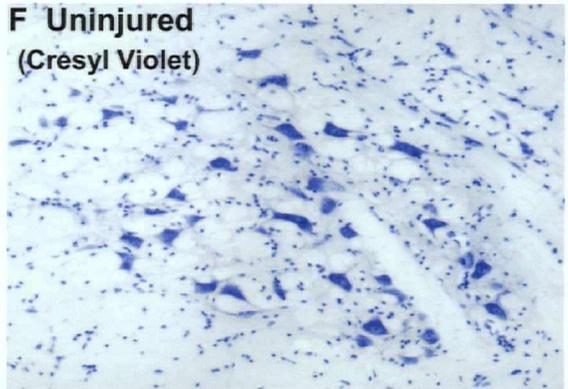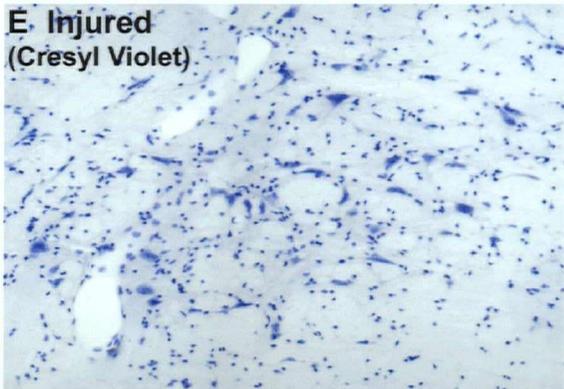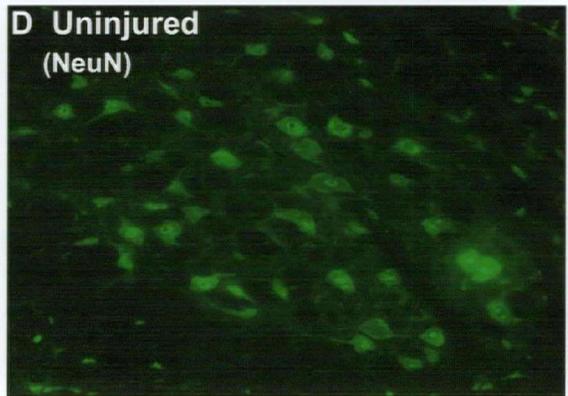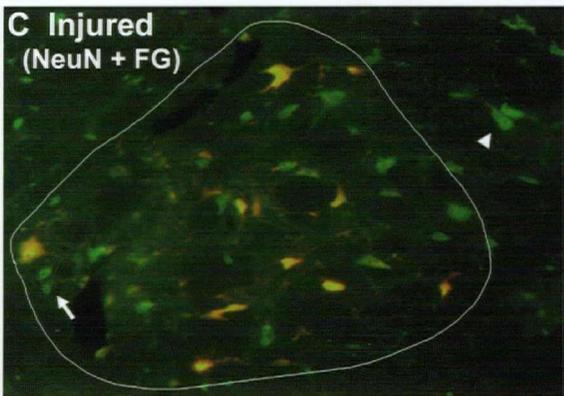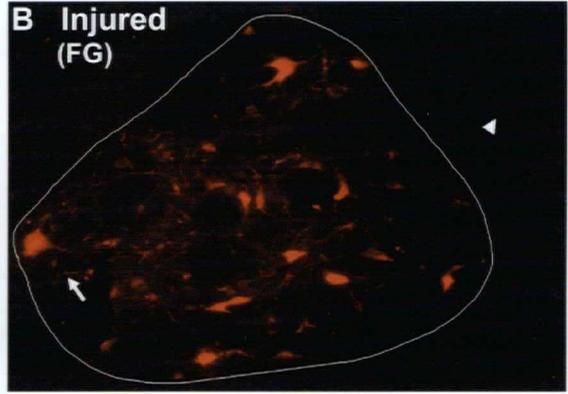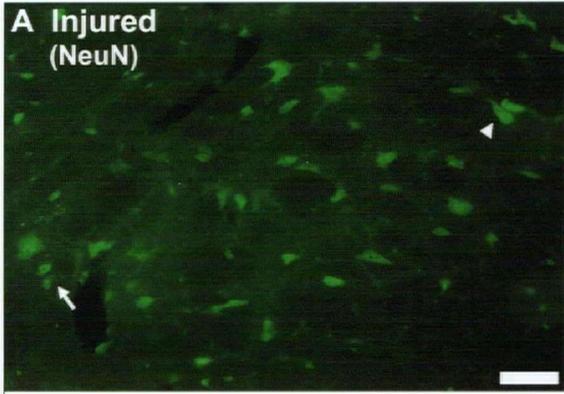
3.5 DISCUSSION

3.5.1. Chapter Summary

This chapter reviews rubrospinal atrophy and survival at a number of time points after cervical axotomy and in a number of experimental paradigms (spinal cord versus cell body treatment). In summary, using stereologic counting techniques and a specific neuronal marker, I observed that rubrospinal neurons do not undergo significant cell loss after cervical axotomy, but rather, undergo severe atrophy which makes them hard to detect using conventional histologic techniques. I found that this atrophy can be reversed with BDNF applied directly to the cell bodies 12 months after injury, but was not reversed with BDNF applied directly to the spinal cord injury site 2 months after injury, despite using different doses of BDNF. I also determined that following a second axotomy, rubrospinal neurons undergo a modest but statistically significant amount of cell death.

3.5.2. Rubrospinal Neuronal Survival Versus Death After Axotomy

The retrograde death of rubrospinal neurons in rat species following cervical axotomy has been reported as approximately 25% to 35% by 2 months after injury (Mori et al., 1997, Goshgarian et al., 1983, Houle and Ye, 1999). Clearly, the significant loss of supraspinal neurons in response to spinal cord injury could have tremendous implications on the prognosis for recovery. My observation that 12 months after cervical axotomy, rubrospinal neurons are atrophic but otherwise present in normal numbers is therefore somewhat at odds with other authors who have reported the death of a significant proportion of adult rubrospinal neurons after axotomy. The response of rubrospinal neurons to axotomy is an issue that has been examined

extensively in the literature, in rats predominantly (Goshgarian et al., 1983, Houle and Ye, 1999, Novikova et al., 2000, Feringa et al., 1988, Mori et al., 1997, Liu et al., 2002) but also in mice (Zhou et al., 1999), and opposums (Xu and Martin, 1990, Wang et al., 1999). While neuronal atrophy appears to occur universally after axotomy, these studies report different rates of cell loss between and amongst species (See table 3.1). Determining whether these represent true intra and inter-species differences is made difficult by variations in histologic technique (eg. retrograde tracing of neurons versus cresyl violet staining) and counting methods (eg. stereologic versus non-stereologic counts).

Influence of Histologic Technique – NeuN vs Cresyl Violet vs Retrograde Tracing

The influence of histologic technique on the reporting of cell death was demonstrated by Feringa et al. (Feringa et al., 1988). In this study, the authors used both horseradish peroxidase (HRP) retrograde tracing and hematoxylin and eosin (H&E) staining to evaluate rubrospinal cell death in Wistar rats undergoing thoracic axotomy. Using HRP, they found no statistically significant difference in the number of injured rubrospinal neurons compared to uninjured at 15 and 25 weeks post-axotomy. However, using H&E staining, they found a significant reduction in rubrospinal neurons in the injured compared to the uninjured red nucleus (42% loss at 52 weeks post-axotomy). Xu and Martin in their study of rubrospinal cell death in adult opposums also commented that the use of cresyl violet staining likely "underestimated the proportion of neurons that survived axotomy" (Xu and Martin, 1990).

Table 3.1. Reported rates of retrograde cell death of rubrospinal neurons after spinal cord injury vary widely in the literature

Note that these studies employ many different animal species, counting techniques, and histologic techniques to arrive at quite varied rates of cell loss after injury.

Authors	Animal	Lesion	Counting Technique	Histology	Cell loss
Egan et al. Acta Neuropathologica, 1977	Rat Male Wistar	C4 or T13 hemisections	Standard - counts of the caudal 200 microns at 1,3,4,7,14,21 days	Cresyl violet	Equal numbers of neurons counted at days 1 and 21 in both groups (cervical or thoracic)
Goshgarian et al. J Comp Neurol, 1983	Female Osborn-Mendel rats	T1 hemisection and C2 hemisection	Standard - counts from 9-219 days	HRP retrograde labeling and neutral red counterstain	With HRP, 63% loss of RSNs; In total (labeled and non-labeled) 35% loss.
McBride et al. J Neuropath Exp Neurol, 1989	Female Wistar rats	T9 transection	Standard - counts of 910 microns - 10 and 20 weeks post	Fluorogold retrograde prelabeling	No significant difference in surviving rubrospinal neurons at 10 and 20 weeks post axotomy.
Xu and Martin, Exp Neurol, 1990	Female opposums	T5 hemisection	Standard - every 10th section - 30 and 60 days post	Fast blue retrograde prelabeling and cresyl violet	With Fastblue, 25% cell loss With cresyl violet, 20% cell loss with cresyl violet
Theriault and Tator, J Comp Neurol 1994	Female Wistar rats	C7-T2 clip compresion or T2-T3 transection	Standard - every 2nd section	Fluorogold or HRP retrograde labeling after injury	With Fluorogold, 27% cell loss at 8 weeks
Mori et al, Exp Neurol, 1997	Female Sprague-Dawley rats	C3/4 hemisection	Standard - every 3rd section - corrected for cell size - 2 and 4 months post	Fluorogold retrograde prelabeling or Nissl-Myelin staining	35-38% cell loss at 2 and 4 months postaxotomy
Feringa et al. Exp Neurol 1998	Rat Female wistar	T9 transection	Standard - counts of caudal and total RSN at 5, 10, 15, 25, 52 weeks	HRP retrograde tracing at T1 H & E staining	With HRP, no significant difference in caudal or total cells at 15 or 25 weeks. With H&E staining, cell death of 22-41%
Zhou et al, NeuroReport, 1999	C57BL/6J mice and Bcl-2 overexpressing	C4-5 hemisection	Optical disector method 1, 2, or 3 months post axotomy	Cresyl violet staining	45% cell loss at 1, 2, and 3 months
Houle and Ye, Neurosci, 1999	Female Sprague-Dawley rats	C3 Hemisection	Standard - counts on alternating sections (25 microns apart) at 1, 4, and 8 weeks post axotomy	True blue retrograde labeling at injury site	25% cell loss at 8 weeks post axotomy.
Liu P et al., Exp Neurol 2003	Female Wistar rats	C2 lateral funiculus incision or brainstem	Standard - counts of every 3rd section (60 microns apart) at 2, 4, and 10 weeks post axotomy	Cresyl violet	15% cell loss at 4 and 10 weeks post axotomy with C2 lesion; 75% cell loss at 10 weeks post axotomy with brainstem lesion
Liu et al, Exp Neurol 2002	Female Sprague-Dawley rats	C3 hemisection	Optical disector method - counts of caudal half - 1 or 2 months post axotomy	Fluorogold retrograde prelabeling or cresyl violet	45% cell loss at 2 months post axotomy

In my own series of animals at both 6 or 12 months post-axotomy, I also observed the influence of histologic technique in the number of rubrospinal neurons counted. With the use of NeuN immunohistochemistry, equal numbers of neurons were present in the injured and uninjured red nuclei, indicating the absence of cell death after axotomy. With the use of cresyl violet staining, however, there were fewer neurons in the injured red nucleus of animals treated with vehicle solution. This decrease in cell numbers would otherwise imply the retrograde death of rubrospinal neurons, if not for the observation that in animals treated with cell body infusions of BDNF, equal numbers of cresyl violet stained neurons were present in the injured and uninjured red nuclei. With the reversal of neuronal atrophy seen with the application of BDNF, the discrepancy in cell counts between vehicle treated and BDNF treated animals on cresyl violet stained sections suggests that severely atrophic neurons are not detected (and thus deemed as dead) using this histologic technique. The reversal of atrophy with BDNF makes it possible to see these neurons again and thus count them. I indeed observed in slides stained first with NeuN and then with cresyl violet that the visualization of small neurons is much enhanced with NeuN (see Figure 3.6). Interestingly, a recent report by Ruitenberg et al. (Ruitenberg et al., 2004) used cresyl violet staining to evaluate rubrospinal neurons 1 and 6 months after cervical axotomy and treatment with adeno-associated virus vector-mediated BDNF gene transfer. While the cell counting data was not made available in the manuscript, the authors report that the BDNF gene transfer effected a reversal of neuronal atrophy and that similar numbers of rubrospinal neurons were counted on cresyl violet stained sections in both injured and uninjured red nuclei, "indicating the absence of massive lesion-induced death of RSNs". This to some extent provides independent substantiation of my findings of rubrospinal neuronal survival one year after cervical axotomy, as I reported in 2002 (Kwon et al., 2002b).

Retrograde tracers have also been used to quantify cell loss after axotomy, with the tracer injected caudally into the spinal cord to label the neuronal population prior to axotomy, and then the loss of retrogradely labeled neurons representative of cell death. Such was the technique used by Mori et al. (FluoroGold) (Mori et al., 1997), McBride et al. (FluoroGold) (McBride et al., 1989), Houle and Ye (True Blue) (Houle and Ye, 1999), and Feringa et al. (horseradish peroxidase)(Feringa et al., 1988). While the strategy of pre-labeling with a retrograde tracer would seemingly have the advantage of distinctively identifying rubrospinal neurons, the interpretation of subsequent cell counts requires one to make a number of assumptions: 1. that the tracer was reliably taken up and retrogradely transported by most if not all the neurons/axons; 2. that the axotomized neurons will actually maintain the tracer for long periods of time, despite all the metabolic changes that occur after axotomy that culminate in their severe atrophy; and 3. that the retrograde tracer does not leach out and get picked up by glial cells which could be mistaken for atrophic neurons. As for the first assumption, Theriault and Tator demonstrated significantly better retrograde prelabeling of rubrospinal neurons with FluoroGold than with HRP, demonstrating that the efficacy of labeling differs amongst the tracers (Theriault and Tator, 1994). Pilot data from our lab suggests that the uptake by rubrospinal neurons of retrograde tracers applied to the spinal cord can indeed suffer from some variability. With regards to the second assumption, the ability of axotomized rubrospinal neurons to maintain each of the varying retrograde tracers over time is unknown. Novikova et al. compared the retrograde labeling of axotomized spinal motoneurons using five different retrograde tracers, including Fast Blue, FluoroGold, FluoroRuby, MiniRuby, and FluoroEmerald (Novikova et al., 1997). They found that the number of Fast Blue-labeled motoneurons remained constant over time (up to 24 weeks), while a time-dependent decrease in the number of neurons labeled with the other tracers

was observed, related to the degradation and leakage of the tracer. Finally, McBride et al. demonstrated that between 10 and 20 weeks post axotomy, there was increased leakage of FluoroGold from rubrospinal neurons and subsequent uptake by surrounding glial cells (which they assumed to be oligodendrocytes) (McBride et al., 1989).

These results all point to the limitations of using retrograde tracers to determine rubrospinal cell death after axotomy. I felt, however, that even if the rubrospinal axons picked up and retrogradely transported FluoroGold in a somewhat inconsistent manner and then maintained the tracer over time in a similarly inconsistent fashion, its presence could at least be used to outline the general area of the red nucleus where the injured neurons resided. Egan et al. pointed out in cresyl violet stained sections that following axotomy, the atrophy of rubrospinal neurons makes it difficult to identify the boundaries of the red nucleus and thus distinguish this neuronal population from surrounding reticular neurons (Egan et al., 1977). Indeed, I found this to be somewhat problematic in my evaluation of rubrospinal neurons 12 months post-axotomy. Therefore, in my studies of rubrospinal neuronal atrophy and cell number at 2 and 6 months post-axotomy, the prelabeling with FluoroGold greatly assisted in outlining the region of the red nucleus within which to count the descending population of injured, atrophic rubrospinal neurons. Indeed, many atrophic rubrospinal neurons identified on NeuN immunohistochemistry in the injured red nucleus had very faint or absent FluoroGold labeling (see Figures 3.6).

3.5.3. Counting Techniques for Evaluating Neuronal Numbers

My reported findings of rubrospinal survival 12 months after cervical axotomy (Kwon et al., 2002b) and of modest cell death (approximately 13%) in response to a second axotomy 6 months after the original cervical axotomy (Kwon et al., 2002c) are based on the application of stereologic counting methods. In summary, the physical disector technique involves outlining the neurons on one section (the "index" section) and comparing them to neurons on the next adjacent section (the "lookup" section) and excluding those neurons seen in both sections. As stated in the Background chapter, it is well recognized that significant controversy surrounds the subject of optimal counting techniques (Benes and Lange, 2001). It is important to note, however, that while rubrospinal atrophy is almost universally observed after axotomy, and cellular atrophy is known to influence the results of standard counting techniques, almost all of the studies that describe the death of rubrospinal neurons after axotomy do not employ either a stereologic counting method or even a mathematical correction to account for the change in cell size (such as the Abercrombie correction).

The loss of approximately 45% of rubrospinal neurons after cervical axotomy in both mice (Zhou et al., 1999) and rats (Liu et al., 2002) was reported by authors that did apply stereologic counting techniques to the injured red nucleus. In both of these studies, cresyl violet staining was used to identify the rubrospinal neurons, which, based on the compative analysis performed in my experiments, appears to be a less reliable technique than NeuN immunohistochemistry for identifying atrophy neurons. The stereologic counting techniques also differed slightly, in that I have used the physical disector method in my studies, while these studies employed the optical disector (also called the optical fractionator) method. In the optical disector technique, one evaluates a section of tissue through its vertical height by sequentially

moving the plane of focus in small increments through the vertical axis, and evaluating the cells as they come into focus. In essence, the optical disector technique counts the cells that come into focus as one moves vertically through the section in very thin slices (planes of focus), then excludes those cells that are in focus in the last plane of focus of the section, as these would presumably be in focus also in the next adjacent section. The technique is applied typically to small portions of the overall population of interest, and in the papers of Zhuo et al. and Liu et al., for each section the red nucleus was divided into quarters and one quarter randomly examined (Zhou et al., 1999, Liu et al., 2002). The optical disector method is more dependent than the physical disector on mathematical calculations that involve the estimated thickness of the section and the area of the sampling compared to the total area of interest, and to some extent, assumes that the cells are evenly distributed throughout that total area of interest (in this case, the red nucleus). My technique of using the physical disector counts is done at a magnification that allows one to count all of the neurons in the red nucleus on a given section. Given the fact that the neurons are not evenly distributed throughout the nucleus, with the dorsomedial "quadrant" housing the most neurons (particularly as one moves rostrally in the nucleus), this would seemingly provide less error. On the other hand, the optical disector technique is not affected by the small extent of tissue distortion that occurs between two physically separate sections. En balance, I think that the differences in these two stereologic counting techniques account for less of the discrepancy between my results and those of Zhou et al. and Liu et al. than the histologic techniques that I employed, with the NeuN immunohistochemistry facilitating the identification of atrophic, injured rubrospinal neurons.

3.5.4. Rubrospinal Neuronal Atrophy After Axotomy

Regardless of histologic technique (NeuN or cresyl violet staining), I observed statistically significant atrophy of axotomized rubrospinal neurons at 2 months, 6 months, and 12 months post-axotomy. Injured rubrospinal neurons measured approximately 65 to 70% of the cross sectional area of their uninjured counterparts. Such atrophy is consistent with numerous other reports of rubrospinal atrophy in response to axotomy in rats, mice, and opposums (Liu et al., 2002, Egan et al., 1977, Kobayashi et al., 1997, Storer et al., 2003, Wang et al., 1999, Xu and Martin, 1990, Zhou et al., 1999). The most rapid extent of rubrospinal neuronal atrophy begins between 7 and 14 days after cervical axotomy (Tetzlaff et al., 1991, Barron et al., 1989). The application of BDNF to either the spinal cord (Liu et al., 2002, Liu et al., 1999) or to the rubrospinal cell bodies themselves (Fukuoka et al., 1997, Kobayashi et al., 1997, Ruitenberg et al., 2004) acutely after injury has been shown to prevent or reverse this atrophy.

In contrast to the effectiveness of acute BDNF application, I found that rubrospinal atrophy was not reversed by the application of BDNF to the injured cervical spinal cord 2 months post-axotomy. The ineffectiveness of BDNF applied to the spinal cord 2 months post-axotomy is consistent with the recently reported findings of Storer et al, who found that BDNF applied to the spinal cord 1 month post axotomy also did not reverse rubrospinal atrophy (Storer et al., 2003). My experiments at two months post-axotomy used exactly the same dose of BDNF (50 ng/μl) and the same technique of gelfoam application (replacing the gelfoam with freshly soaked gelfoam every 15 minutes) as Storer and colleagues. Additionally, I used two substantially higher concentrations of BDNF (1000 ng/μl and 20,000 ng/μl) to evaluate for a dose dependent effect, and again found no reversal of rubrospinal neuronal atrophy. Tobias et al. applied grafts of BDNF and NT-3 secreting fibroblasts into the cervical spinal cord 6 weeks

after hemisection (Tobias et al., 2003). When comparing the cross sectional area of injured against contralateral uninjured neurons, they reported only a slight reversal of rubrospinal atrophy, from 50.8±3% in control animals to 58.7±2% in neurotrophic factor treated animals. Because the grafts contained a combination of BDNF and NT3, it is difficult to delineate from this study which neurotrophic factor this small increase in cell size should be attributed to. Clearly, my results and those of others indicate that the application of BDNF (even in a range of concentrations) to the injured spinal cord at a "chronic" time point after injury has limited to no appreciable effect on rubrospinal cell size.

While the application of BDNF to the injured spinal cord had limited effect on rubrospinal neuronal size 2 months post-axotomy, application to the rubrospinal cell bodies 12 months after cervical axotomy was associated with a near-complete reversal of neuronal atrophy. My reporting of these observations represented the first description of BDNF administration directly to the cell bodies of rubrospinal neurons in such a chronic injury state (Kwon et al., 2002b). These findings, however, were corroborated recently by Ruitenberg and colleagues with the *in vivo* gene transfer of BDNF into rubrospinal neurons axotomized 18 months prior (Ruitenberg et al., 2004). These authors found that 3 months after virus injection (21 months after the original axotomy), the rubrospinal neurons of animals injected with the BDNF-encoding adeno-associated viral vector had a mean cross sectional area of 284±17 μm^2 compared to uninjured neurons measuring 338±9 μm^2 (representing atrophy of approximately 80 to 85% the size of normal rubrospinal neurons). While the reversal of rubrospinal atrophy was not complete, the size of the chronically injured rubrospinal neurons after BDNF treatment was significantly greater than those of animals that received the control virus ($p < 0.006$).

As neuronal atrophy and its reversal or prevention after axotomy is a common outcome measure for studies of experimental therapeutic interventions, it is worth considering the potential physiologic implications of neuronal atrophy (and its reversal). While neuronal body size in metazoan organisms is determined largely by DNA content and transcriptional activity (Cavalier-Smith, 1978), vertebrate neurons possess generally constant amounts of nuclear DNA (McIlwain, 1991, Pearson et al., 1984), and thus transcriptional activity appears to be the most important determinant of cell size (Pena et al., 2001). In support of this, Sato et al. demonstrated in frog motoneurons that the largest cells contained the largest nuclei, which also possessed the highest transcriptional activity (Sato et al., 1994). The size of non-neuronal cells also appears to correlate with transcriptional activity. Schmidt and Schibler found in cells from the liver, lung, kidney, spleen, and thymus of rats and mice that large cells synthesize more RNA than small cells, and that the ratio between RNA (variable) and DNA (fixed) per cell correlated with cell size (Schmidt and Schibler, 1995).

Given this relationship between transcriptional activity and cell size, it would be reasonable to postulate that the atrophy of rubrospinal neurons after axotomy represents a reduction in their transcriptional activity, while the reversal of neuronal atrophy likely represents an increase in transcriptional activity. Exactly which of the genes whose expression changes after axotomy or after neurotrophic factor application are most influential in determining rubrospinal neuronal cell size is a matter of speculation, although genes for structural cytoskeletal proteins such as neurofilament (Verge et al., 1990) are likely candidates. In support of this proposed relationship in axotomized rubrospinal neurons, Fernandes et al. demonstrated that within the injured red nucleus, the largest rubrospinal neurons also possessed the highest level of GAP-43 and Tα1 tubulin mRNA expression, and were typically the neurons

to have regenerated through a peripheral nerve transplant, thus also receiving trophic support from the transplant itself (Fernandes et al., 1999). In studies that evaluate experimental therapies to promote axonal regeneration after spinal cord injuries, it would therefore seem reasonable to view the prevention of neuronal atrophy or its reversal in the chronic setting as a potentially beneficial outcome.

3.5.5. Dosage Considerations for BDNF

As stated in the introductory section of this chapter, the direct application of neurotrophic factors such as BDNF to the spinal cord injury site is a frequently employed experimental treatment strategy. In general, the exogenous administration of BDNF to the spinal cord injury site has been performed in one of three methods: single application (injection or within gelfoam), via continuous infusion, and by cellular expression through ex vivo or in vivo genetic transfer techniques (Novikova et al., 2002, Sayer et al., 2002, Sharma et al., 2000, Jakeman et al., 1998, Lu et al., 2001, Ankeny et al., 2001, Bamber et al., 2001, Blits et al., 2003). Clearly, there are substantial differences in the actual quantity and mechanism of BDNF administration to the injured spinal cord in these studies. An optimal dose/concentration for exogenously applied BDNF has not yet been established, and in keeping with this, a variety of doses and treatment modalities have in fact been employed. These range from as low as 50 ng/µl in gelfoam soaked sponges as described by Houle and colleagues (Houle and Ye, 1999, Ye and Houle, 1997) to as high as 12,500 ng/µl infused over 28 days through an osmotic minipump as described by Jakeman et al. (Jakeman et al., 1998). Fibroblasts genetically altered to produce BDNF have been measured to produce between 12.8 to 94 ng of BDNF/10^6 cells/24 hours *in vitro*, (Liu et al., 1999, Liu et al., 2002, Tobias et al., 2003) but the actual *in vivo* delivery to the cord over time as the transplant incorporates into host tissue is difficult to determine. The study by Jakeman et al.

employed three different concentrations of BDNF in a 28 day osmotic minipump infusion (4150, 8300, and 12,500 ng/µl, providing a total dose of 50, 100, 150 µg/day). The authors reported increased functional recovery in the two higher concentrations of BDNF, including improved plantar stepping, increased early locomotor recovery, and more consistent forelimb and hindlimb coordination. The anatomic correlate behind such functional recovery is difficult to ascertain, but this study points to an influence of BDNF concentration, despite the fact that even the lowest of these concentrations is likely orders of magnitude higher than what is expressed within the native cord after injury (Ikeda et al., 2001).

From a therapeutic standpoint, the issue of dose is obviously one of particular relevance, and for this reason I chose to evaluate three BDNF concentrations in my study, each concentration separated by an order of magnitude. Conveniently, a 1/20 dilution of my stock BDNF solution (20,000 ng/µl) provided the 1,000 ng/µl concentration of BDNF that Bregman and colleagues had used to supplement a fetal tissue transplant in a over-hemisection model of acute spinal cord injury (Bregman et al., 1997, Bregman et al., 1998) This concentration of BDNF prevented the atrophy of acutely axotomized rubrospinal neurons (at the thoracic level) and promoted growth of serotonergic, noradrenergic, and corticospinal axons within the fetal tissue transplants. A 1/20 dilution of this concentration provided the 50 ng/µl that Houle and colleagues have employed in numerous studies of the rubrospinal system after cervical spinal cord injury (Houle and Ye, 1999, Ye and Houle, 1997). The method of applying the BDNF in gelfoam and then replacing the BDNF-soaked gelfoam sponge with fresh neurotrophic factor every 15 minutes over the first hour was reported to attenuate rubrospinal death after second axotomy performed 4 weeks after initial injury (Houle and Ye, 1999), and so I chose to employ this same regimen and BDNF concentration in my study. I decided against using an osmotic

minipump infusion of BDNF into the spinal cord injury site two months after axotomy as an additional treatment arm for a number of reasons. For one, I felt that the lowest BDNF concentration (50 ng/μl) was likely supra-physiologic in nature. Secondly, BDNF infused directly to the cervical spinal cord through osmotic mini-pumps 12 months after cervical axotomy did not reverse rubrospinal neuronal atrophy in the hands of Nao Kobayashi and Wolfram Tetzlaff (n=3 animals, unpublished data) and thus instigated my subsequent investigations into cell-body treatment at this chronic time point, which I later reported on (Kwon et al., 2002b). Finally, the efficiency of such osmotic minipump infusions over time has been shown to be modest, making it difficult to predict exactly how much trophic factor actually gets delivered (Jones and Tuszynski, 2001).

3.5.6. Administration of BDNF to the Cell Body and to the Injured Spinal Cord

Obviously, one of the most striking observations from this chapter is the difference between the spinal cord and the cell body administration of BDNF in terms of the reversal of rubrospinal neuronal atrophy. While the time frame differed between these two experimental paradigms (2 months post-axotomy for the spinal cord application of BDNF versus 12 months post-axotomy for the cell body application of BDNF), it is probably reasonable to postulate that the effective reversal of neuronal atrophy with cell body application of BDNF at 12 months could have been reproduced at 2 months post-axotomy. In their in vitro study of sympathetic neurons, Toma et al. found that the increased effectiveness of cell body administration of NGF compared to axonal administration was not the result of differences in TrkA receptors, but rather to the spatial localization of the NGF receptor-ligand complex (Toma et al., 1997). Nonetheless, in the absence of effectiveness of the spinal cord application of BDNF at two months post-axotomy I considered whether there were in vivo differences in TrkB receptor

expression between the chronically injured cell bodies and spinal cord. My results of these investigations are described in Chapter 6.

- CHAPTER 4 -

REGENERATION ASSOCIATED GENE EXPRESSION

IN THE CHRONICALLY INJURED RUBROSPINAL SYSTEM

4.1. SUMMARY

The intrinsic ability of neurons to regenerate axons after injury is thought to be closely linked to the coordinated expression of a multitude of genes, collectively termed regeneration associated genes, or RAGs. The failure of CNS neurons to regenerate axons after injury is thought to be in part related to the inability to mount and sustain a sufficient RAG expression response. In this chapter I review the expression of two important RAGs, GAP-43 and $T\alpha 1$ tubulin in rubrospinal neurons two and 12 months after cervical axotomy. Using *in situ* hybridization, I observed that the expression of GAP-43 and of $T\alpha 1$ tubulin was low in injured rubrospinal neurons as compared to the uninjured contralateral control neurons at both the two and 12 month time points. The application of BDNF to the spinal cord injury site 2 months post-axotomy did not stimulate an increase in GAP-43 or $T\alpha 1$ tubulin expression over that which was induced by the refreshment injury performed during BDNF application (Kwon et al., 2004a). However, the application of BDNF directly to the rubrospinal cell bodies 12 months post-axotomy did stimulate an increase in GAP-43 and $T\alpha 1$ tubulin expression (Kwon et al., 2002b). These results suggest that the chronically injured rubrospinal neurons maintain responsiveness to BDNF at the level of the cell body but not at the injured spinal cord.

4.2. INTRODUCTION

As discussed in the Background chapter, the cell body response to axotomy is felt to be an element of the neuron's axonal regenerative competence after injury. That neurons within the CNS possess an ability to extend their axons into the permissive environment of peripheral nerves after injury was demonstrated first by Tello and Ramon y Cajal almost a century ago (Ramon y Cajal S, 1928), and reiterated with more contemporary methods in work performed by Richardson, Aguayo, and colleagues (Richardson et al., 1980). The somewhat limited nature of this intrinsic regenerative nature in mature CNS neurons, however, lies in stark contrast to the fairly robust regenerative abilities of motor and sensory neurons projecting through the peripheral nervous system. While the injury environments encountered by neurons of the CNS and PNS are clearly different in their permissiveness to regeneration (the latter being far more conducive), it is also apparent that CNS neurons fail to mount or to maintain some of the gene expression changes thought to be necessary for a peripheral regenerative response (Fernandes et al., 1999, Tetzlaff and Bisby, 1990). The genes thought to be related to the regenerative response encode a wide spectrum of proteins, including transcription factors, cytoskeletal proteins, growth cone proteins, and cell adhesion molecules (reviewed by Fernandes and Tetzlaff, 2000). The identification of these proteins and their enhancement may augment the regenerative capacity of the injured neuron and thus represents a potential therapeutic target for spinal cord repair (reviewed by Plunet et al., 2002).

While a large number of genes likely undergo changes after axotomy, those that have been associated with axonal growth propensity have been termed "regeneration associated genes", or RAGs (Plunet et al., 2002). The failure of neurons within the CNS to incite a sufficient and sustained up-regulation of RAGs is considered to embody their "intrinsic" inability

to regenerate after axotomy. The two RAGs that our laboratory have studied most extensively are GAP-43 and Tα1 tubulin, the descriptions of which are detailed in the Background chapter, Sections 1.5.4 and 1.5.5.

In brief, the importance of RAG expression in axonal regeneration after CNS injury is implied by the correlation between their upregulation and the regenerative propensity of neurons both in the CNS and the PNS. For example, rubrospinal neurons of the CNS transiently upregulate GAP-43 and tubulins after cervical axotomy but not thoracic axotomy, and correspondingly regenerate axons into permissive peripheral nerve transplants engrafted into the cervical but not thoracic spinal cord (Tetzlaff et al., 1991, Tetzlaff et al., 1994). Fernandes et al. observed on a cell-by-cell basis that those rubrospinal neurons that were capable of regenerating through a peripheral nerve transplant after cervical axotomy were those that upregulated GAP-43 expression (Fernandes et al., 1999). Peripheral axotomy of DRG neurons increases GAP-43 expression while central axotomy does not (Chong et al., 1994), and correspondingly the regeneration of the central axons is promoted only by a preceding peripheral axotomy (the "conditioning lesion") (Richardson and Issa, 1984, Neumann and Woolf, 1999), demonstrating the correlation between this gene and axonal regeneration for neurons of the PNS. A relationship between GAP-43 expression and axonal elongation has been demonstrated across numerous species, such as the optic nerves of fish, toads, and rabbits (reviewed by Skene, 1989). As I am interested in evaluating measures that reflect the regenerative capacity of rubrospinal neurons in the chronic injury state, I examined GAP-43 and Tα1 tubulin mRNA expression using *in situ* hybridization.

4.3. OVERVIEW OF EXPERIMENTAL QUESTIONS AND HYPOTHESES

In this chapter, I evaluated the mRNA expression of GAP-43 and Tα1 tubulin in rubrospinal neurons to test the following hypotheses:

1.   Investigators who have applied BDNF directly to the injured spinal cord have done so in various doses/concentrations.   Given that the expression of GAP-43 and Tα1 tubulin decreases in rubrospinal neurons after axotomy (Kobayashi et al., 1997, Storer and Houle, 2003), and that BDNF administration to the spinal cord injury site appears to diminish in its ability to increase RAG expression (Storer et al., 2003) and promote a regenerative response after a delay in intervention (Tobias et al., 2003), *I hypothesized that the loss of effectiveness in promoting RAG expression in the "chronic" setting might be related to providing the appropriate dose/concentration of the neurotrophic factor.*

To test this hypothesis, animals underwent a cervical axotomy, then two months later, BDNF in 3 exponentially increasing concentrations (50, 1000, and 20,000 ng/µl) was applied within gelfoam to the refreshed injury site (in addition to PBS-treated controls).   *In situ* hybridization for GAP-43 and Tα1 tubulin mRNA was then performed on injured and uninjured rubrospinal neurons.

2. Given that the infusion of BDNF into the vicinity of the rubrospinal cell bodies was shown to promote RAG expression in acutely injured rubrospinal neurons (Kobayashi et al., 1997), *I hypothesized that the cell-body administration of BDNF could promote GAP-43 and Tα1 tubulin expression when applied chronically after cervical axotomy.*

To test this hypothesis, animals underwent a cervical axotomy, then twelve months later, BDNF was infused through an osmotic minipump cannula stereotactically placed just lateral to

the red nucleus.   *In situ* hybridization for GAP-43 and Tα1 tubulin mRNA was then performed

on injured and uninjured rubrospinal neurons.

4.4 RESULTS

4.4.1. RAG Expression Two Months Post Axotomy with BDNF Applied to Cord

Two months after cervical axotomy, the lesion site was re-exposed and extended rostrally approximately 1.0 mm (the "refreshment injury"). Gelfoam soaked with PBS or with one of three concentrations of BDNF (50, 1000, 20,000 ng/μl) was inserted into the lesion. *In situ* hybridization was performed on 20 μm thick sections through the caudal half of the red nucleus. Counterstaining of rubrospinal neurons was performed with ethidium bromide.

The *in situ* hybridization signal in the injured neurons was expressed as a ratio of that in the uninjured neurons. For GAP-43 mRNA expression (n=7 animals), this ratio was on average 1.6 to 1.8 in the injured neurons. This upregulation in GAP-43 expression that was similar amongst all four experimental groups (PBS, Low, Medium, and High Concentration BDNF). (Figure 4.1) For Tα1 tubulin expression (n=8 animals), this ratio was, on average, close to 1.0 and again was not noticeably different amongst the four groups. (Figure 4.2) Compared to the well-recognized decrease in the expression of these genes in the cervically axotomized rubrospinal system (Kobayashi et al., 1997, Storer and Houle, 2003), these values represent an increase in gene expression that is similar in all groups, suggesting that this may be more a result of the refreshment injury (ie. second axotomy) than the BDNF treatment.

Figure 4.1. GAP-43 expression in the injured red nucleus is increased compared to uninjured after spinal cord application of BDNF and PBS two months after axotomy

Two months after cervical axotomy and spinal cord application of PBS or BDNF (in one of three concentrations) GAP43 expression appeared in the injured rubrospinal neurons to increase above contralateral uninjured neurons (approximately 1.7-1.8 times). No noticeable difference between PBS and BDNF-treated animals was observed, suggesting that a BDNF effect was lacking and that the increase in GAP-43 expression was related to the refreshment injury. The darkfield image of the ISH silver grains is represented in red, while the sections are counterstained with ethidium bromide and represented in green (false colours). Scale Bar, 50 µm
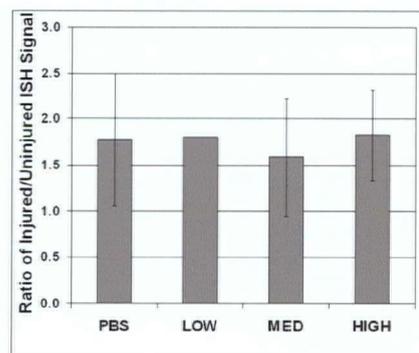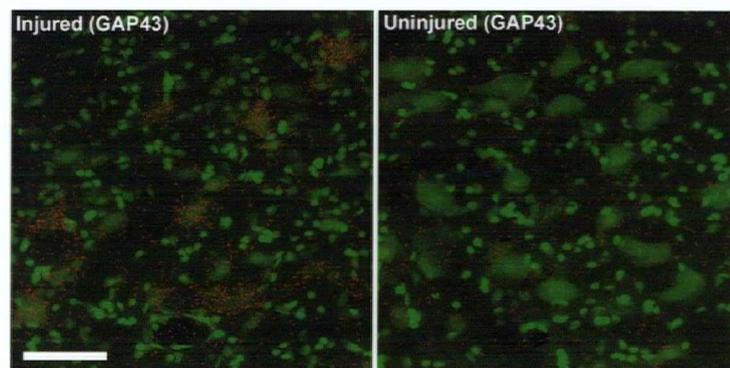
Figure 4.2. Tα1 tubulin expression in the injured red nucleus is increased compared to uninjured after spinal cord application of BDNF and PBS two months after axotomy

Two months after cervical axotomy and spinal cord application of PBS or BDNF (in one of three concentrations) Tα1 tubulin expression appeared to increase to levels similar to the contralateral rubrospinal neurons in all animals tested, with again, no noticeable difference between PBS and BDNF treated animals. This suggests that the gene expression changes are related to the refreshment injury, and not the application of this neurotrophic factor. The darkfield image of the ISH silver grains is represented in red, while the sections are counterstained with ethidium bromide and represented in green (false colours). Scale Bar, 50 μm
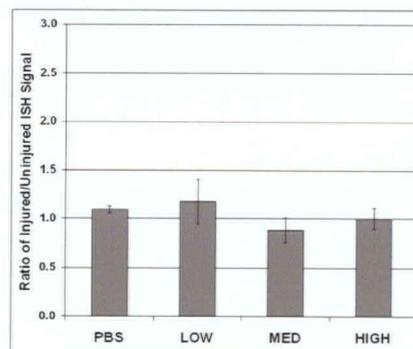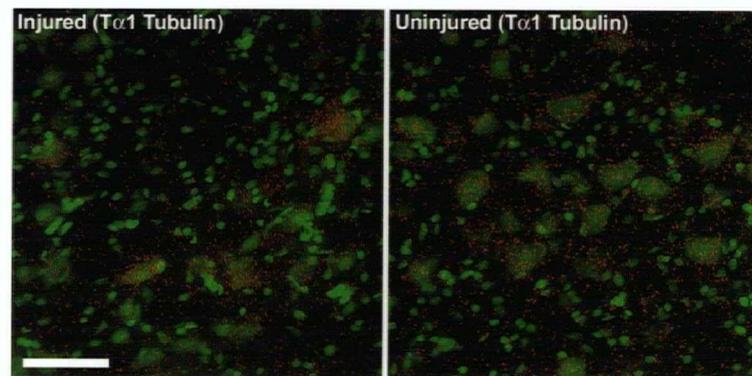
4.4.2. RAG Expression Two Months Post Axotomy with BDNF Applied to Brainstem

12 months after cervical axotomy, an osmotic mini-pump cannula was stereotactically inserted into the brainstem to infuse either BDNF or its vehicle solution alone into the vicinity of the rubrospinal neurons. *In situ* hybridization was performed on 20 μm thick sections through the caudal half of the red nucleus. Quantification of these findings was not performed, as these experiments were conducted simply to identify whether rubrospinal neurons this long after injury (one year) were still able to mount some form of a regenerative gene expression response when treated with BDNF.

Consistent with the findings at 2 months post-injury, 12 months after cervical axotomy the ISH signal for GAP-43 and Tα1 tubulin in injured rubrospinal neurons was low in the control animals treated with vehicle solution alone. (Figures 4.3 and 4.4) For GAP-43, the ISH signal in the injured red nucleus was comparable to the low ISH signal of the uninjured neurons, while for Tα1 tubulin, the ISH in the injured red nucleus appeared lower than that of the uninjured red nucleus. Given what I found at 2 months post-axotomy and what has been described about the acute upregulation of these genes after cervical axotomy, it is reasonable to postulate that these 12 month chronically injured neurons similarly mounted a transient regenerative response after axotomy that abated by 4-8 weeks post-injury and remained quiescent over the next 10 to 11 months. BDNF infusion at 12 months post-axotomy stimulated an increase in ISH signal for GAP-43 and for Tα1 tubulin. For GAP-43, this effect was observed in some animals treated at 18 months post-injury.

Figure 4.3. BDNF infusion to the red nucleus 12 and even 18 months after cervical axotomy promotes an increase in GAP-43 expression

Twelve months after cervical axotomy, the cell body application of BDNF increased GAP-43 expression in the injured red nucleus to levels greater than that observed in the uninjured red nucleus. Note that the effect was achieved even in animals that were 18 months post-axotomy (bottom row). The infusion of the vehicle solution alone did not promote GAP-43 expression. Scale bar, 50 μm.
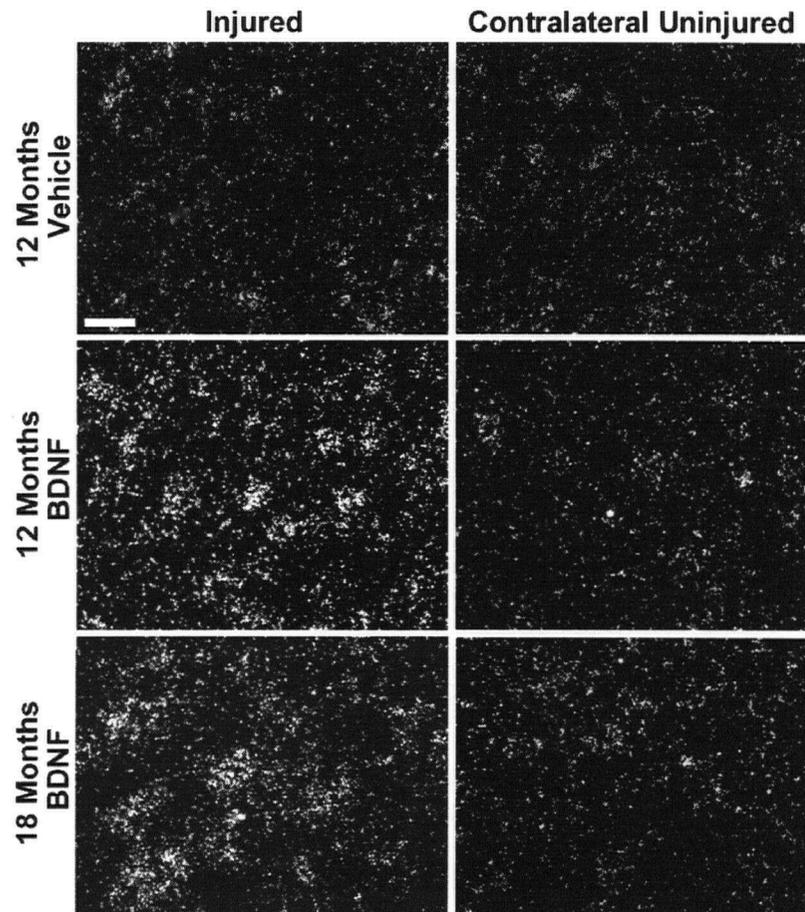
Figure 4.4. BDNF infusion to the red nucleus 12 months after cervical axotomy promotes an increase in Tα1 tubulin expression

Twelve months after cervical axotomy, the cell body application of BDNF increased Tα1 tubulin expression in the injured red nucleus, restoring it to levels comparable to that of the uninjured red nucleus. The infusion of the vehicle solution alone did not promote Tα1 tubulin expression.   Scale bar, 50 μm.
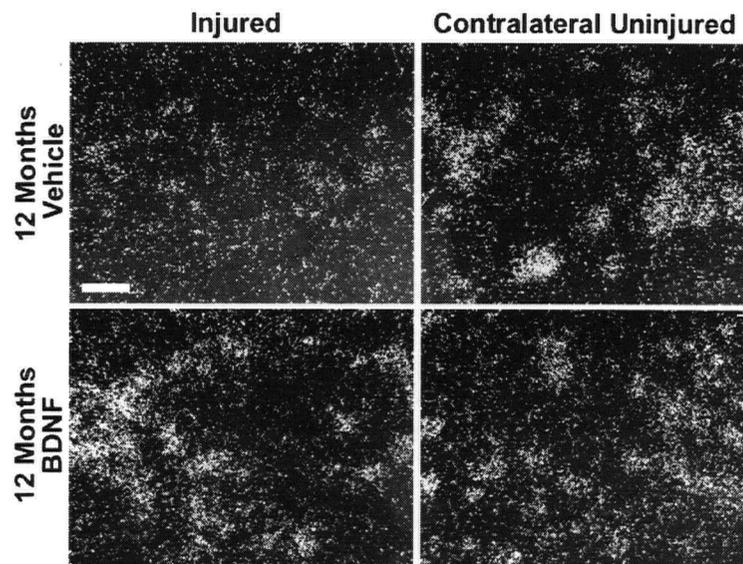
4.5 DISCUSSION

4.5.1. Chapter Summary

Similar to Chapter 3, this chapter reviews the effects of BDNF treatment at two different time points and in two different modalities: spinal cord application two months post-axotomy, and cell body application 12 months post-axotomy. My observations that BDNF application to the injured spinal cord did not stimulate either GAP-43 or Tα1 tubulin expression over that of the refreshment injury at two months post-axotomy suggest that the rubrospinal axons were not responsive to spinal cord application of this neurotrophic factor, and is consistent with the failure to reverse neuronal atrophy with this mode of application (Chapter 3). My observations that BDNF application to brainstem stimulates GAP-43 and Tα1 tubulin expression over that of vehicle application suggest that the rubrospinal neuronal bodies remain responsive to the application of BDNF 12 months after axotomy.

To put the findings of this chapter into perspective, it is important to review the changes in GAP-43 and Tα1 tubulin expression that occur over time after cervical axotomy. It has been repeatedly shown that GAP-43 and Tα1 tubulin expression in rubrospinal neurons increases acutely after cervical axotomy but then diminishes over the subsequent weeks (Fernandes et al., 1999, Kobayashi et al., 1997, Tetzlaff et al., 1991). More recently, using a similar cervical axotomy model, Storer and Houle demonstrated an acute rise in rubrospinal expression of GAP-43 within 1 day of injury, and a further increase of this gene expression and that of βII tubulin by 3 days post-axotomy (Storer and Houle, 2003). However, by 28 days post-axotomy, GAP-43 expression had returned to original baseline expression levels, and βII tubulin expression had fallen more than 20% below original levels. This early increase in RAG expression within rubrospinal neurons likely represents an initial regenerative attempt by these CNS neurons, while

the inability to sustain this response over the ensuing weeks is reflective of the axonal regeneration failure observed within the CNS after injury.

4.5.2. RAG Expression In Response to Refreshment Injury and Spinal Cord Application of BDNF

Two months post-axotomy I observed an increase in GAP-43 and Tα1 tubulin expression in all animals undergoing a refreshment injury and gelfoam application with PBS or BDNF. With no apparent differences in the GAP-43 and Tα1 tubulin expression response observed amongst these animals (ie. PBS versus BDNF), it would appear that chronically injured rubrospinal neurons do not initiate changes in the expression of these RAGS specifically in response to BDNF applied to the axons within the injured spinal cord. Rather, the change in RAG expression observed in the injured red nucleus is likely the result of the refreshment injury itself that was performed as part of the insertion of the BDNF-soaked and PBS-soaked gelfoam.

Indeed, the role of the refreshment injury is suggested by Storer and Houle in their observations of the red nucleus following cervical re-axotomy 4 weeks after the initial injury (Storer and Houle, 2003). They reported that the second injury initiated another rise in GAP-43 and βII tubulin expression that was more rapid than the increase in their expression after the initial injury. In the case of GAP-43, the magnitude of this increase in mRNA expression after a second axotomy was even greater than the magnitude of the increase after the first axotomy. The increase I observed in GAP-43 expression amongst all the animals (PBS and BDNF) to approximately 1.8 times that of uninjured rubrospinal neurons is similar to the results of Houle and colleagues who reported an increase of at least 2.0 times that of uninjured (Storer and Houle, 2003). Similarly for Tα1 tubulin, with a level of expression falling below that of uninjured by as early as 14 days post axotomy (Kobayashi et al., 1997), the reported expression in my current

study of approximately 1.0 times that of uninjured in the two month chronic state likely represents an acute upregulation due to the second axotomy.

While the implication from the findings from Storer and Houle at 4 weeks post-axotomy and my observations at 2 months post-axotomy is that chronically injured rubrospinal neurons retain the ability to upregulate the expression of certain RAGs in response to another injury, what remains somewhat unanswered is how much of the gene expression changes are attributable to neurons that are axotomized *again* versus those that terminated just rostral to the initial injury and are axotomized *for the first time* during the refreshment injury. My analysis of the red nucleus is performed only on the caudal half which is predominantly the lumbar-projecting region of the nucleus, so it would be anticipated that most of the neurons evaluated were injured during the original axotomy and thus re-injured during the refreshment injury.

The method by which the quantification of ISH signal was performed warrants some discussion as it pertains to the interpretation of the results. The silver grains from the darkfield images were overlaid upon the ethidium bromide counterstained rubrospinal neurons which were outlined using SigmaScan Pro ImageAnalysis 5.0 Software (Systat Software, Inc, Point Richmond, CA). The extent to which each neuron is filled with silver grains is then calculated by the SigmaScan software and converted into an Excel spreadsheet where it is averaged across the neurons that were outlined. This average (minus background ISH signal) from the injured neurons is then compared against the similarly derived average (minus background ISH signal) from the contralateral uninjured neurons. While the algorithm is conceptually simple, it does not account for differences in neuronal cross sectional area. Given that the cross sectional area of the rubrospinal neurons was quite uniform across all four groups of animals (PBS, Low, Medium, and High Dose BDNF), as described in Chapter 3, I feel that the final interpretation of

this semi-quantitative analysis is valid and meaningful. Had there been substantial differences between the mean cross sectional areas between treatment groups (for example, BDNF significantly reversing neuronal atrophy), then a more sophisticated quantification algorithm that accounted for the size of each neuron would be required.

Also consistent with my *in situ* hybridization data and the ineffectiveness of BDNF applied to the spinal cord at two months post-axotomy, Storer et al. found BDNF to be ineffective at promoting GAP-43 and βII tubulin expression within the red nucleus when applying this neurotrophic factor to the spinal cord 4 weeks after cervical hemisection (Storer et al., 2003). The injury model (unilateral rubrospinal tract disruption), refreshment injury, and mode of BDNF application (gelfoam soaked in 50 ng/μl BDNF) were identical between our studies, although my BDNF intervention occurred at 2 months rather than 4 weeks post-axotomy. Of note, Storer et al. observed that while BDNF did not stimulate GAP-43 and βII tubulin expression in rubrospinal neurons injured 4 weeks previously, the application of 10 ng/μl GDNF did (Storer et al., 2003). This demonstrates that the failure of rubrospinal neurons to initiate RAG expression changes was not solely a function of the chronically axotomized state, but rather a neurotrophic-specific phenomenon. The potential role of TrkB receptor expression in this lack of BDNF effect is explored in Chapter 6.

4.5.3. RAG Expression In Response to Cell Body Application of BDNF

I observed an increase in GAP-43 and Tα1 tubulin mRNA expression in rubrospinal neurons injured 12 months previously when BDNF was applied directly to the cell bodies. These observations suggest a retained responsiveness of the injured red nucleus to cell-body application of this neurotrophic factor. The small numbers of animals, however (n=7) make it difficult to perform a truly quantitative comparison between the experimental groups however. Kobayashi

et al. reported that cell body application of BDNF after acute injury promoted the expression of these genes (Kobayashi et al., 1997). The demonstration of RAG expression with cell body application of BDNF 12 months after injury had not been described prior to my reporting of this in 2002 (Kwon et al., 2002b). The upregulation of GAP-43 and Tα1 tubulin expression in these chronically injured neurons is consistent with the reversal of their neuronal atrophy (Chapter 3) and the increased regeneration into peripheral nerve transplants (Chapter 5).

4.5.4. RAG Expression and the Promotion of Axonal Regeneration

While I specifically examined GAP-43 and Tα1 tubulin within the rubrospinal system, I recognize that these are only a fraction of the molecular changes that likely occur in response to axotomy. Our current appreciation of the full extent of this battery of gene expression changes and how they interact with each other to ultimately produce axonal outgrowth is fairly rudimentary, although micro-array technology will likely accelerate our understanding of this in the future.

GAP-43 is a growth associated protein localized to the growth cone where, by virtue of its interactions with calmodulin, PI(4,5)P$_2$, G proteins, and the actin cytoskeleton, it influences axonal growth and synaptic plasticity (reviewed by Benowitz and Routtenberg, 1997 and Caroni, 2001). The correlation between increased GAP-43 expression and axonal growth and sprouting has made GAP-43 a commonly used indicator of the neuronal growth propensity (reviewd by (Caroni, 1997) and (Skene, 1989)). One should be aware, however, that there do exist examples in which GAP-43 was neither sufficient (Buffo et al., 1997) nor necessary (Strittmatter et al., 1995) for axonal growth. Bisby and colleagues demonstrated that even without a noticeable increase in GAP-43 expression, mouse motoneurons were capable of initiating collateral sprouting in response to muscle inactivity or partial muscle denervation (Bisby et al., 1996).

Andersen and Schreyer demonstrated that axonal regeneration not only occurred from DRG neurons expressing GAP-43 but also from DRG neurons devoid of GAP-43 immunoreactivity (Andersen and Schreyer, 1999). Axonal regeneration, however, did not occur uniformly, with rapid growth observed only from those neurons constituitively expressing GAP-43. These results imply that while GAP-43 may not be an absolute requirement for axonal regeneration within this neuronal system, its expression is correlated with the ability to promote rapid axonal growth. Bomze at al. demonstrated *in vivo* that the concomitant over-expression of both GAP-43 and CAP-23 (a related growth cone protein) resulted in significant regeneration of the central DRG axons into peripheral nerve transplants, while this failed with the over-expression of each gene alone (Bomze et al., 2001). Still, the most extensive regeneration was observed after a conditioning peripheral axotomy, indicating that over-expression of only two genes (GAP-43 and CAP-23) does not completely substitute for the coordinated expression of a multitude of RAGs that are stimulated by the conditioning peripheral nerve lesion.

The results of these above studies illustrate that axonal growth can differ both in its nature (eg. collateral sprouting versus long distance regeneration) and its rate (eg. slow versus rapid growth), and that cell body influences these differences in part by the pattern of RAG expression. This was perhaps most eloquently demonstrated by Smith and Skene, who found that the constituitive gene expression of mature DRG neurons facilitated "arborizing" neurite outgrowth, but changes in gene transcription induced by peripheral axotomy could "switch" the neurons into promoting "elongated" axonal outgrowth instead (Smith and Skene, 1997). As stated earlier, such a peripheral axotomy is known to initiate important changes in RAG expression. Conceptually, one of the obvious therapeutic goals for "axonal regeneration strategies" in the setting of spinal cord injury is to promote the long-distance growth of axons

across the injury site to re-innervate distal targets. It should not, however, be overlooked that other forms of axonal regeneration such as the "arborizing" neurite outgrowth illustrated by Smith and Skene (Smith and Skene, 1997) or the collateral sprouting illustrated by Bisby et al (Bisby et al., 1996) may also play an important role in enhancing local plasticity at the spinal cord injury site. The genetic mechanism underlying these forms of growth therefore also carry some relevance. As such, further characterization of RAG expression within CNS neurons after injury and the elucidation of mechanisms by which to stimulate the expression of those genes necessary for axonal growth of all forms will be important for the future development of therapeutic interventions.

- CHAPTER 5 -

AXONAL REGENERATION OF

CHRONICALLY INJURED RUBROSPINAL NEURONS

5.1. SUMMARY

While rubrospinal neuronal survival and size (Chapter 3) and RAG expression (Chapter 4) are thought to be important elements of the intrinsic regenerative competence, ultimately one is most interested in examining whether axonal regeneration is possible in the chronic injury setting. BDNF was applied to the spinal cord 2 months after cervical axotomy in 3 different doses, and an autologous segment of peripheral nerve was transplanted into the spinal cord injury site. Alternatively, BDNF was infused into the vicinity of the rubrospinal neuronal cell bodies 12 months after cervical axotomy and a peripheral nerve autograft was again transplanted. I found that BDNF application to the spinal cord did not promote axonal regeneration from chronically injured rubrospinal neurons two months after cervical axotomy (Kwon et al., 2004a). However, with cell body application of BDNF as late as 12 months after injury, I observed a significant increase in chronically injured rubrospinal neurons that regenerated axons into this peripheral nerve transplant compared to PBS-treated control animals (Kwon et al., 2002b). These findings suggest that in the chronic injury state, rubrospinal neurons are capable of promoting axonal regeneration in response to BDNF applied directly to the cell bodies, but not to their injured axons within the spinal cord.

5.2. INTRODUCTION

Outside of the efforts to spare spinal cord tissue with neuroprotective therapies from secondary injury, it has long been felt that to effect an improvement in neurologic function in patients with spinal cord injuries (particularly those whose injuries occurred long ago), it would be essential to have axons that are disrupted at the injury site regenerate across it and reconnect with distal targets. This remains the ultimate objective for axonal regeneration strategies, and, from a conceptual point of view, will likely need to be met to some extent if we are to achieve the goal of restoring individuals with spinal cord injuries to full physical function in the future. The development of therapies to promote such axonal growth continues to be a major focus of neuroscientific attention.

As stated in the Background chapter, there are numerous strategies that have been explored to promote axonal regeneration within the injured spinal cord. The challenge is largely two-fold: one being the failure to mount and sustain a sufficient RAG expression response (discussed in detail in Chapter 4), and the other being elements within the injured spinal cord that impede axonal growth, thus establishing a non-permissive environment. It is widely believed that the optimal therapeutic result will be achieved by combinatorial interventions that address both of these challenges. As such, in this chapter I have combined the use of the neurotrophic factor BDNF (conceptually to augment the intrinsic regenerative competence) with the local transplantation of autogenous peripheral nerve grafts (conceptually to provide a permissive environment) in order to evaluate axonal regeneration of chronically injured rubrospinal neurons.

5.2.1. Neurotrophic Factors and Axonal Regeneration

Because of their widespread effects within the nervous system, particularly with respect to neuronal survival and axonal growth, neurotrophic factors have represented an appealing therapeutic strategy for spinal cord injury. Numerous methods of delivering neurotrophic factors to the injured spinal cord have been established, including direct injection, within gelfoam, continuous infusion, and gene therapy approaches (both *ex vivo* and *in vivo*), the latter aimed at promoting the more prolonged delivery of neurotrophic factors within the CNS. The effectiveness of neurotrophic factor application as a therapeutic strategy relies heavily upon the bioavailability of the protein and the presence of the appropriate receptors on the target tissue.

The exogenous application of BDNF to the injured spinal cord has been evaluated by numerous investigators in the past. Particularly relevant to this chapter on rubrospinal axonal regeneration, the study of Liu and colleagues reported long-distance regeneration of rubrospinal axons across a cervical hemisection lesion with the acute transplantation of fibroblasts genetically modified to secrete BDNF (Liu et al., 1999). The authors also demonstrate anterogradely labeled rubrospinal axons crossing through the fibroblast graft and re-entering the host spinal cord distal to the injury site, where some fibers even re-entered the grey matter (possibly contributing to the observed improvements in motor function). In contrast, however, the same BDNF-fibroblast transplantation performed 6 weeks after cervical hemisection did not promote rubrospinal axonal regeneration through or distal to the transplant, nor did it promote significant motor recovery (Tobias et al., 2003).

5.2.2. Modifying the Inhibitory Environment of the Injured CNS

While neurotrophic factors are thought to enhance the neuron's ability to regenerate its injured axon (either directly by acting upon the neuron and its axon or indirectly by acting upon surrounding cells that in turn support axonal growth), the other principal strategy to promote axonal regeneration within the injured CNS is to provide the injured axons with a more permissive environment for growth. Conceptually this can be achieved with cellular transplantation strategies that "bridge" the injury (Murray, 2004), and by therapies that neutralize the inhibitory elements within the injured CNS, which are largely related to the glial scar and to CNS myelin (reviewed by Silver and Miller, 2004 and Grados-Munro and Fournier, 2003).

Amongst the various cellular substrates available for transplantation, the use of peripheral nerve grafts and their Schwann cells have been extensively studied. The transplantation of peripheral nerve grafts into the spinal cord was rationalized by the recognition that axonal regeneration of PNS neurons occurred readily within a presumably permissive Schwann cell environment. Of note, the important study of Richardson and Aguayo over two decades ago that illustrated the long-denied regenerative ability of CNS neurons employed a peripheral nerve graft implanted into the spinal cord (Richardson et al., 1980b). Since then, numerous authors have demonstrated the regeneration of CNS axons into peripheral nerve transplants or Schwann cell grafts applied acutely into the injury site (reviewed by Bunge, 2000). However, similar to the decline in effectiveness seen during the chronic injury setting with BDNF-secreting fibroblasts (Tobias et al., 2003), axonal regeneration into peripheral nerve grafts transplanted with a 3 to 4 week delay after injury has also not been met with success (Decherchi and Gauthier, 2000, Houle, 1991).

5.3. OVERVIEW OF EXPERIMENTAL QUESTIONS AND HYPOTHESES

In this chapter, I evaluated the regeneration of rubrospinal axons into peripheral nerve transplants to test the following hypotheses:

1. Given that BDNF administration to the spinal cord injury site appears to diminish in its ability to promote axonal regeneration after a delay in intervention (Tobias et al., 2003), and that various doses/concentrations of BDNF have been applied to the injured spinal cord, *I hypothesized that the loss of effectiveness in promoting axonal regeneration might be related to providing the appropriate dose/concentration of the neurotrophic factor.*

To test this hypothesis, animals underwent a cervical axotomy, then two months later, BDNF in 3 exponentially increasing concentrations (50, 1000, and 20,000 ng/μl) was applied within gelfoam to the refreshed injury site (in addition to PBS-treated controls). A pre-degenerated autologous nerve graft was then transplanted into the injury site, and the extent of axonal regeneration of both chronically and acutely injured rubrospinal neurons into the nerve graft was evaluated with retrograde tracers.

2. Given that the infusion of BDNF into the vicinity of the rubrospinal cell bodies was shown to promote axonal regeneration of acutely injured rubrospinal neurons into peripheral nerve transplants (Kobayashi et al., 1997), *I hypothesized that the cell-body administration of BDNF could promote the axonal regeneration of chronically injured rubrospinal neurons.*

To test this hypothesis, animals underwent a cervical axotomy, then twelve months later, BDNF was infused through an osmotic minipump cannula stereotactically placed just lateral to the red nucleus. A pre-degenerated autologous nerve graft was then transplanted into the injury

site, and the extent of axonal regeneration of both chronically and acutely injured rubrospinal

neurons into the nerve graft was evaluated with retrograde tracers.

5.4. RESULTS

5.4.1. Axonal Regeneration Two Months Post-Axotomy with BDNF Applied to Cord

The thoracolumbar descending population of rubrospinal neurons was retrogradely labeled with FluoroGold injected at T1. Seven days later, the dorsolateral aspect of the cervical spinal cord at C3/4 was cut to unilaterally sever the rubrospinal tract. Two months later, the cervical axotomy site was refreshed, BDNF or PBS applied in gelfoam (as described in detail in the methods section) and a 35 mm segment of predegenerated tibial nerve was inserted into the spinal cord injury site and secured with 10-0 prolene sutures. Two months later, BDA was applied to the free end of the nerve graft to retrogradely label neurons whose axons grew to the end of the peripheral nerve transplant. A week later the animals were sacrificed and counts of double-labeled (chronically injured) and single-labeled (acutely injured) neurons were performed.

Regeneration of Chronically Injured Neurons – Double Labeled

After intervention with PBS or BDNF at the injury site and peripheral nerve transplantation, the number of double-labeled rubrospinal neurons was counted to quantify neurons that were injured two months previously (labeled with FluoroGold), and that were able to extend an axon to the tip of the nerve transplant (to obtain the BDA). (Figure 5.1) The numbers of double labeled neurons in each group were 6.2±1.5, 6.9±2.6, 10.5±5.5, and 10.5±3.1 for the PBS, Low, Medium, and High BDNF groups respectively. (Figure 5.2) There was no significant difference in double labeled cells between the groups on one way ANOVA (p=0.71). Even pooling all of the BDNF-treated animals and comparing the number of double-labeled cells (9.25±2.1; n=20) with that of the PBS treated animals (6.2±1.5; n=6) did not reveal a significant difference (p=0.44). The data suggested that perhaps there was a BDNF effect with the higher

doses, but when pooling the medium and high dose BDNF groups (10.5±2.9; n=13) and comparing this to the PBS alone (6.2±1.5, n=6) or to the pooled PBS and low dose BDNF group (6.5±1.52; n=13) there were no significant differences (p=0.17 and p=0.12 respectively). This indicates that BDNF, at any of the three doses, did not promote axonal regeneration of chronically injured rubrospinal neurons two months after axotomy when applied to the injury site.

Regeneration of Acutely Injured Neurons – Single Labeled

The refreshment injury which extends the spinal cord lesion rostrally is likely to injure *for the first time* a number of axons that terminated just proximal to the original injury (ie. above C3/4). Such axons would not have picked up the FluoroGold injection at T1, and thus, if successfully regenerating through the transplanted peripheral nerve graft, would only be single labeled with the BDA. The single-labeled neurons were counted, in part to determine if BDNF had an acute effect on the regenerative capacity of these neurons, and in part to confirm that the microsurgical placement of the nerve graft was such that at least some form of axonal regeneration was possible through the cord-graft interface.

The numbers of single labeled neurons in each group were 13±6.0, 22.1±5.0, 29.3±5.2, and 29.0±3.4 for the PBS, Low, Medium, and High BDNF groups respectively. There was no significant difference in single-labeled cells between the four groups on one way ANOVA (p=0.10). However, when pooling the BDNF-treated animals and comparing the number of single-labeled cells (26.7±2.6; n=20) with that of the PBS treated animals (13.0±6.0; n=6) there was a statistically significant difference (p=0.01). (Figure 5.2) This would suggest that BDNF did promote the axonal regeneration of acutely injured rubrospinal axons, in keeping with the data of Liu et al. in their acute transplantation of BDNF-secreting fibroblasts (Liu et al., 1999).

There was no significant difference in the number of single-labeled rubrospinal neurons between the three BDNF groups on one way ANOVA (p=0.46).

Figure 5.1.   Double labeling paradigm (FluoroGold and BDA) to evaluate regeneration into peripheral nerve transplants two months after cervical injury.

FluoroGold labeling of descending rubrospinal neurons (A) is represented here in the green channel (false colour) for illustrative purposes.   Neurons retrogradely labeled with BDA from extending axons through the peripheral nerve transplant are shown in (B).   Overlaying these images (C) demonstrates yellow double-labeled neurons (arrows), indicating that they were both injured 2 months prior to implantation and then successfully regenerated through the graft. Note also the presence of single labeled BDA neurons (asterisks) – such neurons likely had axons that ended just proximal to the original injury and then were axotomized for the first time with the refreshment injury. (Scale Bar = 50 μm)
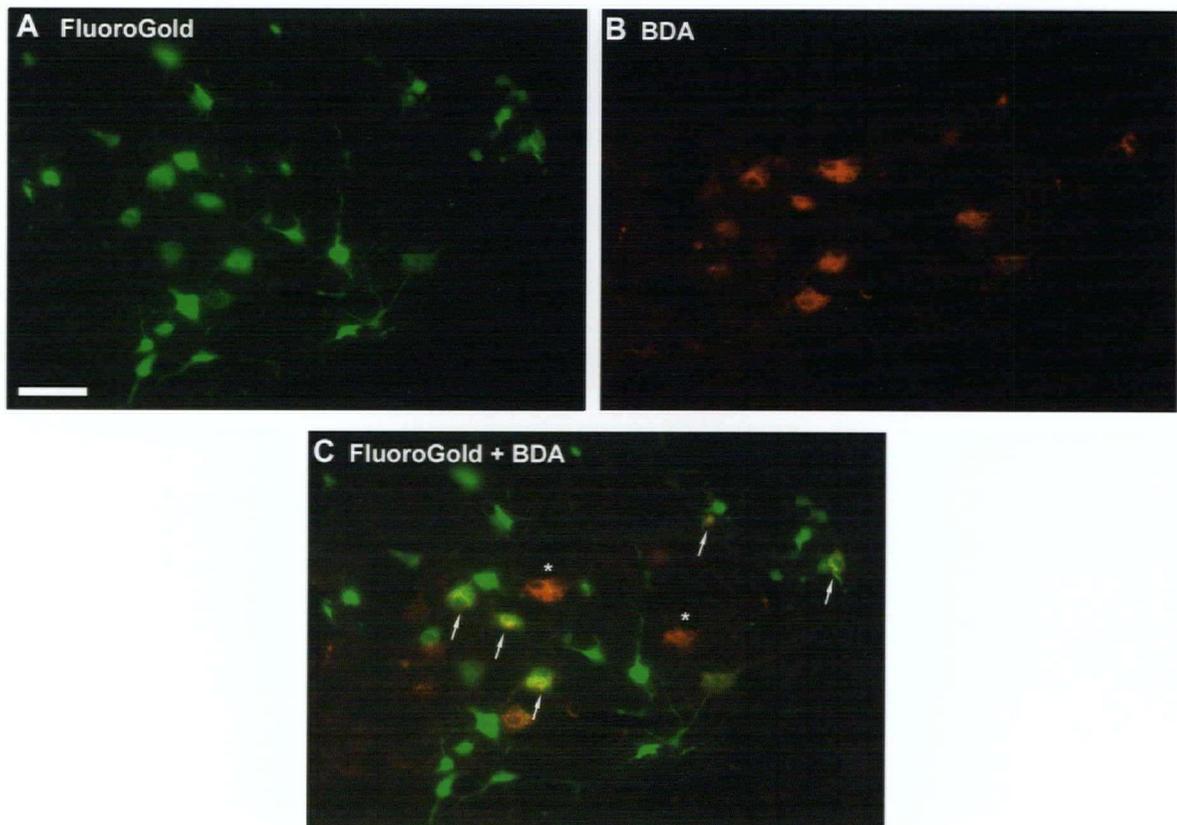
Figure 5.2. Axonal regeneration data from animals treated two months after axotomy at the spinal cord injury site with PBS or BDNF at three different doses (LOW, MEDIUM, HIGH).

A two-month chronic time point, there was no significant difference in the number of chronically injured neurons that regenerated into the peripheral nerve transplants (double-labeled cells – light grey) amongst the groups. Even pooling the number of double-labeled cells from the low, medium, and high dose BDNF groups and comparing against the PBS group did not result in a significantly greater number of chronically injured neurons regenerating with BDNF treatment (p=0.44). Conversely, pooling the number of single-labeled cells from the low, medium and high dose BDNF groups and comparing this to the PBS group revealed a significantly greater number of acutely injured neurons regenerating with BDNF treatment (p=0.01).
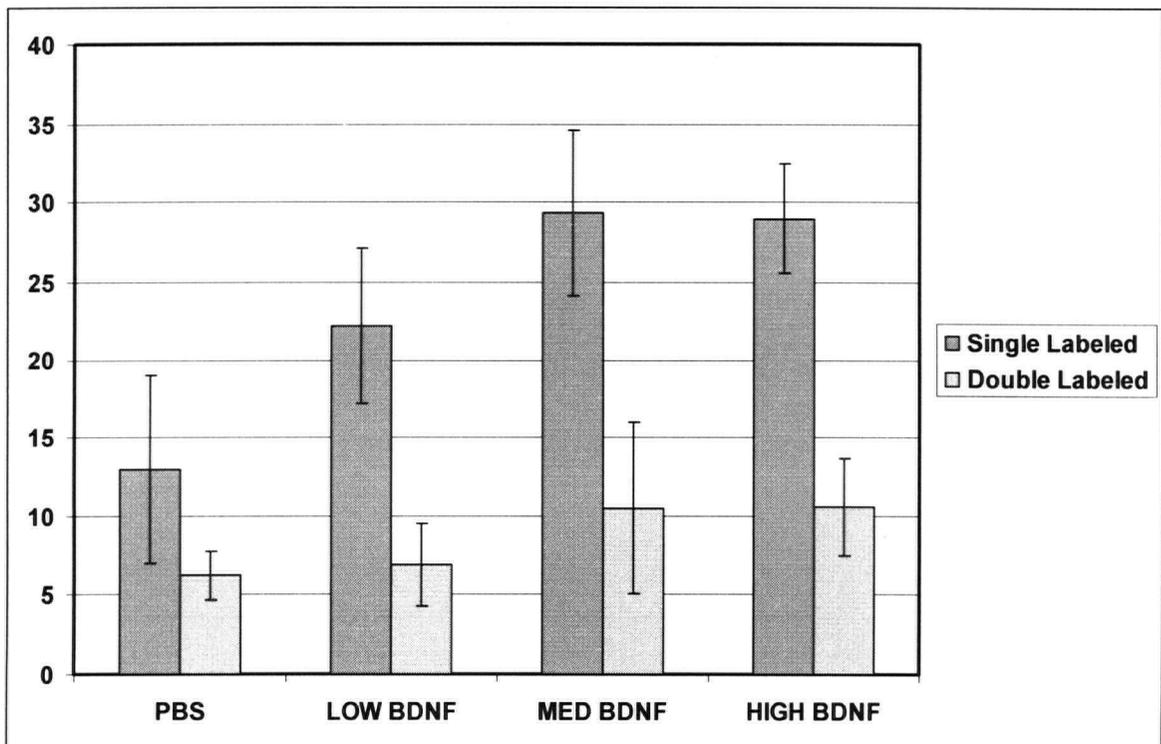
5.4.1 Axonal Regeneration Twelve Months Post-Axotomy with BDNF Applied to Brainstem

Similar to the axonal regeneration experiments at 2 months post-axotomy, lumbar-descending rubrospinal neurons were retrogradely labeled prior to cervical axotomy. In these experiments, the axonal tracer FastBlue was injected at C8 prior to cutting the dorsolateral funiculus at C3/4. One year later, I injected FluoroGold below T1 to retrogradely label any residual rubrospinal axons that might have escaped injury with the cervical axotomy. No retrograde FluoroGold labeling of rubrospinal neurons was ever observed, thus confirming the completeness of the unilateral rubrospinal axotomy. A predegenerated 35 mm segment of tibial nerve was transplanted into the spinal cord injury site and the BDNF applied via osmotic minipump into the vicinity of the rubrospinal cell bodies. DiI or BDA was applied to the free tip of the graft to retrogradely label axons that regenerated to the end. (Figures 5.3 and 5.4) The animals were sacrificed two months after transplantation to evaluate the number of double labeled neurons.

The number of double-labeled neurons in the BDNF-treated animals (n=14) was 28.6±8.3, and in the vehicle-treated animals (n=6) was 11.0±4.4. This was a statistically significant difference (p=0.038), indicating that cell-body treatment with BDNF did promote the axonal regeneration of chronically injured rubrospinal neurons. The number of single-labeled neurons in the same BDNF and vehicle-treated animals was 71.1±18.8 and 22.2±6.6 respectively, which again demonstrated a significantly greater number of regenerating acutely injured rubrospinal neurons with BDNF treatment (p=0.013) Figure 5.5 The spinal cord of an animal with BDNF treatment and BDA at the tip of the peripheral nerve transplant was sectioned sagittally to illustrate the interface between the cord and peripheral nerve transplant. (Figure 5.6)

Figure 5.3. Double labeling paradigm (FastBlue and DiI) to evaluate regeneration into peripheral nerve transplants twelve months after cervical injury.

In concept, the double-labeling strategy employed here is similar to that used at two months (illustrated in Figure 5.1) except that the rubrospinal neurons were initially labeled with FastBlue instead of FluoroGold, and in this case, DiI was placed at the tip of the peripheral nerve graft to retrogradely label neurons that regenerated axons through the transplant. The FastBlue image of rubrospinal neurons labeled retrogradely prior to axotomy (left) was overlaid upon the fluorescent DiI image of rubrospinal neurons (right). The overlaid image demonstrates red DiI single labeled rubrospinal neurons which are presumably acutely injured (arrowheads), and double labeled neurons which are chronically injured (arrows). Scale bar, 50 μm.
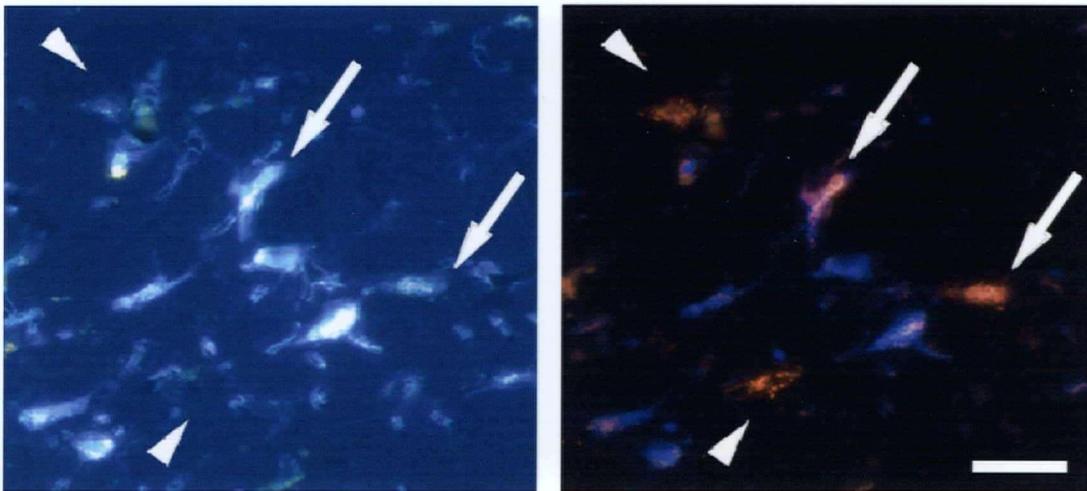
Figure 5.4. Double labeling paradigm (FastBlue and BDA) to evaluate regeneration into peripheral nerve transplants twelve months after cervical injury.

In this case, BDA was applied to the free tip of the peripheral nerve graft to retrogradely label rubrospinal neurons with axons regenerating through the transplant. Similar to Figure 5.1, the images are imported into an RGB figure, with the image of the BDA-labeled rubrospinal neurons in the red channel and the FastBlue in the blue channel. The BDA-labeled neurons therefore appear pink in the overlaid image. Note the presence of both double-labeled neurons (arrow) and single-labeled neurons (asterisks), the former representing chronically injured neurons, the latter representing acutely injured neurons that regenerated through the graft.
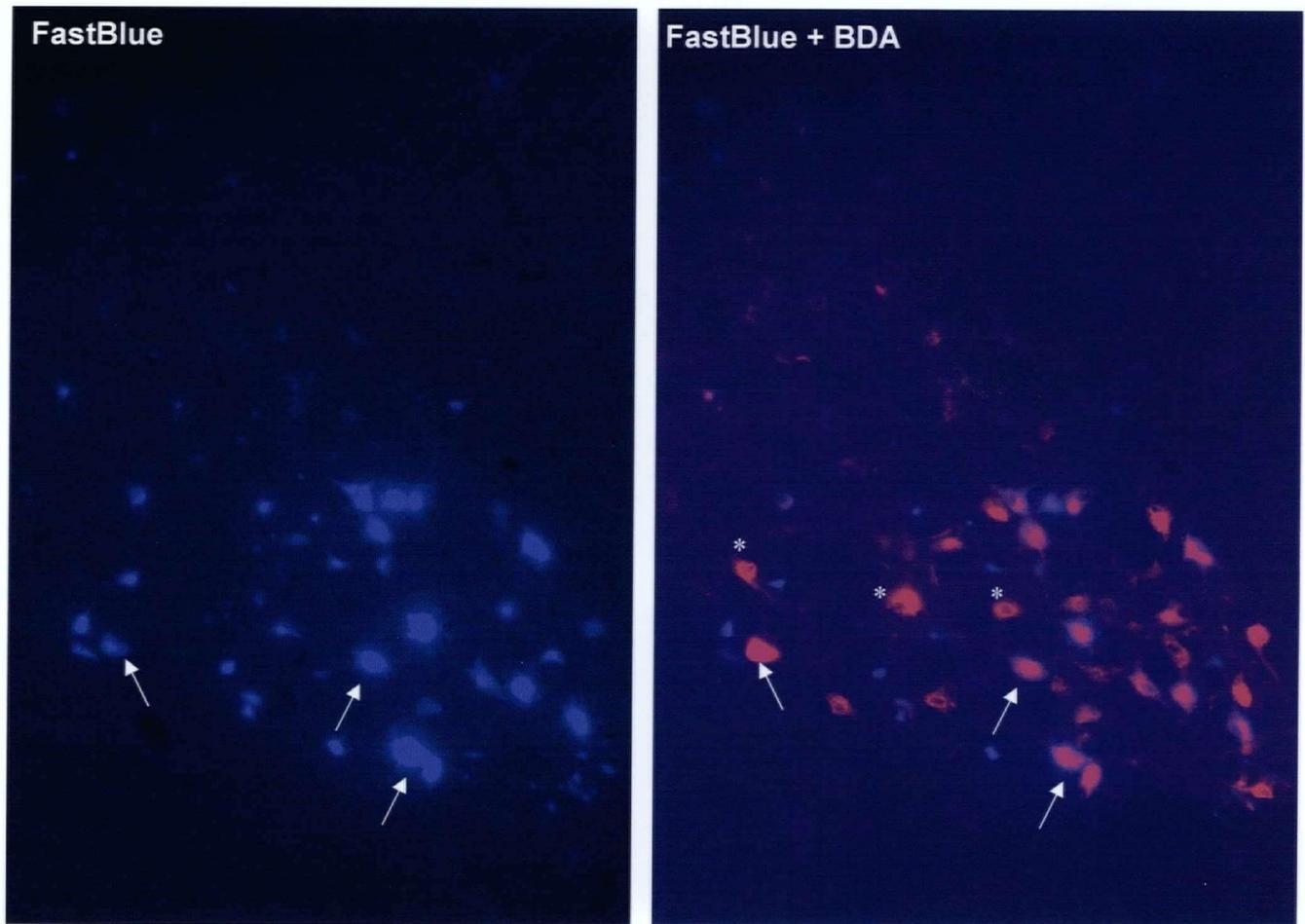
Figure 5.5. Axonal regeneration data from animals treated with cell body administration of BDNF or vehicle 12 months after axotomy.

At the 12 month time point, the animals treated with BDNF infusions to the vicinity of the red nucleus achieved significantly greater numbers of both double and single labeled neurons than vehicle treatment ($p < 0.05$ for both).
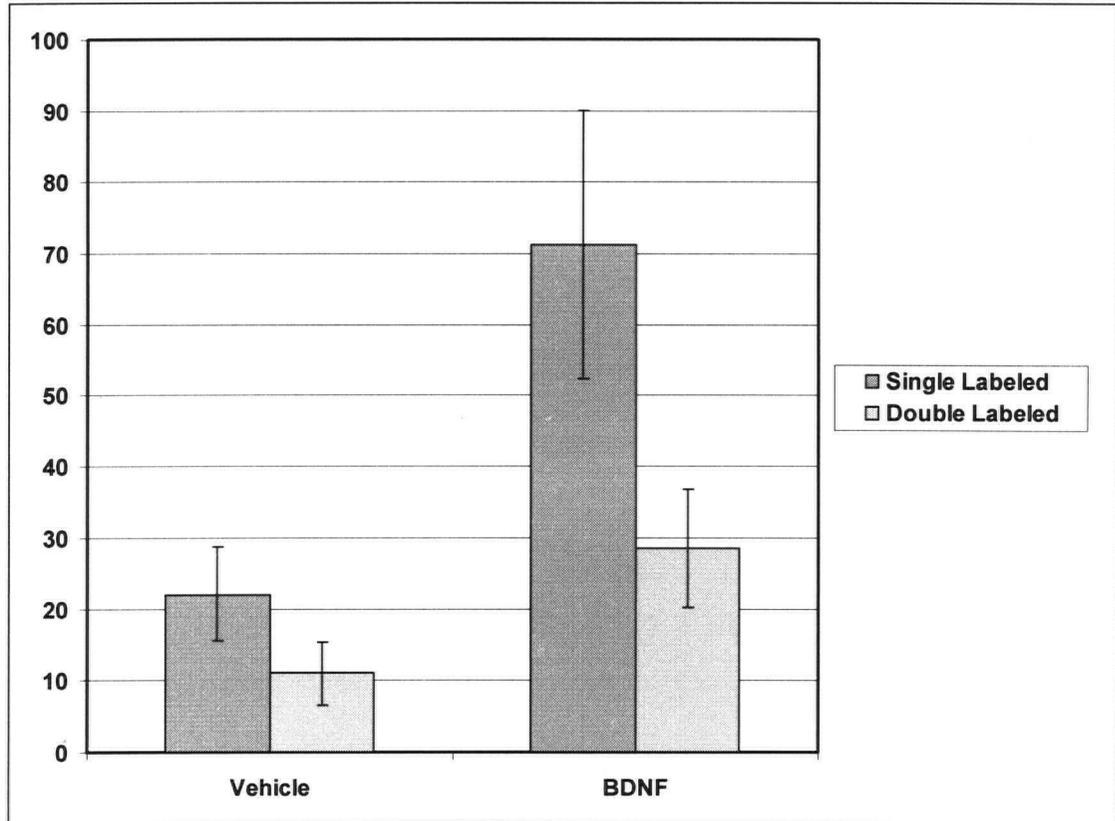
Figure 5.6. Sagittal section of spinal cord at the interface between the cord and peripheral nerve graft in a 12 month chronically injured animal.

For illustrative purposes, the spinal cord of an animal treated with BDNF 12 months after cervical axotomy was sagittally sectioned to examine the interface between the cord and graft. (A) This animal had BDA placed at the tip of the peripheral nerve transplant and demonstrated the greatest number of double-labeled neurons of all the BDNF-treated animals. Sections were processed with the ABC kit to visualize the BDA labeled axons, and then counterstained with cresyl violet.   Note the large BDA labeled axons (arrows) in the position of the rubrospinal tract (B) entering into the peripheral nerve transplant (C).   Scale bar, 50 μm.
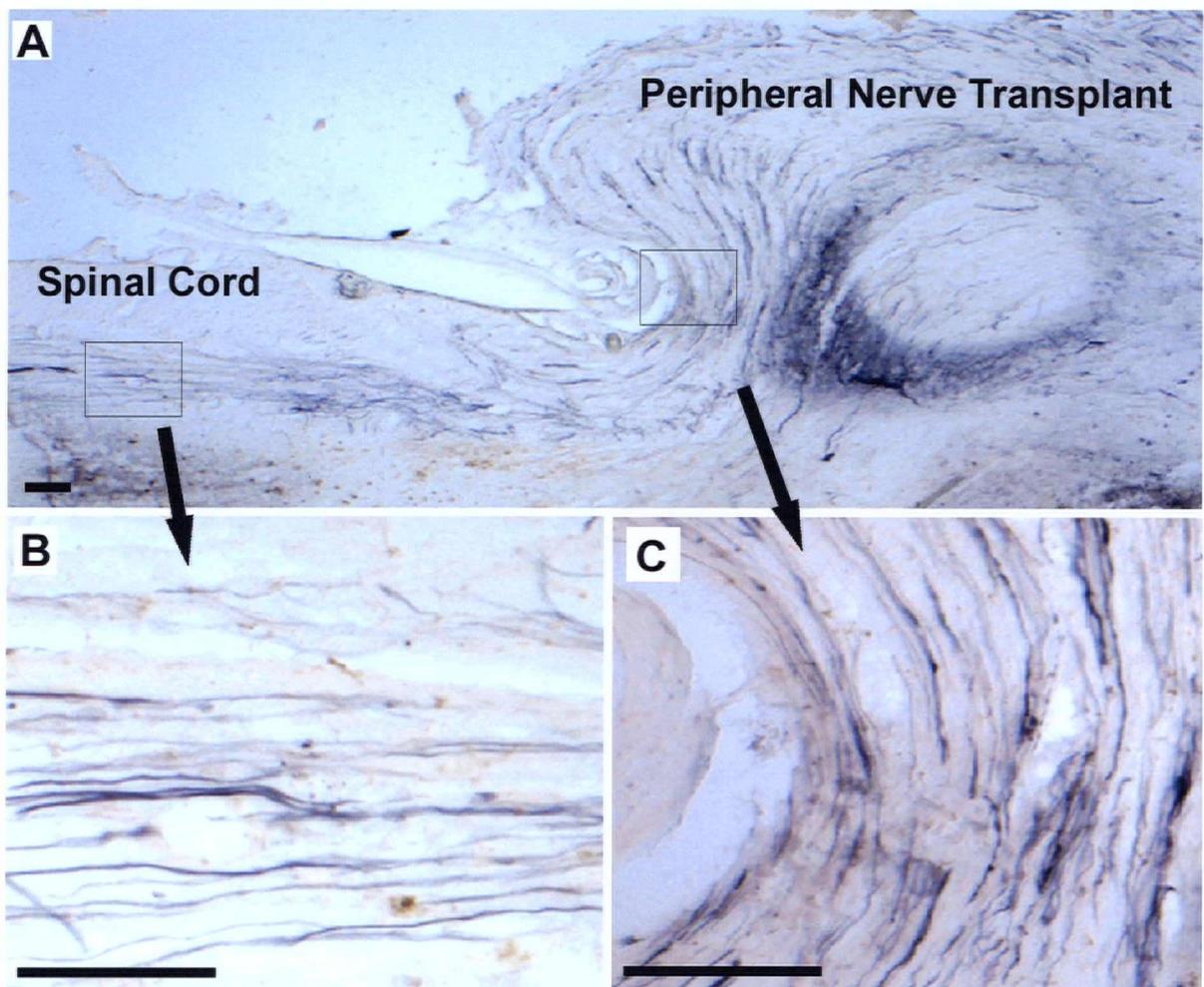
5.5. DISCUSSION

5.5.1. Chapter Summary

The results from these peripheral nerve transplant experiments demonstrate that rubrospinal neurons chronically injured at the cervical spinal cord level can regenerate their axons in response to BDNF administered directly to the cell bodies (12 months post-injury) but not to their injured axons (2 months post-injury). These findings are consistent with the reversal of neuronal atrophy and the upregulation of GAP-43 and $T\alpha 1$ tubulin expression with cell body and not spinal cord application of BDNF (discussed in Chapter 3 and 4).

5.5.2. Axonal Tracing for the Evaluation of Axonal Regeneration After Chronic Injury

A number of retrograde tracers were used in these experiments to evaluate axonal regeneration of chronically injured rubrospinal neurons. As we and others have pointed out, the in vivo study of axonal regeneration can be a challenging task (Kwon et al., 2002d, Steward et al., 2003). One of the major drawbacks with injury models that employ *partial* spinal cord lesions is the potential for axonal sparing. The rubrospinal tract runs in the dorsolateral aspect of the spinal cord; in this position, it can be reliably transected with an incision through the dorsolateral funiculus. This is an injury model that we (Kobayashi et al., 1997, Kwon et al., 2002b, Kwon et al., 2002c) and others (Liu et al., 2002, Liu et al., 1999, Tobias et al., 2003) have used extensively to study rubrospinal regeneration. The partial nature of this dorsolateral funiculus injury is particularly useful for chronic regeneration experiments, as the animals must survive with the injury for often prolonged periods of time. Such animals quickly regain normal bowel/bladder function and grossly normal lower extremity locomotor function. It is acknowledged, however, that with variability in the method of performing the axotomy and the stretching of the axons, some

sparing of axons might occur. In my 12 month chronic experiments, this issue was addressed with the second retrograde tracer injection at T1 (FluoroGold); with this, I did not see retrogradely labeled rubrospinal neurons in the injured red nucleus, indicating that the performance of my dorsolateral funiculus incision was consistent and sufficiently aggressive to reliably cut the entire rubrospinal tract unilaterally This confirmatory step was performed in the 12 month chronic injury experiments; the experiments at 2 months post-axotomy were performed subsequent to this, and I felt that my cumulative experience with the injury model made it unnecessary to continue with this second retrograde tracer injection to rule out sparing.

The defining feature of the double-labeling tracer paradigm in the peripheral nerve transplant experiments is that it allows one to distinguish axonal regeneration originating from acutely versus chronically injured neurons. In this paradigm, axons descending to the thoracolumbar spinal cord receive the tracer and their neurons are retrogradely labeled; they are then cut at C3/4. If they then successfully regenerate through the peripheral nerve graft and pick up the second retrograde tracer placed at its free end, they will then be "double-labeled". Neurons that are single-labeled with only the second retrograde tracer and not the first likely had axons that ended just proximal to the original C3/4 axotomy and were injured for the first time during the refreshment injury and insertion of the nerve graft.

Being able to make this distinction between axonal regeneration from acutely and chronically injured axons is obviously important if we are to evaluate the effectiveness of therapies for chronic spinal cord injury. Unfortunately, such a distinction is often difficult to make from studies of therapeutic interventions at chronic time points. For example, Tuszynski and colleagues transplanted fibroblasts genetically modified to secrete NGF into a dorsal hemisection spinal cord injury model, 3 months after original injury (Grill et al., 1997). They

reported impressive invasion of cerulospinal and primary afferent sensory axons but not of corticospinal axons – growth that was qualitatively similar to that which they observed in an acute injury paradigm (Tuszynski et al., 1996). However, these chronic experiments did not employ a double-labeling tracing paradigm, and as such, it is difficult to tell whether the axons they observed growing into the graft were actually chronically injured or if they were acutely injured at the time of the graft placement (Grill et al., 1997). This absence of a double-labeling tracing paradigm has made the distinction between acute and chronic neuronal regeneration similarly difficult in other chronic injury studies, including studies of BDNF-secreting fibroblasts inserted into a cervical hemisection injury 5 weeks after injury (Jin et al., 2000), minced autologous peripheral nerve inserted into a dorsal hemisection injury 25 weeks after injury (Ferguson et al., 2001), and olfactory ensheathing cell transplants inserted into a thoracic transection 4 weeks after injury (Lu et al., 2002).

In this regard, I have demonstrated that some rubrospinal neurons, injured 12 months previously, were capable of regenerating through peripheral nerve transplants. The BDNF infusion within the vicinity of the rubrospinal cell bodies enhanced the regenerative capacity of these chronically injured neurons, as evidenced by the significantly greater numbers of double-labeled neurons in BDNF-treated compared to vehicle-treated animals. Interestingly, there were a small number of double-labeled neurons in the animals treated with vehicle solution alone. The results of Houle would suggest that no regeneration occurs from rubrospinal neurons into peripheral nerve transplants injured 1 month prior (Houle, 1991). My somewhat contradictory observations may be related to the placement of the minipump cannula and infusion into the vicinitiy of the red nucleus, with the resultant inflammatory reaction promoting a growth response from the rubrospinal neurons. Such a growth response to inflammation was illustrated

by Lu and Richardson, who incited an inflammatory reaction within the DRG by injecting it with *Corynebacterium parvum* or macrophages and then demonstrated an increased DRG axonal regeneration propensity through a crush injury of the dorsal root (Lu and Richardson, 1991). Benowitz and colleagues have demonstrated a similar phenomenon in the eye, whereby an injury to the lens incites an inflammatory reaction (with macrophages being a critical component) that increased GAP-43 expression within retinal ganglion cells and promoted axonal regeneration in the crushed optic nerve (Leon et al., 2000, Yin et al., 2003). I have not further characterized the complex nature of the inflammatory response incited by the cannula of the osmotic minipump and how this might affect the growth propensity of rubrospinal neurons, although I recognize that such study might help to delineate some of the pathways by which inflammation influences axonal regeneration.

Perhaps better characterized is the effect of the second axotomy on chronically injured rubrospinal neurons. At two months post-axotomy, I observed that the GAP-43 and Tα1 tubulin ISH signals were increased to a similar extent in all animals, whether they received the repeated applications of BDNF (in one of three concentrations) or PBS within gelfoam at the spinal cord injury site (Chapter 4). I similarly found a small increase in cell size in all of these animals compared to animals that did not have a refreshment injury (Chapter 3). Storer and Houle have also demonstrated that this second axotomy promotes a significant rise in GAP-43 and βII tubulin expression. I would postulate that this growth response may be responsible for the regeneration of chronically injured rubrospinal neurons two months post-axotomy. The fact that there was no difference in double-labeled neurons between all animal groups on one way ANOVA or even between the PBS and all pooled BNDF treated animals suggests that this observed regeneration was related to the effects of the second axotomy.

5.5.3. BDNF and the Promotion of Axonal Regeneration

As discussed briefly in Chapter 3, numerous investigators have applied BDNF to the injured spinal cord either by direct infusion/application or by genetically modified cell lines (ex vivo gene therapy). Such studies have demonstrated a reduction in retrograde rubrospinal atrophy and loss (Liu et al., 2002, Diener and Bregman, 1994), the promotion of rubrospinal axonal regeneration (Liu et al., 1999), and the improvement of motor function (Liu et al., 1999, Namiki et al., 2000, Jakeman et al., 1998, Li et al., 2003, Ikeda et al., 2002) in animals treated during the acute phase of injury with BDNF. The inference from these studies reporting improved motor function is that the BDNF application encouraged some form of axonal growth or plasticity or accommodation which then mediated the observed neurologic recovery. Determining the anatomic correlate behind functional recovery, however, is an extremely difficult process and in most cases can only be postulated, recognizing that lower animals such as rodents have remarkable spinal cord plasticity and intrinsic locomotor ability to compensate for partial or even complete spinal cord injuries (Barbeau and Rossignol, 1994). Generally speaking, the exact contribution that a single neuronal system such as the rubrospinal system makes to a particular motor function is only known to a rudimentary extent (Kuchler et al., 2002).

My current experiments examined only axonal regeneration within the peripheral nerve transplant and did not evaluate intraspinal regeneration of rubrospinal axons (ie. across the injury site) nor did it evaluate functional recovery. As indicated previously, intraspinal regeneration of rubrospinal axons was observed through a graft of BNDF-secreting fibroblasts when implanted acutely after spinal cord injury (Liu et al., 1999) but not when implanted 6 weeks after spinal cord injury (Tobias et al., 2003). My observations of axonal regeneration 12 months after

axotomy with cell body treatment of rubrospinal neurons builds on the work of Kobayashi et al. who demonstrated increased axonal regeneration with such cell body treatment in an acute spinal cord injury model (Kobayashi et al., 1997). Conceptually, the cell body application of BDNF may be more directly able to effect the necessary changes within the neuronal soma at a chronic time point than the spinal cord application of BDNF. Such a statement is supported by my observations that at two months post-axotomy, spinal application of BDNF did not stimulate rubrospinal axonal regeneration while cell body application twelve months after axotomy did. The influence of TrkB receptor expression on these findings is discussed further in Chapter 6. Nevertheless, while the cell body treatment provides proof of principle that axonal regeneration might be stimulated by intervening at this level, a method for inducing such changes with an intervention at the spinal cord would clearly be more desirable. Intuitively, the surgical manipulation of the already injured spinal cord in order to apply a therapy (eg. BDNF) would be far less dangerous to an individual patient than an intracranial, intraparenchymal injection into the otherwise uninjured brain. Interestingly, an in vivo gene therapy strategy was recently described by Koda et al., who administered a BDNF-encoding adenovirus vector into an acute thoracic spinal cord transection (Koda et al., 2004). Using both anterograde and retrograde labeling, the authors reported rubrospinal axonal regeneration across the injury site. They also reported marker gene (lacZ) expression within the rubrospinal neurons, demonstrating successful retrograde transfection of these neurons with the implication that their BDNF production was increased. Even though the authors of this study report axonal regeneration across a complete thoracic spinal cord transection, the description of the spinal cord after injury raises questions about the completeness of the injury, particularly around the periphery. Nonetheless, the ability to achieve gene expression changes and possibly also increased BDNF secretion within the cell

bodies with an intervention directed to the acute injury site is indeed interesting, and hopefully further studies of this treatment will be performed at chronic time points to establish whether retrograde transfection of chronically injured rubrospinal neurons to enhance their BDNF production will be possible.

The discussion surrounding the successful axonal regeneration of rubrospinal neurons with acute BDNF fibroblast engraftment compared to 6 week post-injury intervention (Liu et al., 1999, Tobias et al., 2003) and the failure of BDNF in 3 doses to promote axonal regeneration 2 months post-injury described herein would imply that the sooner the BDNF were applied, the better. Bregman and colleagues have demonstrated that this might not be the case, and that the situation may in fact be somewhat more complicated (Coumans et al., 2001, Liu et al., 1999). Following a complete thoracic spinal cord transection injury, they transplanted fetal tissue and infused BDNF at a concentration of 1000 ng/µl via osmotic minipump either acutely, or at 2 or 4 weeks post-injury. They found that the growth of serotonergic axons into the transplant was greatest in animals that received the transplant and BDNF 2 weeks after injury, rather than acutely. Retrograde labeling of rubrospinal neurons was also demonstrated in animals receiving delayed transplants, although a quantification of rubrospinal growth at each time point of intervention (acute, 2 weeks, 4 weeks) was not provided. It is important to note that although total serotonergic fiber length distal to the lesion was improved in animals treated 2 and 4 weeks post-injury compared to those treated acutely, the response at 4 weeks post-injury was notably less than at 2 weeks post-injury, suggesting that while a two week delay in intervention appeared favorable, further delaying the treatment would not be beneficial. As an explanation for the improved response at 2 weeks post injury, they proposed that the inflammatory response might be more active acutely than "subchronically" and hence the spinal cord environment more

amenable to axonal regeneration at the latter time points. Also, they proposed that injured neurons themselves at the latter time points might mount an augmented regenerative response after a second axotomy, a hypothesis that would be supported to some extent by the observations of Storer and Houle who found that rubrospinal upregulation of GAP-43 and βII tubulin was actually greater and more rapid after a second cervical axotomy than after the initial injury (Storer and Houle, 2003). While the findings of Coumans et al. raises questions about the optimal time period to intervene for the promotion of axonal regeneration (Coumans et al., 2001), they cannot necessarily be extrapolated to neuroprotective therapies, which are generally shown to be most effective when applied as soon as possible after injury.

5.5.4. Peripheral Nerve Transplants and Rubrospinal Axonal Regeneration

The long-recognized permissiveness of peripheral nerves to axonal growth may be largely attributable to the Schwann cells, and the relative ease with which they can be acquired and expanded in culture has made them attractive candidates for promoting regeneration in spinal cord injured patients (Bunge, 2000). An important study in 1996 by Cheng et al. (Cheng et al., 1996) demonstrated corticospinal tract regeneration and functional recovery after bridging a rat spinal cord transection with 18 tiny intercostal nerve grafts stabilized with a fibrin glue containing acidic fibroblast growth factor. One of the authors of this paper has recently reported the application of this intercostal nerve graft transplant paradigm in a chronic thoracic transection injury model, in which axonal regeneration and partial functional recovery was observed in treated animals (Fraidakis et al., 2004). To extend these research findings into the clinical setting, a human case report of such autologous nerve grafting with acidic fibroblast growth factor was recently published (Cheng et al., 2004). The authors reported on a patient with an incomplete thoracic spinal cord laceration (stabbing victim) who underwent this grafting

procedure approximately 4 years after injury and subsequently achieved measureable improvement in lower extremity motor function. While the implication of this study is that axonal regeneration occurred through the nerve graft, it is difficult to rule out that the recovery was mediated by fibers from the intact part of the spinal cord.

It should be recognized that cellular substrates, including Schwann cells, used for transplantation represent more than just a passive substitute for the non-permissive CNS environment and its inhibitory elements. Cells used in transplantation paradigms constituitively express, or can be genetically modified to secrete a host of neurotrophic factors and thus they themselves can influence the regenerative competence of injured CNS neurons (Zompa et al., 1997). Schwann cells are certainly no exception to this, as they are known to express neurotrophic factors, cell adhesion molecules, and extracellular matrix molecules important for axonal regeneration (Guenard et al., 1993) and have also been genetically modified to increase their secretion of such neurotrophic factors as BDNF (Menei et al., 1998) and NGF (Tuszynski et al., 1998). As such, while conceptually providing a permissive environment for axonal growth (in essence, overcoming the extrinsic barriers to axonal growth) the application of cellular substrates such as Schwann cells within peripheral nerve transplants is not mutually exclusive from the strategy of enhancing the intrinsic regenerative capacity of CNS neurons (with neurotrophic factors, for example).

In this regard, the transplantation of Schwann cells or peripheral nerves would seemingly be an ideal "combinatorial" therapeutic strategy to promote axonal regeneration, addressing both some of the intrinsic and extrinsic obstacles. The corollary to this however, is that as permissive as an environment as they might be, Schwann cells (and other cellular substrates for that matter) still ultimately require that the neurons themselves mount a regenerative response in response to

the injury and treatment. The chronic spinal cord injury setting, therefore, imposes a particular challenge, as illustrated by my findings that rubrospinal axonal regeneration was not stimulated by the application of BDNF to the spinal cord injury site, despite the varying doses of BDNF applied. These findings are consistent with the failed rubrospinal regeneration in response to BDNF-secreting fibroblasts 6 weeks after injury (Tobias et al., 2003). Also consistent with this, Decherchi et al. found that the implantation of an autologous peripheral nerve transplant 3 weeks after cervical spinal cord injury led to 80% *less* axonal regeneration than when the graft was implanted acutely (147±44.5 double-labeled cells acutely versus 21.7 ±5.6 double-labeled cells chronically) (Decherchi and Gauthier, 2000). Houle similarly found that a delay in autologous peripheral nerve transplantation of 4 weeks resulted in no axonal regeneration of rubrospinal neurons (or any other supraspinal neuron for that matter) into the transplant (Houle, 1991).

While these reports describe the failure of axonal regeneration from chronically injured rubrospinal neurons in response to spinal cord application of BDNF and peripheral nerve transplants, the change in responsiveness over time to particular therapies is not a phenomenon exclusive to the rubrospinal system and to these particular therapies. One of the most studied experimental therapies in spinal cord injury research is the neutralization of inhibitory epitopes within CNS myelin with antibodies. Pioneering work by Schwab and colleagues demonstrated long-distance corticospinal axonal regeneration and subsequent functional recovery in response to the acute administration of IN-1 antibody to mitigate the inhibitory effects of a protein now known as NOGO-A (Schnell and Schwab, 1990, Bregman et al., 1995, Chen et al., 2000). However, a delay in applying the IN-1 antibody by 8 weeks after injury led to 98% less corticospinal regeneration than at 2 weeks post-injury (von Meyenburg et al., 1998). Along the same lines, Houle and Ye reported that the administration of bFGF to a cervical hemisection

injury 8 weeks after injury promoted 50% less regeneration of various supraspinal neurons than the same intervention at 4 weeks post-injury (Houle and Ye, 1997).

This disparity between the extent of axonal regeneration observed with acute versus delayed application of BDNF and acute versus delayed transplantation of a peripheral nerve graft illustrate that a therapy may lose its effectiveness in promoting axonal regeneration with a delay in intervention. Obviously, this has fairly important implications for individuals with chronic spinal cord injuries whose hopes for an imminent therapy for their condition are generally founded in the encouraging results of studies that report axonal regeneration in acute injury models. As such, this provides strong rationale to evaluate therapies at chronic time points after spinal cord injury. My findings of rubrospinal axonal regeneration 12 months post-injury with cell body application of BDNF is promising proof of principle that axonal regeneration may be possible after prolonged delays in intervention. Of the significant issues to be yet resolved is how such an intervention can be applied in a less invasive fashion, and what combinatorial therapies are required to promote the regeneration of chronically injured rubrospinal neurons across the injury site and into the distal spinal cord. In the context of combinatorial therapies, a recent study by Bunge and colleagues has demonstrated that after a contusion injury, the application of a Schwann cell graft (as a strategy to "bridge" the inhibitory spinal cord environment) in combination with means of elevating intracellular cAMP levels (as a strategy to boost the neuronal regenerative propensity) promoted regeneration of serotonergic axons and locomotor recovery (Pearse et al., 2004). Another recent study by Tuszynski and colleagues also demonstrated the potential utility of combinatorial approaches (Lu et al., 2004). These authors reported that the combined application of cAMP and NT-3 in conjunction with the transplantation of bone marrow stromal cells into a dorsal column injury promoted axonal

regeneration beyond the spinal cord injury site. Such regeneration was not observed with the application of cAMP or NT-3 alone. These exciting findings which demonstrate the potential for combinatorial therapies in the acute injury setting may prove to be beneficial for promoting regeneration of chronically injured neurons.

- CHAPTER 6 -

TRKB RECEPTORS WITHIN CHRONICALLY

INJURED RUBROSPINAL NEURONS AND AXONS

6.1. SUMMARY

In this chapter, I review the expression of full length TrkB receptors within the chronically injured rubrospinal system, with the assumption that the presence of TrkB receptors would make the rubrospinal system most directly responsive to the exogenous application of BDNF. Using immunohistochemistry, I documented the presence of full length TrkB receptors on the cell bodies of rubrospinal neurons both 2 and 12 months after cervical axotomy (Kwon et al., 2002b). Conversely, I found that anterogradely labeled rubrospinal axons at the cervical injury site did not contain full length TrkB immunoreactivity (Kwon et al., 2004a). These differences in the expression of full length TrkB receptors on the injured axons within the spinal cord and the neuronal cell bodies are consistent with the lack of BDNF effectiveness applied to the spinal cord and its effectiveness when infused into the vicinity of the red nucleus.

6.2. INTRODUCTION

The effectiveness of the exogenous administration of neurotrophic factors as a therapeutic strategy for CNS injury ultimately depends on the presence of the appropriate receptors within the target tissue. The Trk neurotrophin receptors are a family of highly related tyrosine kinases that demonstrate specificity in their binding to members of the classic neurotrophin family (discussed in more detail in the Background chapter, section 1.6.1). TrkB receptors bind with high affinity to BDNF and NT-4/5, and as such their expression is of particular relevance to these experiments in which I have applied BDNF to promote axonal regeneration of the chronically injured rubrospinal system. TrkB receptors contain both an extra and intracellular domain, with the kinase moieties found intracellularly (reviewed by Barbacid (Barbacid, 1994)). Both full length and truncated forms of the TrkB receptor have been described, with the latter lacking the intracellular kinase domain while maintaining the extracellular binding domain (and thus remaining specific to BDNF and NT-4/5 binding) (Middlemas et al., 1991).

6.2.1. Expression of TrkB Receptors in the Uninjured CNS

The expression of TrkB receptor mRNA has been widely demonstrated in the brain and spinal cord (Klein et al., 1989, Klein et al., 1990), and immunoreactivity to the extracellular domain of the TrkB receptor has also been shown to be widespread in the CNS (Yan et al., 1997). Of specific relevance to my experiments, Kobayashi et al. used in situ hybridization to show that virtually all uninjured rubrospinal neurons of the adult rat express mRNA for full-length TrkB receptors (Kobayashi et al., 1997), while robust immunoreactivity to the extracellular domain of the TrkB receptor was demonstrated by Yan et al. on the cell bodies of

adult rat rubrospinal neurons (Yan et al., 1997). Within the spinal cord, in situ hybridization studies have identified mRNA of full length TrkB receptors in the neurons of grey mater, but not in the glial cells of the white matter (Frisen et al., 1992, Liebl et al., 2001, King et al., 2000). A predominantly non-neuronal expression of the truncated isoforms of TrkB is seen within the uninjured spinal cord (King et al., 2000, Frisen et al., 1993). A similar cellular pattern of immunoreactivity is observed within the spinal cord to antibodies directed against the intracellular kinase domain of the TrkB receptor (Skup et al., 2002).

6.2.2. Expression of TrkB Receptors in the Injured CNS

While the expression of TrkB receptors on the native rubrospinal system is obviously of importance, it is the expression of these receptors in response to spinal cord injury that is most relevant to the effectiveness of exogenous BDNF administration as a potential treatment strategy. Additionally, in the context of how such a neurotrophic intervention might be influenced after injury, it is worth noting the changes that occur at both the supraspinal and the spinal cord level as they pertain to the neuronal cell bodies and injured axons respectively. Kobayashi et al. demonstrated with in situ hybridization that one week after cervical axotomy, the expression of full length TrkB receptor mRNA in injured rubrospinal neurons had decreased by approximately 30% compared to uninjured, and that this decline in expression continued in the second week as neuronal atrophy progressed (Kobayashi et al., 1997). Later time points beyond 2 weeks were not evaluated. Frisen and colleagues demonstrated in both cats and rats that within 3 weeks of a spinal cord axotomy, the mRNA expression of truncated TrkB receptors increased significantly in the scar tissue and white matter adjacent to the injury (Frisen et al., 1992, Frisen et al., 1993). The authors noted that the mRNA increase was specifically of the truncated form of TrkB, was restricted to glial cells, and was limited to the area immediately surrounding the axotomy, with

no alterations in TrkB expression in sections above or below the injury (Frisen et al., 1993). Similar findings were reported by Liebl et al using a contusion injury model, in which they reported a high level of truncated TrkB mRNA expression in ependymal cells and astrocytes that lined the injury site, with the absence of full length TrkB expression at the injury site (Liebl et al., 2001). Consistent with this, King et al. demonstrated a dramatic increase in truncated TrkB receptor immunoreactivity at the interface between damaged and undamaged spinal cord after a hemisection injury (King et al., 2000). This increase began within days of injury, peaked at 2 weeks post injury, and remained for at least 4 weeks (later time points were not evaluated).

Conceptually, if the expression of full length TrkB receptors were to diminish over time in injured rubrospinal neurons (as shown by (Kobayashi et al., 1997), this neuronal system could become less responsive to the exogenous application of BDNF. The aforementioned changes in TrkB expression within the spinal cord after injury, with an increase in truncated and decrease in full length isoforms, might also influence the effectiveness of BDNF applied exogenously to this region. What these studies have not demonstrated, however, is the immunoreactivity to full length TrkB of the rubrospinal neurons both at the level of their cell bodies and in their axons within the spinal cord. Recognizing the demonstrated decrease in the full length TrkB mRNA expression in rubrospinal neurons after injury (Kobayashi et al., 1997), it might be convenient to consider that the localization of the protein would be diminished throughout the neuron and axon. However, Lu et al. demonstrated in the corticospinal system that after subcortical injury, immunoreactivity to full length TrkB receptors was found on the cell bodies of corticospinal neurons, but was absent on their projecting axons at the level of the injury (Lu et al., 2001).

In this chapter I immunohistochemically evaluated full length TrkB receptor expression in the chronically injured rubrospinal system, working under the assumption that the expression

of full length TrkB receptors is important for the responsiveness of rubrospinal neurons and axons to BDNF application, and acknowledging that the expression of TrkB receptors (both full length and truncated isoforms) appears to change after cervical axotomy and may potentially differ between the neuronal cell body and its projecting axon. Of particular interest in this research question was to seek some rationale for the observed differences between cell body and spinal cord application of BDNF in terms of the effects on rubrospinal neuronal atrophy (Chapter 3), RAG expression (Chapter 4), and axonal regeneration (Chapter 5).

6.3. OVERVIEW OF EXPERIMENTAL QUESTIONS AND HYPOTHESES

In this chapter, I evaluated full length TrkB receptor immunoreactivity on the cell bodies and axons of chronically injured rubrospinal neurons to test the following following hypotheses:

1. Given that the cell body administration of BDNF to chronically injured rubrospinal neurons reversed neuronal atrophy, increased GAP-43 and Tα1 tubulin expression, and promoted axonal regeneration, *I hypothesized that chronically injured rubrospinal neurons maintain full length TrkB receptors on their cell bodies as the means of remaining responsive to this neurotrophic factor.*

To test this hypothesis, animals underwent a cervical axotomy, then two and twelve months later the presence of full length TrkB receptors on the injured rubrospinal neurons was evaluated with a polyclonal antibody to the intracellular kinase domain of the receptor (SC-12, Santa Cruz Biotechnology, Santa Cruz, CA).

2. Given that the spinal cord administration of BDNF at three exponentially different doses did not reverse neuronal atrophy, increase GAP-43 and Tα1 tubulin expression, or promote axonal regeneration of chronically injured rubrospinal neurons, and that others too have demonstrated the ineffectiveness of BDNF administration to the spinal cord, *I hypothesized that full length TrkB receptors are not maintained on the axons of chronically injured rubrospinal neurons at the injury site.*

To test this hypothesis, animals underwent a cervical axotomy, and then two months later the injured and uninjured rubrospinal tracts were anterogradely labeled. Immunohistochemistry with the a polyclonal antibody to the intracellular kinase domain of the receptor (SC-12, Santa Cruz Biotechnology, Santa Cruz, CA) was then performed on horizontal sections of the spinal cord to evaluate TrkB immunoreactivity on rubrospinal axons adjacent to the injury site.

6.4 . RESULTS

To evaluate TrkB receptor expression on rubrospinal cell bodies, immunohistochemistry on 20 micron thick cryostat sections of the caudal half of the red nucleus was performed, using a rabbit polyclonal TrkB antibody specific for the intracellular domain of the receptor, a biotinylated secondary antibody, and the ABC kit for visualization. To evaluate TrkB receptor expression on the injured axons, the rubrospinal axons were first anterogradely labeled with BDA (both on the injured and uninjured side). 20 micron thick horizontal sections were taken through the spinal cord injury site, so that both the injured and uninjured rubrospinal tracts could be visualized on the same section. Immunohistochemistry to the TrkB receptor was then carried out as above, but with a fluorescent secondary antibody (Alexa 488, Molecular Probes, Eugene Or). Colocalization between the TrkB and BDA (as visualized with streptavidin conjugated to Cy3) was evaluated with confocal microscopy.

Twelve months after cervical axotomy, immunoreactivity to full length TrkB receptors was observed on the cell bodies of both injured and uninjured rubrospinal neurons (Figure 6.1). These animals had BDNF or vehicle alone infused into the vicinity of the rubrospinal neurons via osmotic minipump. Despite the atrophy of the vehicle treated animals, the immunoreactivity to TrkB was apparent.

Similar immunoreactivity was observed on the cell bodies of injured and uninjured rubrospinal neurons 2 months after cervical axotomy. These animals had BDNF or PBS applied to the spinal cord injury site within gelfoam. Again, there was significant atrophy of the injured neurons, but despite this atrophy they appeared to maintain full length TrkB immunoreactivity (Figure 6.2). The TrkB immunoreactivity on the injured rubrospinal neurons did not appear to

be grossly different between the PBS and BDNF-treated animals (see Figure 6.2), although the quantification of the immunoreactivity was not attempted.

Anterograde labeling of the rubrospinal tract on both sides of the spinal cord was consistently achieved with the BDA injection performed using the coordinates as described by Houle et al (Houle and Jin, 2001). (Figure 6.3) After anterogradely labeling the rubrospinal tract (both injured and uninjured), there appeared to be very consistent co-localization between the BDA labeled rubrospinal axons and the full length TrkB immunoreactivity on the uninjured side of the spinal cord at the C3/4 level. Conversely, on the injured side of the spinal cord at this level, TrkB immunoreactivity was not observed to colocalize with the anterogradely labeled axons, suggesting that at the injury site, chronically injured rubrospinal axons had lost their TrkB receptors. (Figure 6.4) Overall, TrkB staining on the injured side of the spinal cord appeared to be substantially less than that observed on the uninjured side (See Figure 6.4 – Panels B versus E, and Figure 6.8 – Panel A versus B). At 63x magnification, it was frequently not possible to see multiple BDA-labeled axons on the horizontal sections, but on cross sectional analysis at the C1 level, colocalization between the BDA and TrkB was seen on every anterogradely labeled axon. (Figure 6.5) The lack of TrkB receptors on rubrospinal axons adjacent to the injury site was seen in all groups of animals, while robust colocalization between axons and TrkB was seen on the uninjured axons in all groups of animals. Control slides in which the TrkB primary antibody was incubated with its blocking peptide in a 1:1 ratio or in which the TrkB primary antibody was not applied demonstrated no specific binding (Figure 6.5).

Figure 6.1. Full length TrkB receptor immunohistochemistry is maintained on rubrospinal neuronal cell bodies 12 months after axotomy.

Full length TrkB receptor immunoreactivity is demonstrated here on the cell bodies of rubrospinal neurons from animals treated with cell body administration of the vehicle solution (top row) or BDNF (bottom row), twelve months after cervical axotomy. Note that the vehicle-treated injured rubrospinal neurons remain extremely atrophic (A) compared to the contralateral uninjured neurons (B) yet maintain immunoreactivity for full length TrkB receptors. Conversely, note the reversal of atrophy in injured rubrospinal neurons treated with BDNF (C), making them comparable in size to the contralateral uninjured neurons (D). Scale bar, 50 μm.
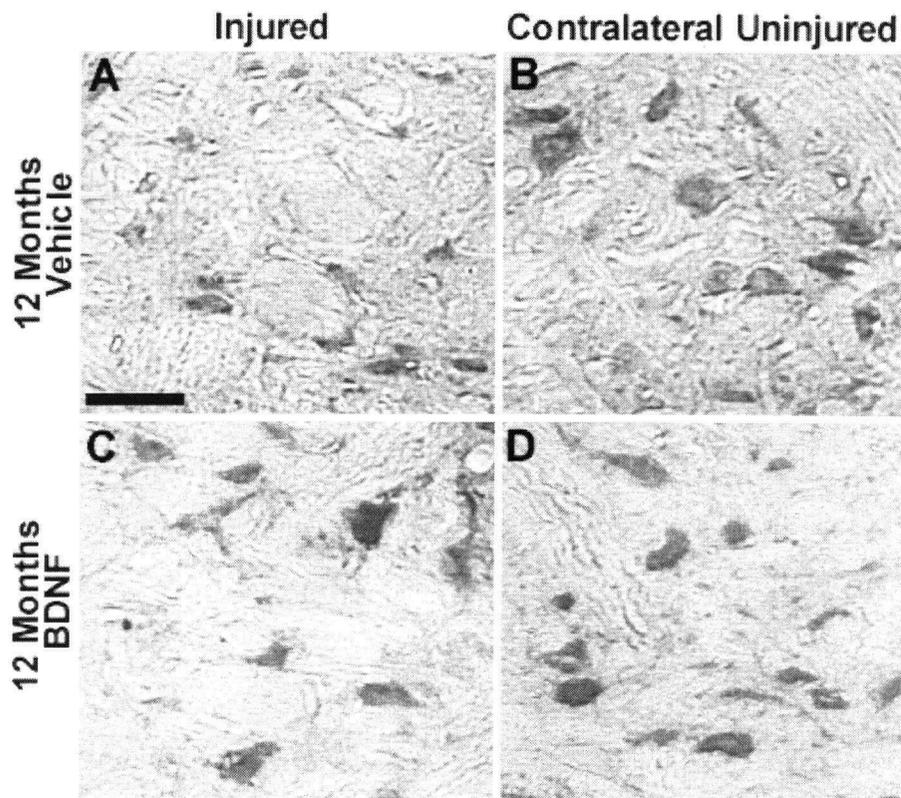
Figure 6.2. Full length TrkB receptor immunohistochemistry is maintained on rubrospinal neuronal cell bodies 2 months after axotomy.

Full length TrkB receptor immunoreactivity is demonstrated here on the cell bodies of rubrospinal neurons from animals treated with spinal cord administration of PBS (top row) or BDNF (bottom row), two months after cervical axotomy. Similar to that seen at 12 months post-axotomy (Figure 6.1), injured neurons are atrophic compared to their uninjured contralateral counterparts yet maintain TrkB immunoreactivity. Note that BDNF treatment did not reverse the atrophy, but TrkB immunoreactivity on the cell bodies is again maintained. All BDNF treated animals at each of the three concentrations of BDNF (50, 1,000, and 20,000 ng/μl) demonstrated similar TrkB immunoreactivity. Scale bar, 50 μm.
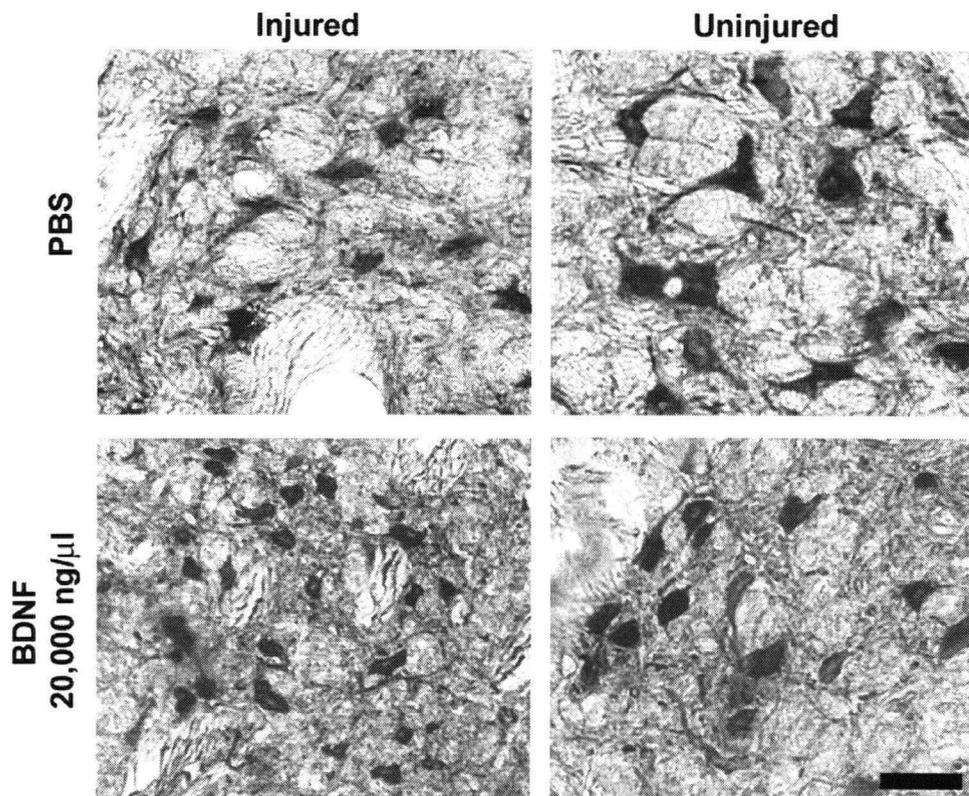
Figure 6.3. Anterograde labeling of with BDA provides consistent visualization of the rubrospinal tract within the dorsolateral funiculus of the spinal cord.

A 5x (A) and 10x (B) image of the spinal cord in cross section demonstrates the BDA labeling of the rubrospinal tract within the dorsolateral funiculus. This cross section is from the spinal cord just rostral to the segment of cord harvested for horizontal sections. Scale bar, 200 μm.
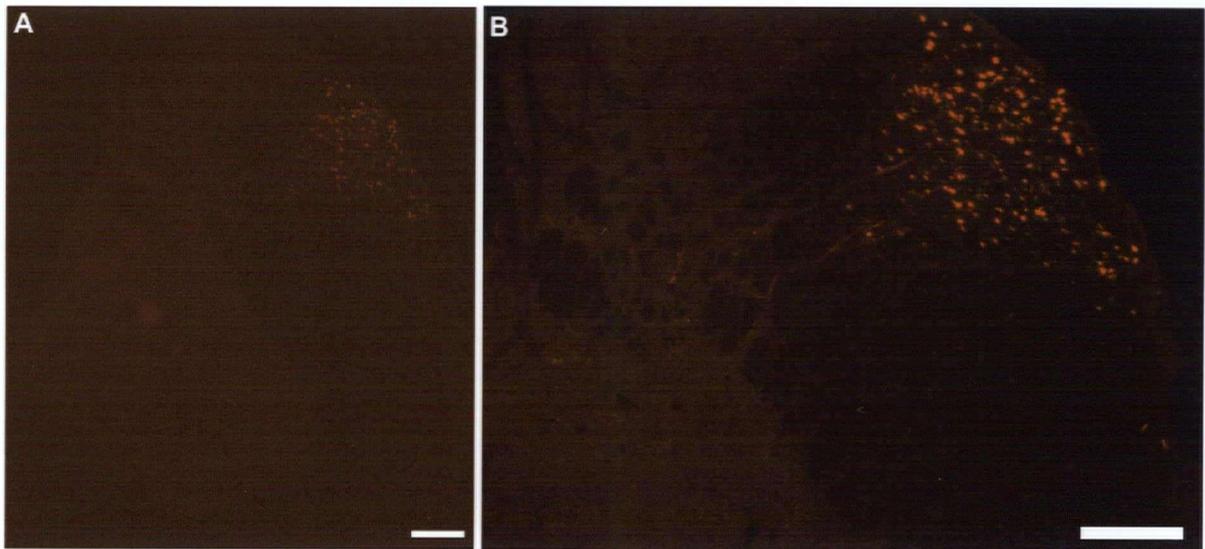
Figure 6.4. TrkB immunoreactivity is not maintained on the rubrospinal axons at the site of spinal cord injury, 2 months after the injury

Colocalization of TrkB immunoreactivity (green) and BDA anterograde labeling (red) of the rubrospinal tract two months after injury. Panels A to F are taken from a PBS-treated animal, with panels A to C demonstrating an anterogradely labeled axon at the injury site and panels D to F demonstrating an anterogradely labeled axon on the uninjured side of the same section. For both injured and uninjured, the BDA labeled axon, the TrkB immunohistochemistry, and the merged, overlaid images are shown. The arrowheads (injured) and arrows (uninjured) are reference points to highlight the absence of TrkB colocalization on the injured axon and the close co-localization of TrkB on the uninjured axon. The immunoreactivity to TrkB appeared to be less on the injured side of the spinal cord at the injury site (B) compared to the opposite, uninjured side of the spinal cord (E) (Scale bar = 25 μm).

Uninjured

Injured

D BDA

A BDA

E TrkB

B TrkB

F Merged

C Merged
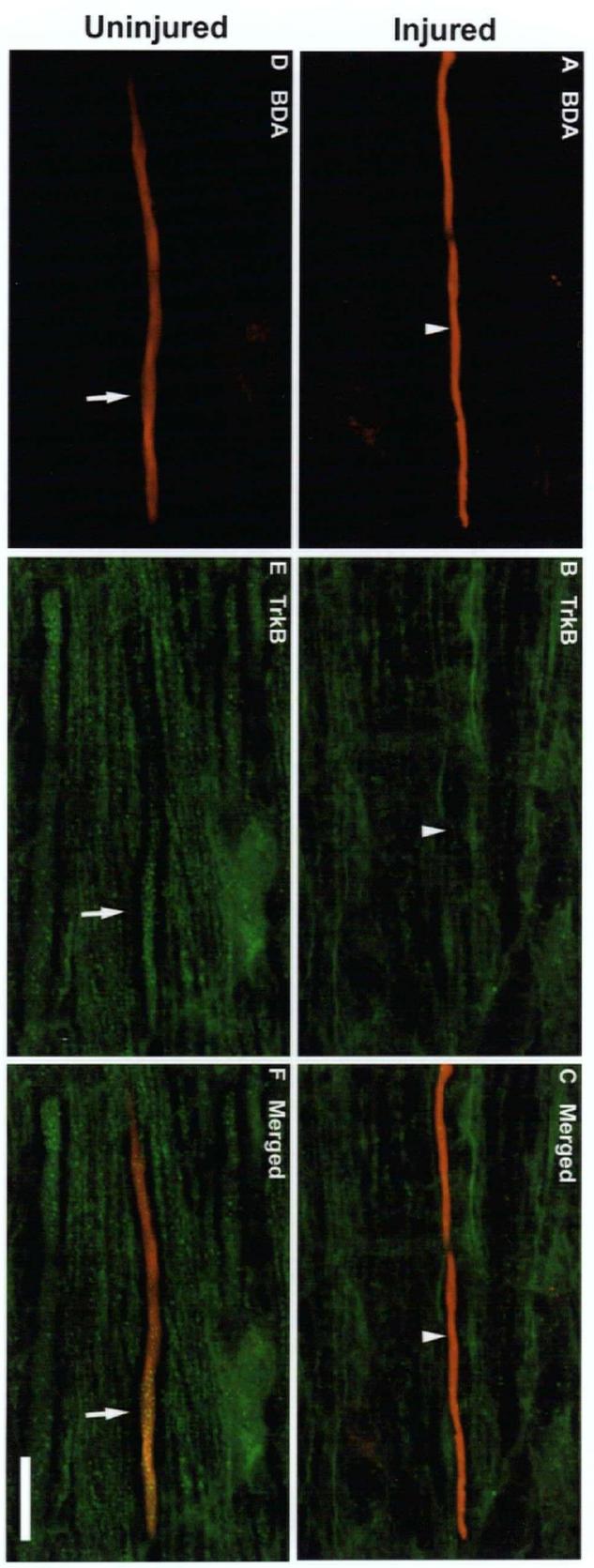
Figure 6.4.

Figure 6.5. TrkB immunoreactivity closely colocalizes with BDA labeled rubrospinal axons on cross-sectional images of the spinal cord at C1, well proximal to the injury site.

These are the BDA (A), TrkB (B) and overlaid images of a cross section of the uninjured rubrospinal tract at approximately C1 (well proximal to the axotomy). Note that in panel A, only a fraction of the axons pick up the anterograde BDA tracer. However, every anterogradely labeled axon colocalizes with TrkB on the merged image (C) (small arrows). (Scale bar = 25 μm).
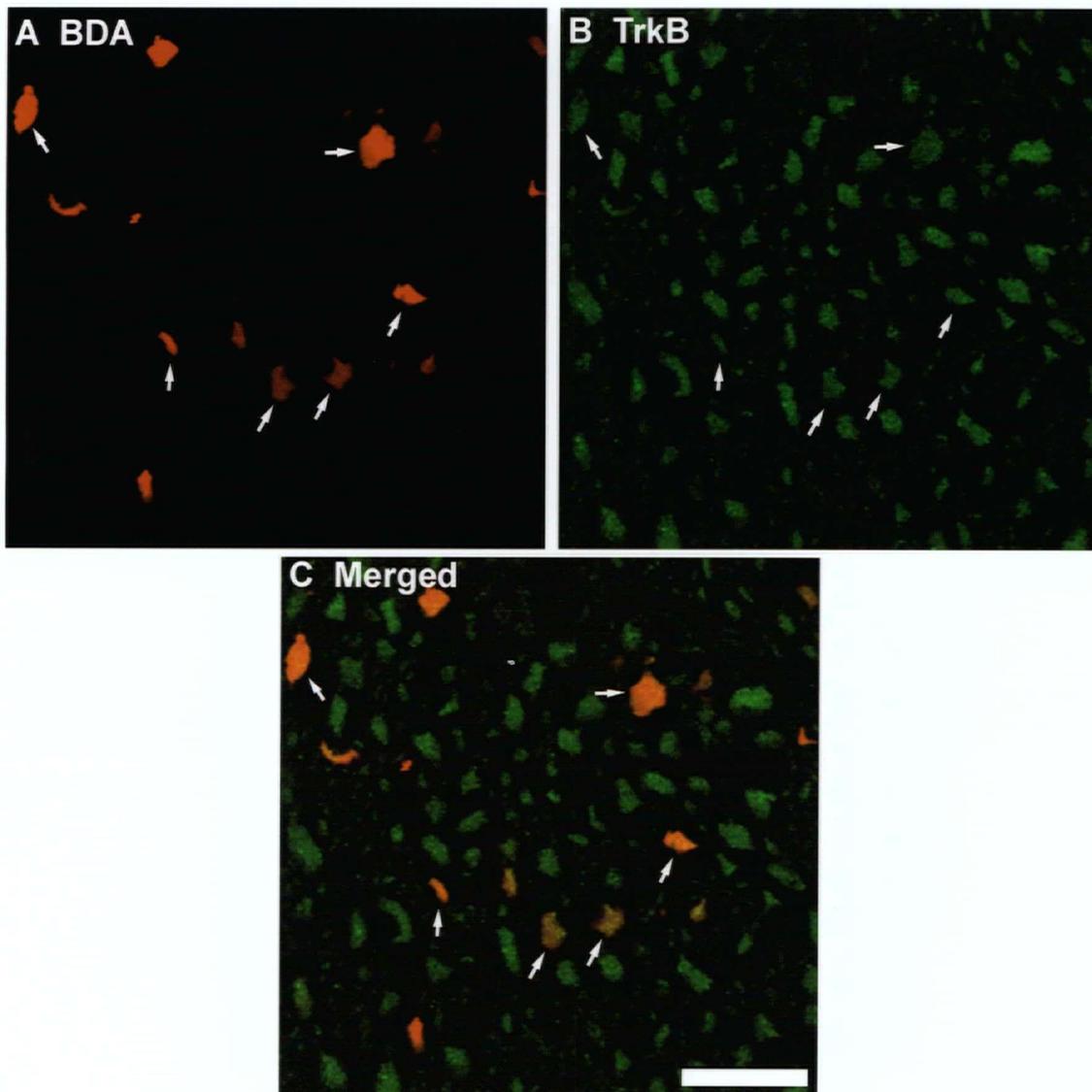
Figure 6.6.   Control TrkB immunohistochemistry slides demonstrate no specific binding.

After incubation of the primary TrkB antibody with its blocking peptide, there is no TrkB staining of the tissue (A).   Also, with no primary antibody, there is no unspecific green staining from the secondary antibody (B).   (Scale bar = 25 μm).
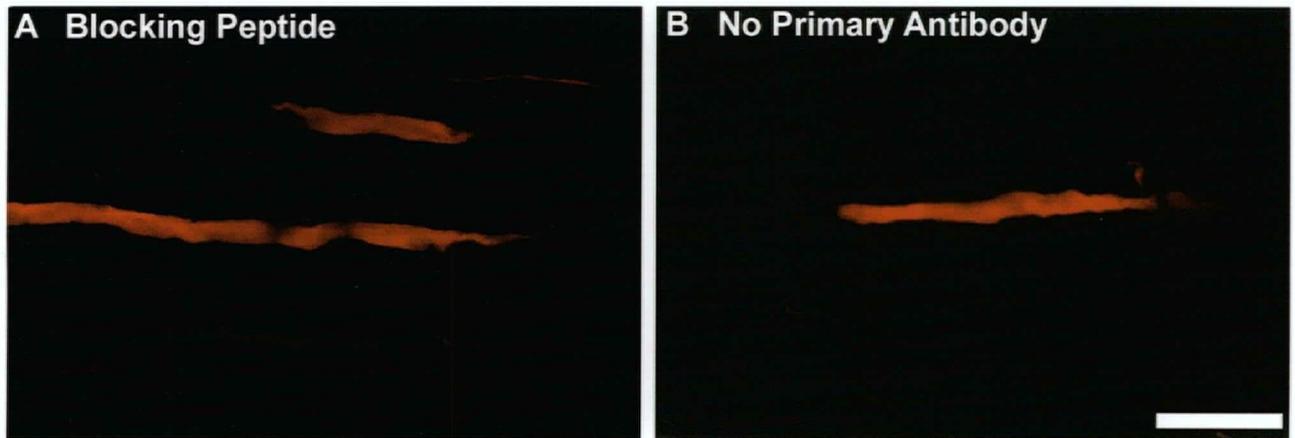
6.5 DISCUSSION

6.5.1. Chapter Summary

In this chapter, I sought to characterize the localization of full length TrkB receptors in the chronically injured rubrospinal system, in part to seek some biological rationale for the differences in effectiveness between cell body and spinal cord application of BDNF. I found that anterogradely labeled rubrospinal axons at the spinal cord injury site did not contain full length TrkB receptors, while the uninjured axons on the contralateral side of the spinal cord did, as evidenced by the strong colocalization between TrkB and BDA on the uninjured side of the spinal cord. Additionally, I found that the cell bodies of injured rubrospinal neurons maintained full length TrkB immunoreactivity at two and twelve months after cervical axotomy.

In Chapters 3, 4, and 5, I observed that the application of BDNF to the injured spinal cord 2 months after cervical axotomy was not effective in reversing rubrospinal neuronal atrophy, did not increase GAP-43 and Tα1 tubulin expression, and did not promote axonal regeneration into peripheral nerve transplants. Conversely, the application of BDNF to the cell bodies at an even more chronic time point (12 months post-axotomy) was effective in reversing neuronal atrophy, increasing GAP-43 and Tα1 tubulin expression, and promoting axonal regeneration into peripheral nerve transplants. The expression of full length TrkB receptors on the rubrospinal cell bodies but not on the rubrospinal axons at the chronic spinal cord injury site provides some explanation for these findings. Furthermore, the expression of full length TrkB receptors on the uninjured rubrospinal axons on the contralateral side of the spinal cord might explain why BDNF applied acutely after injury to the spinal cord appears to be effective in promoting axonal regeneration (Liu et al., 1999).

6.5.2. Anterograde Labeling of Rubrospinal Tract

Anterograde labeling of the rubrospinal tract was performed via an intraparenchymal injection of BDA to the medial aspect of the red nucleus where the rubrospinal axons emerge and project into the spinal cord. The coordinates for this injection were those published by Houle et al. in their study of rubrospinal axonal dieback from a cervical hemisection injury (Houle and Jin, 2001). These authors demonstrated effective anterograde labeling of the rubrospinal tract and were able to quantify the extent of axonal dieback weeks after the cervical spinal cord injury. My analysis of cross sections of the spinal cord demonstrated pronounced anterograde labeling in the dorsolateral funiculus of the spinal cord, confirming that the technique worked in my hands as well. On these cross sections, however, it appeared that the BDA tracer was picked up by only a fraction of the total number of rubrospinal axons. Given that there are thousands of neurons in the red nucleus, one would expect there to be many more axons labeled at the upper part of the cervical spinal cord if the BDA tracer had been picked up by the entire population of axons. Indeed, on the horizontal sections, at 63x magnification, it was often not possible to find multiple BDA labeled axons in the same field of view. One can note on these horizontal sections, particularly on the uninjured side of the spinal cord, that there appears to be "streaks" of green TrkB staining that run in parallel with the anterogradely labeled axons (see Figure 6.4, panel E). These streaks of TrkB staining are likely representative of rubrospinal axons that were simply not labeled with BDA, as is suggested by the cross sectional imaging. (Figure 6.5)

6.5.3. TrkB Receptors on the Axons and Cell Bodies of Rubrospinal Neurons

My observations that TrkB receptors are maintained on the cell body but are not found on the axons at the spinal cord injury site are consistent with the reported findings of Lu et al. in their evaluation of corticospinal neurons (Lu et al., 2001). Using very similar techniques in which corticospinal axons were anterogradely labeled with BDA and colocalization with TrkB immunoreactivity was evaluated with confocal microscopy, these authors demonstrated the maintenance of TrkB receptors on the corticospinal neuronal cell bodies but not on the axons in the spinal cord after a dorsal hemisection injury. BDNF applied to the injured spinal cord did not promote the growth of corticospinal axons (devoid of TrkB receptors), but when administered subcortically – presumably making the neurotrophic factor available to the cell bodies which possessed TrkB receptors – the BDNF was effective at preventing retrograde neuronal death after axotomy. This correlation between the presence of TrkB receptors and responsiveness to BDNF in the injured corticospinal system is very consistent with my findings in the injured rubrospinal system.

Given the changes that I observed in TrkB immunoreactivity within the injured spinal cord, it is interesting to consider how long it takes for such changes to occur. Conceptually, if the loss of TrkB receptors at the injury site occurs over a long period of time, that time period would define a window of opportunity to apply neurotrophic factors to the as yet responsive axons. Kobayashi et al. demonstrated with in situ hybridization that TrkB receptor expression within rubrospinal neurons decreased by 30% within one week of cervical axotomy (Kobayashi et al., 1997). Clearly, changes occur quickly in TrkB transcription, but without doing immunohistochemical evaluations on the injured spinal cord at various time points after injury, it is not possible to know how that change in gene expression is reflected by changes in protein

levels at the distal tip of the axon. Of note, Liebl et al. reported that TrkB expression within rubrospinal neurons did not decrease significantly after thoracic contusion injury of the spinal cord, but in this study, with a dorsal contusion injury, it is uncertain how many of the laterally place rubrospinal axons were in fact disrupted to begin with (Liebl et al., 2001). Alternatively, the disparity between the results of Kobayashi and Liebl could be explained by different responses to injury at the cervical and thoracic levels. While my cervical partial transection model is less representative of a clinical spinal cord injury than a contusion injury, the certainty with which the rubrospinal tract is cut allows us to answer the question of what is happening at the cell body level in response to injury.

Furthermore, if TrkB receptors are lost on the axons at the injury site, it would be interesting to know how far back along the axon such changes exist. *Are the TrkB receptors lost for a few hundred microns, millimeters, or along the entire axon up to the axon hillock?* If the receptors were lost for only a short distance, than perhaps spinal cord application of BDNF might work if one could get the neurotrophic factor to diffuse proximally enough to access the remaining TrkB receptors. Alternatively, if the receptors were lost all the way up to the axon hillock, then no amount of BDNF diffusion from the injury site would likely be effective. Unfortunately, my current experiments do not allow us to definitively answer this question. The greatest difficulty arises in distinguishing between those axons on the injured side of the spinal cord that are themselves injured, and those that are actually terminating on their targets proximal to the injury site. I evaluated TrkB receptor expression on axons adjacent to the injury site itself, as I felt that only these axons could reliably be defined as actually being injured. More proximal to this, when the injury site cannot be visualized, it is more difficult to know if a given BDA labeled axon is injured or is terminating prior to reaching the axotomy site. This problem

is schematically illustrated in Figure 6.7. One could argue that you could simply follow an axon from the injury site back towards the cell body, but on horizontal sections, the axons often move out of the plane of the section within a few hundred microns, making it impossible to follow a given axon for a prolonged length of spinal cord. The cross sectional images at C1 suggest that there is less TrkB receptor expression within the dorsolateral funiculus on the injured side compared to uninjured. (Figure 6.8) Nevertheless, colocalization of BDA labeled axons and TrkB receptors is also seen on the injured side of the spinal cord well proximal to the injury (around C1), which again raises the problem illustrated in Figure 6.7. A thorough evaluation of serial cross sections moving back from the spinal cord injury site might be helpful in resolving this question.

Figure 6.7. The current experimental paradigm of evaluating injured rubrospinal axons on horizontal sections makes it difficult to determine how far proximally the loss of TrkB receptors occurs.

With the lack of TrkB colocalization with rubrospinal axons at the injury site, it would be interesting to determine how far proximally this loss of receptors occurs. Unfortunately, the problem with looking proximally in the spinal cord is determining whether the actual axon being evaluated is injured or uninjured further caudally.

Looking at these two axons proximal to the injury, it is not possible to distinguish between the intact and the injured axon.

Injured axon at the axotomy site
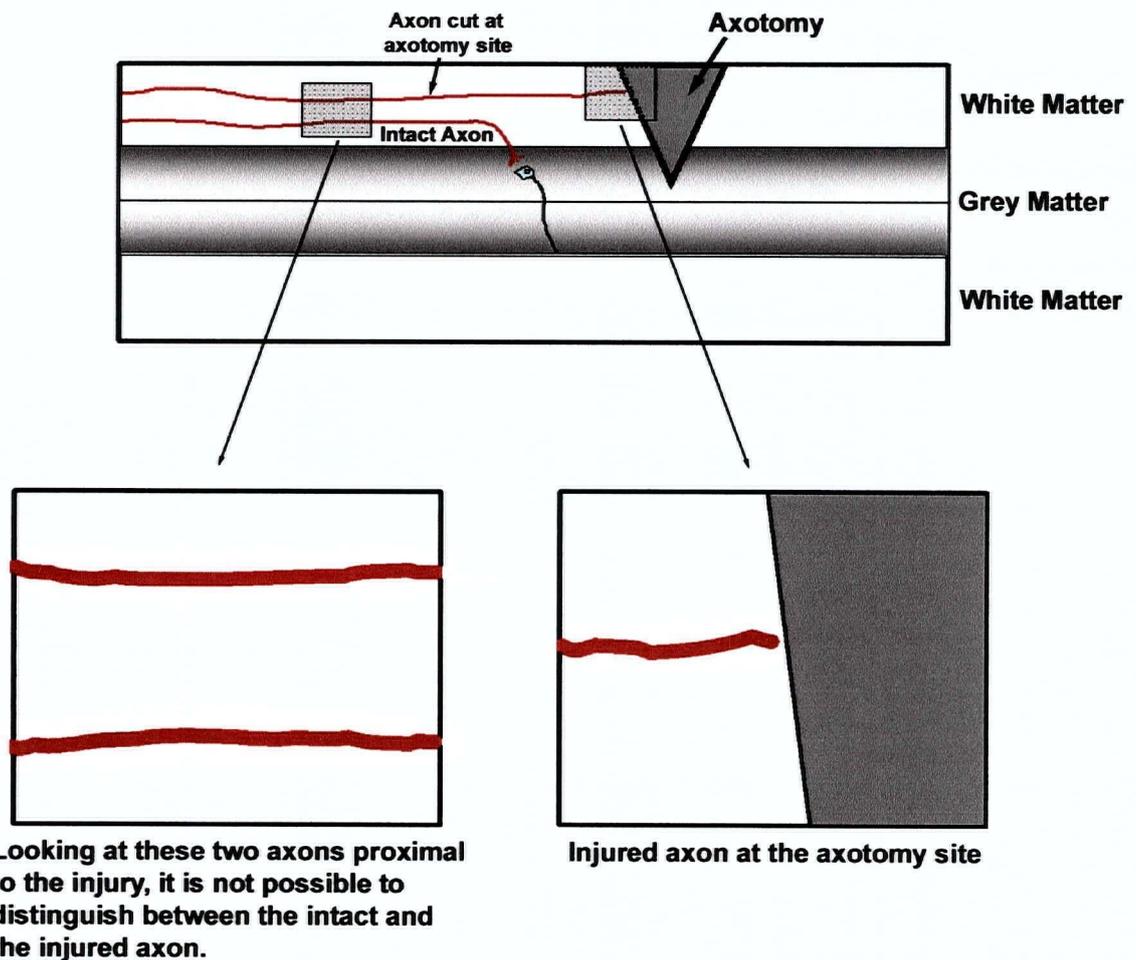
Figure 6.8. **Immunoreactivity to full length TrkB appears to be less on the injured side of the spinal cord compared to uninjured well proximal to the injury site (at C1).**

With regards to the question of how far proximally the loss of TrkB receptors extends away from the injury site, at C1, there appears to be less TrkB immunoreactivity on the injured than uninjured side of the spinal cord. This would suggest that the loss of TrkB receptors extends well proximal to the injury site, although it is impossible to know on the injured side of the spinal cord which axons are intact or cut distally (as described in Figure 6.6). Serial analysis of cross sections more proximal to the injury site would be helpful to resolve this issue. Scale bar, 25 μm.
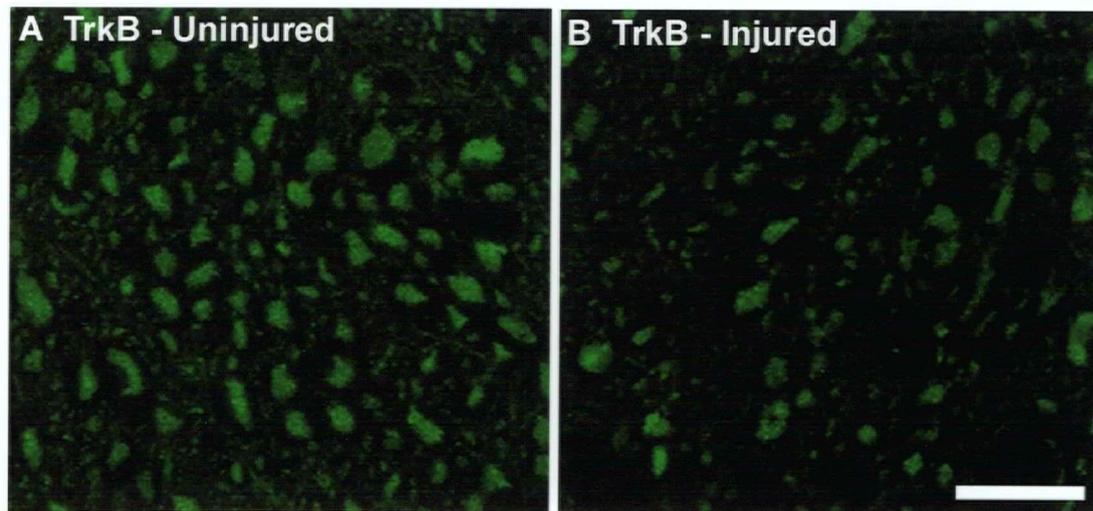
- CHAPTER 7-

GENERAL DISCUSSION

7.1. SUMMARY

In this thesis, I have described a number of experiments that have been performed to evaluate the rubrospinal system in a chronic injury state after cervical axotomy. For the most part, the experiments were performed at two and twelve months after axotomy, and they attempted to determine changes that occurred over time at both the level of the rubrospinal cell bodies and at their injured axons. Brain derived neurotrophic factor (BDNF) was applied to either the rubrospinal cell bodies or to their axons to determine the responsiveness of this neuronal system in the chronic injury state.

My findings can be summarized as follows. Two months after cervical axotomy, rubrospinal neurons undergo significant atrophy and exhibit limited expression of GAP-43 and Tα1 tubulin, genes thought to be important for axonal regeneration. Rubrospinal neurons appear to maintain full length TrkB receptors on their cell bodies, and while their uninjured axons within the cervical spinal cord also contain TrkB receptors, the injured axons at the level of the spinal cord axotomy do not. Consistent with this, BDNF applied to the spinal cord injury site at three exponentially increasing concentrations did not reverse rubrospinal cell atrophy, did not stimulate GAP-43 and Tα1 tubulin expression, and did not promote axonal regeneration of rubrospinal axons into the permissive environment of a peripheral nerve transplant. At 12

months after cervical axotomy, a stereologic evaluation of rubrospinal neurons demonstrates that rubrospinal neurons are in fact alive, but very atrophic. Similar to the findings at 2 months post-injury, the rubrospinal neurons 12 months post-injury display limited expression of GAP-43 and Tα1 tubulin but do maintain full length TrkB receptors on their cell bodies. At this chronic time point, the administration of BDNF to the injured cell bodies reversed neuronal atrophy, stimulated GAP-43 and Tα1 tubulin expression, and promoted axonal regeneration into peripheral nerve transplants.

While much of the discussion of the results has been covered in the individual chapters, a number of broad issues surrounding the nature of my experimental model and the implications of the findings warrant further discussion. In the following sections, I will attempt to place these findings into a larger context, and consider some of the future directions for such research.

7.2. MODELING OF CHRONICITY IN SPINAL CORD INJURY

Currently, there are over 250,000 individuals living in North America who have suffered a spinal cord injury some time ago, whose neurologic status is essentially stable, and who are now considered to be chronically injured (Sekhon and Fehlings, 2001). This number of chronically injured individuals by itself provides compelling rationale to study axonal regeneration strategies in chronic injury paradigms. These patients, particularly those with complete loss of motor and sensory function whose neurologic deficits are stable (ie. no longer expected to improve on their own), are considered to be the most likely candidates for initial clinical trials into experimental therapies that might induce some axonal regeneration, as it felt that they have the "least to lose, and the most to gain". Given this fact, it is somewhat ironic that all axonal regeneration strategies are tested experimentally in the acute injury setting, and few are ever evaluated after a delay in intervention to model a chronic injury situation. It may be tempting to consider intervening with an axonal regeneration therapy acutely in patients with complete motor and sensory paralysis, given that their prognosis for distal lower extremity recovery is expected to be poor (Marino et al., 1999). Despite this, these patients often do achieve some local motor recovery, mediated by preserved spinal cord tissue around the zone of injury, even in the absence of distal recovery. This is an issue of critical importance to those with cervical spinal cord injuries. For example, for someone who presents acutely with a C6 level of complete quadriplegia but with a flicker of triceps movement (mediated by the C7 nerve root), the very real possibility that he or she may naturally regain functional triceps power over time is enormously important, even though the chance of recovering lower extremity function is negligible (functional triceps power would enable the patient to transfer in and out of bed independently and use a non-motorized wheelchair). As such, until the patient spontaneousy

achieved his/her full recovery potential (of course, with the help of standard medical, surgical, and rehabilitative care) and establishes a stable neurologic impairment, it would be extremely risky to undertake an intervention that could potentially diminish perserved tissue at the injury site. An example of this would be the surgical manipulation required to transplant a cellular substrate into the injured cord. This spontaneous recovery typically occurs over the first 18 months that follow the traumatic incident, again, reinforcing the need for research in spinal cord injury models with a delay in intervention (Marino et al., 1999).

Having outlined the compelling need for experimental research that accounts for a delay in therapeutic intervention, it is nonethelesss unclear how to reproduce the chronic condition in a rat model of spinal cord injury. One of the issues of some uncertainty is the actual definition of "chronic" as it applies to the neurobiology of spinal cord injury. At an operational level, for the purposes of animal modeling and testing of experimental treatments, it might be reasonable to suggest that an injury becomes chronic when treatments that work acutely no longer remain effective. While this may have some conceptual appeal, this time frame of effectiveness may vary widely amongst therapies. Alternatively, the definition of chronicity could apply to the molecular, biochemical, and morphologic changes that occur within the relevant neuronal systems after spinal cord injury.

I performed my experiments largely at two different time points after injury: 2 months and 12 months. Clearly, waiting 12 months post-injury is, from a practical perspective, an extremely difficult endeavour, as the animal care is costly and the animals, having passed over half their lifespan, are relatively fragile to the anesthetic and surgical procedures necessary to test these interventions. I therefore proposed 2 months post-axotomy as a reasonable period of time to wait before intervening with the BDNF treatment. With respect to changes that occur at the

spinal cord injury site, Hill et al. reported the establishment of a clearly defined cavity 3 weeks after a contusion spinal cord injury, which was fully developed and stable at 14 weeks post-injury and remained so for the subsequent 6 months (Hill et al., 2001). Following a sharp hemisection injury, Houle and Jin reported dieback of rubrospinal, vestibulospinal, and reticulospinal axons that occurred primarily within the first week post-injury and then remained fairly stable for the next 13 weeks (Houle and Jin, 2001). In terms of the molecular changes that occur at the level of the cell body, GAP-43 and $T\alpha1$ tubulin expression increases acutely after cervical axotomy but then diminishes over the subsequent 4 to 7 weeks (Fernandes et al., 1999, Fernandes and Tetzlaff, 2000, Kobayashi et al., 1997, Tetzlaff et al., 1991) More recently, Storer and Houle have demonstrated that 4 weeks after cervical axotomy, the acute increases in GAP-43 and βII tubulin expression have abated and the levels of expression are either at or below that of the contralateral uninjured neurons (Storer and Houle, 2003). I feel, therefore, that the selection of a two-month post-axotomy period in the rat provides a reasonable reproduction of a chronic spinal cord injury, both with respect to the changes that occur at the level of the cell body and at the spinal cord. Additionally, the results of my experiments in which the rubrospinal system was essentially unresponsive to BDNF applied at the spinal cord two months after injury provides some support to the operational definition of chronicity at this time point. Of note, a similar failure to upregulate GAP-43 and βII tubulin expression in rubrospinal neurons or to reverse their atrophy was recently reported by Storer et al. when applying BDNF to the injury site only 4 weeks post-axotomy (Storer et al., 2003).

Given that our laboratory and others have characterized some of the changes that occur in the rubrospinal system two months after cervical axotomy, and have identified aspects of the system that influence its responsiveness to neurotrophic therapy, we would propose that it

represents a reasonably useful "chronic" spinal cord injury model to study the effectiveness of axonal regeneration strategies. Useful features of this as a model include the assuredness in performing a complete transection of the rubrospinal tract, the ability to both anterogradely and retrogradely label the system, and the ability to use changes in neuronal cross sectional area, RAG expression, and axonal regeneration into peripheral nerve transplants as outcome measures. The generalizability of the findings in the rubrospinal system to other neuronal systems after chronic injury is uncertain, but is certainly relevant to any clinical application of an experimental therapy for patients with chronic spinal cord injury.

7.3. ADMINISTRATION OF BDNF AS A THERAPEUTIC STRATEGY FOR SPINAL CORD INJURY

In these experiments, we applied BDNF to the brainstem or spinal cord in an effort to establish its effect on the chronically injured rubrospinal system and ultimately to shed insights into the challenges of promoting axonal regeneration in patients with chronic spinal cord injury. Such *in vivo* exogenous administration of BDNF as a therapeutic strategy raises interesting questions. As full length TrkB receptors appear to be important for mediating the biological effect of BDNF, my line of reasoning was to study TrkB receptor expression within the rubrospinal system. The findings of full length TrkB receptors on the rubrospinal cell bodies and on uninjured rubrospinal axons fits into a fairly rational model in which the administration of BDNF binds to TrkB receptors and exerts its effects on gene expression, neuronal size, and axonal regeneration. This is supported by my observations after BDNF application to rubrospinal cell bodies 12 months post-axotomy. Conversely, the direct administration of BDNF to injured rubrospinal axons that lack full length TrkB receptors fails to elicit a change in gene expression, neuronal atophy, and axonal regeneration. This is supported by my observations after BDNF application to the spinal cord 2 months post-axotomy.

While all of this seems fairly rationale, it is recognized that the infusion or application of such enormous doses of BDNF is a crude experimental paradigm, although likely more selective than the systemic and intrathecal administration of BDNF that, for example, has been tested in patients with amyotrophic lateral sclerosis (Ochs et al., 2000, BDNF Study Group, 1999). The side effects reported in these studies (eg. diarrhea, muscle weakness, agitation, and sleep disturbances) highlight the widespread distribution of TrkB receptors both within and outside the nervous system. One could also postulate that the BDNF that we infused into the vicinity of the

red nucleus stimulated surrounding glial cells or neurons which then secreted other factors (possibly even BDNF) that acted in a paracrine fashion to effect the changes we observed in the 12 month chronically injured rubrospinal neurons. For example, microglia/macrophages that are attracted to the cannula might have been stimulated by the BDNF to secrete other neurotrophic factors, as they have been shown to do *in vitro* (Elkabes et al., 1996). Indeed, the small extent of axonal regeneration observed in chronically injured animals that received intracranial infusions of vehicle solution alone suggest that elements of an inflammatory or glial response to cannula insertion may have stimulated the rubrospinal neurons. The failure to observe a reversal of atrophy, an increase in RAG expression, and the promotion of axonal regeneration with the spinal cord application of BDNF in the chronic setting could be similarly explained by a failure to elicit such a response in surrounding tissue. Resolving the actual mechanism by which the BDNF is acting would of course shed further light into the obstacles that must be overcome to promote axonal regeneration in a chronic injury setting.

7.4. FUNCTION OF TRKB RECEPTORS IN THE CHRONICALLY INJURED RUBROSPINAL SYSTEM

If we were to make the reasonable assumption that it is in fact the presence of full length TrkB receptors in the rubrospinal system that makes it responsive to BDNF, it is interesting to consider how they are actually working to effect this response, particularly given that we are applying the neurotrophic factor to the cell body or to the axon. How does BDNF activation of full length TrkB receptors on the cell body translate into the changes in cell size, gene transcription, and axonal regeneration that we observed? How different is this from the activation of these receptors on the axons (for example, in the acute injury setting)? As long as the TrkB receptors are present, would cell body and spinal cord administration of BDNF have the same effect?

Much has been published on the downstream signalling that follows Trk receptor activation, reflecting a widespread interest in the biologic mechanisms by which neurotrophic factors exert their actions. Most of this literature is on the TrkA receptor, but there is likely much overlap in the intracellular pathways activated by TrkA and TrkB receptors. Neurotrophin binding to the extracellular domain of Trk receptors induces receptor dimerization and kinase activation. The phosphorylation of specific cytoplasmic tyrosine residues then attracts PLC-γ and adaptor proteins such as SHC and SH2-B (Patapoutian and Reichardt, 2001). Atwal et al. demonstrated that for TrkB receptors, SHC binding is coupled to the activation of the extracellular signal regulated kinase (ERK) protein kinase pathway and to the activation of the survival factor Akt via the phosphatidylinositol-3-OH kinase (PI3K) pathway (Atwal et al., 2000). Downstream effects of the activation of these pathways include the elevation of

intracellular calcium, or changes in gene transcription. The exact molecular mechanisms by which this ultimately translates into the outcome measures that we studied – notably, the increases in cross sectional area of neurons, ISH for GAP-43 and Tα1 tubulin, and axonal regeneration – is not entirely clear.

It is, nonetheless, intriguing to consider that the intracellular signalling pathways initiated in rubrospinal neurons by BDNF administration at the cell body might actually be different than what occurs when it is delivered to the injured spinal cord – ie. that the activation of TrkB receptors on the cell bodies initiates different signalling pathways than activation of TrkB receptors on the axons. If this were the case, the cell body administration of BDNF would actually represent a fundamentally different treatment paradigm than the spinal cord administration of BDNF (and not one distinguished only by the effectiveness after a delay in intervention). Indeed, while such *in vivo* data is not currently available, Watson et al. studied DRG neurons in compartmented cultures and reported that the different intracellular signalling pathways could be initiated by the same stimulus depending on if the stimulus occurred at the cell body or at the axonal level (Watson et al., 2001). In this study, the authors found that neurotrophic factor administration to the cell bodies activated CREB via the stimulation of both Erk1/2 and Erk5. Neurotrophic factor administration to the axons activated both Erk1/2 and Erk5 locally within the axon, but retrograde CREB activation at the cell body caused by Erk5 only, implying that changes in gene transcription at the nuclear level were mediated by different intracellular pathways depending on where the neurotrophic stimulation occurred (cell body versus axon) (Watson et al., 2001). Conversely, local activation of Erk1/2 at the level of the axon may itself contribute to axonal outgrowth independent of a cell body response, possibly as the result of the phosphorylation of microtubule-associated proteins and modulation of other

cytoskeletal proteins (Atwal et al., 2000).   These studies point to differences between the intracellular sequelae of neurotrophic factor administration at either the cell body or axon - a distinction that certainly could have some relevance to my current observations within the rubrospinal system. My findings at the two month time point after axotomy would suggest that the lack of a regenerative response seen with spinal cord application of BDNF was related to the loss of TrkB receptors. However, even if TrkB receptors were still maintained on the chronically injured axons, one could also consider the possibility that for the outcome measures that we were studying (neuronal cross sectional area, GAP-43 and Tα1 tubulin expression, and axonal regeneration), TrkB activation at the cell body (via BDNF administration) simply activated the necessary signalling pathways in the chronic setting that TrkB activation at the axons did not.

Along the same lines, it is interesting to consider how such a retrograde signal (eg. TrkB activation via BDNF) at the axon is transferred back to the cell body in the acute, let alone the chronic state. Ultimately, this has a profound influence on the therapeutic strategy of providing neurotrophic factors directly to the injured spinal cord, assuming that successful long-distance axonal regeneration requires some form of participation from the cell body. A number of potential mechanisms have been proposed for the means by which neurotrophic factor stimulation at the axonal level initiates a signal that is retrogradely transmitted back to the cell body (reviewed by Miller and Kaplan, 2001 and 2002 and by Ginty and Segal, 2002). Binding of the neurotrophic factor to the Trk receptor may cause internalization of the Trk receptor, with or without the neurotrophic factor, into a vesicle which is then retrogradely transported back to the cell body (Watson et al., 1999, Bhattacharyya et al., 1997). The internalization of the Trk receptor occurs in such a manner as to leave the intracellular domains of the receptor on the outside of the vesicle where they can continue to initiate downstream signalling (thus being

described as "signalling endosomes" (Beattie et al., 1996, Ye et al., 2003). Alternatively, there may be a rapid "wave" of Trk receptor activation initiated by the neurotrophin binding but then propogated along the course of the axon without vesicular transport (Senger and Campenot, 1997). Finally, intracellular signalling proteins such as PI-3 kinase might be activated at the axon level and serve as the retrograde activation signal to the cell body (Kuruvilla et al., 2000).

While these methods of retrogade signalling are not mutually exclusive, it is possible that these pathways themselves are affected in the chronic injury state. For example, given all the other molecular changes that have occurred in chronically injured rubrospinal neurons after cervical axotomy, what is to say that the retrograde transport mechanisms required to move signalling endosomes back to the cell body have remained intact? It is interesting, however, to note that Storer and Houle found that while the application of BDNF to the spinal cord 1 month after axotomy did not lead to changes in rubrospinal cross sectional area or GAP-43 expression, the application of GDNF did (Storer et al., 2003). GDNF binds to GDNF-Family Receptor-α 1 (GFRα1) then complexes with Receptor Tyrosine Kinase (RET), which, like TrkB receptors, possesses a number of intracellular tyrosine kinase domains that serve as docking sites for adaptor proteins (Airaksinen and Saarma, 2002). Similar to TrkB receptors, phosphorylation of RET allows for SHC binding and subsequent activation of many of the same intracellular signalling cascades, including the PI3K, ERK, and MEK5 (Takahashi, 2001). If one were to make the assumption that these kinase pathways function similarly whether they are activated by the autophosphorylation of TrkB receptors or the autophosphorylation of RET, then the reversal of rubrospinal atrophy with the application of GDNF (Storer et al., 2003) would indicate that the mechanisms by which retrograde signalling is accomplished are indeed intact in this chronic setting (one month post-injury).

The implications of this discussion on the development of therapeutic strategies involving neurotrophic application in the chronic injury setting are clear: if the failure of BDNF application is solely related to the expression of TrkB receptors on the axons at the chronic injury site, then there would be a compelling rationale to pursue a strategy by which TrkB expression might be augmented, possibly by genetic manipulation. If, however, dysfunction of other intracellular mechanisms downstream from Trk activation would preclude effective retrograde transmission of such a signal, then establishing neurotrophic factor therapy at the chronic injury setting becomes exponentially more complex. Targeting the cell body directly, as I have done with the intracranial infusion of BDNF, might then represent a more appealing strategy. From a purely practical point of view, however, the clinical application of such an intervention would be difficult to justify, as it would require inserting a needle through otherwise uninjured brain tissue. Also, it would be difficult to anticipate the effect of BDNF or the inflammatory response to the insertion device on other neuronal systems nearby. Clearly, an intervention at the spinal cord that could retrogradely effect the necessary changes within the cell body for axonal outgrowth would be more desirable. The recent report of Koda et al in which a BDNF-encoding adenovirus was applied to the acute spinal cord injury site and was found to successfully transfect rubrospinal neurons (Koda et al., 2004) demonstrates that technologies may become available to effect the necessary regenerative changes at the cell body level with an intervention directed at the chronically injured spinal cord.

7.5. FUTURE DIRECTIONS

This research has provided some insight into the chronic spinal cord condition, particularly as it applies to the rubrospinal system. While these findings shed some light on the challenges that may be faced in the development of therapeutic strategies to promote axonal regeneration in the chronic injury setting, clearly, much remains to be done to realize this goal.

1. What is happening to TrkB expression within the injured rubrospinal system?

Given the interest in applying neurotrophic factors within the spinal cord, it would be useful to better understand what is happening to TrkB expression within the injured rubrospinal axons. As stated in chapter 6, it is unclear how far back towards the cell body the loss of TrkB receptors on injured rubrospinal axons extends. For this, it would be useful to sample the chronic injury site in cross section instead of longitudinally (as was performed in my experiments) in order to evaluate the anterogradely labeled rubrospinal axons serially as one moves away from the injury site. In such an experiment, one could determine at fairly precise distances from the injury site how many BDA labeled axons also contained TrkB immunoreactivity, and how many did not. For example, if all BDA labeled axons 2 mm rostral to the injury contained TrkB immunoreactivity, then one would infer that the loss of TrkB receptors is only a very local phenomenon confined to the injury site alone. If, however, the ratio BDA and TrkB labeled axons compared to the total number of BDA labeled axons was low or continued to decrease as one moved more rostrally, it might be inferred that the TrkB receptors were lost along the entire length of the axon. (Figure 7.1)

Figure 7.1. **Experimental paradigm to characterize the loss of TrkB receptors on rubrospinal axons.**

Serial cross sectional analysis of anterogradely labeled rubrospinal axons with TrkB immunohistochemistry may help to determine whether the loss of TrkB receptors occurs only adjacent to the axotomy site (below, left) or along the entire length of the axon (below, right). By counting the number of anterogradely labeled axons and then determining how many of them are co-labeled with TrkB, one may be able to make this distinction. If the loss of receptors is restricted only to the injury site, then presumably, further rostrally, all of the labeled axons will also contain TrkB immunoreactivity (below left). If, however, the loss of receptors occurs along the entire length of the axon, back to the axon hillock, as one moves rostrally, there will be a combination of both BDA labeled axons with TrkB (those that aren't injured distally) and BDA labeled axons without TrkB (those that are injured distally).
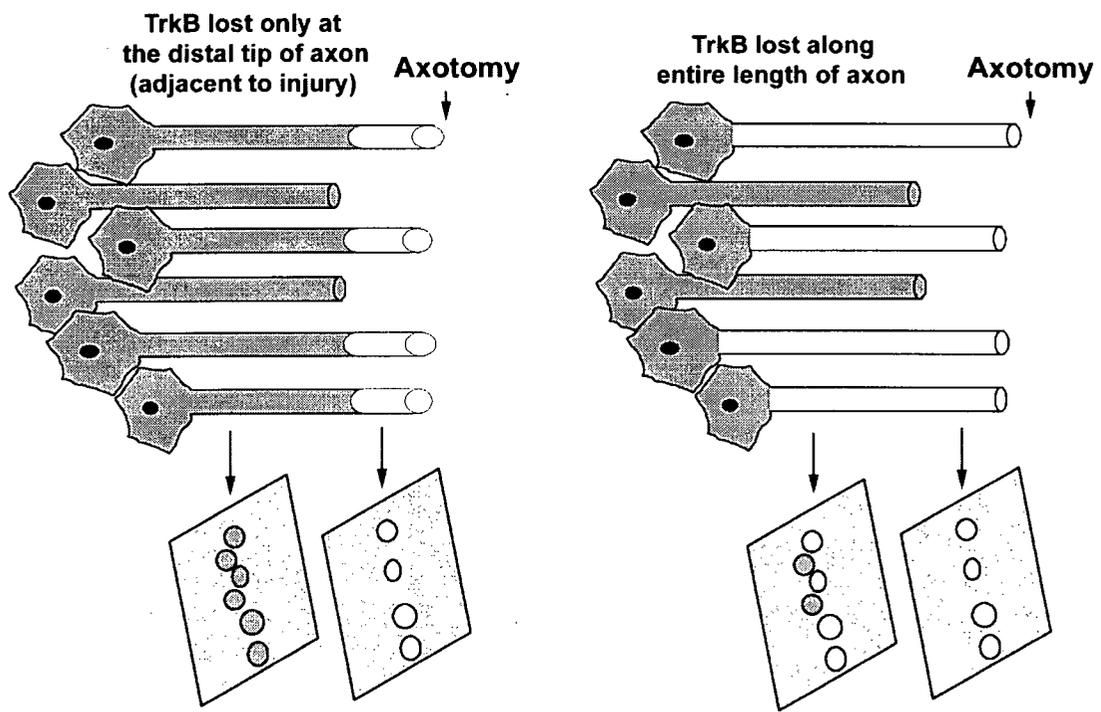
Serial cross-sections of spinal cord, starting from the axotomy site and moving rostrally

2. *What is the true therapeutic potential of cell body application of BDNF in the chronic injury state?*

My experiments demonstrated that cell body application of BDNF 12 months after cervical axotomy could promote axonal regeneration into a free-ending peripheral nerve transplant. Clearly, it would be interesting to know whether this intervention can lead to intraspinal regeneration or sprouting, either alone or in conjunction with a strategy to address the inhibitory injury environment, such as a bridging peripheral nerve graft or olfactory ensheathing cells. BDNF infusion into the parenchyma of the motor cortex, for example, was found to promote sprouting of corticospinal fibers after thoracic spinal cord injury, although regeneration into peripheral nerve transplants was not observed (Hiebert et al., 2002). Pilot studies in our lab are underway with the transplantation of bridging peripheral nerve grafts within the spinal cord to evaluate the ability of cell body application of BDNF to drive rubrospinal axonal regeneration into these grafts and back into the injured spinal cord. Furthermore, it would be of interest to evaluate whether a combinatorial strategy such as this could lead to some functional recovery, although in such experiments it is often difficult to ascribe a certain functional change to the regeneration of a specific axonal tract. Nonetheless, the role of the rubrospinal tract in upper and lower extremity function is a topic of interest in our lab and in others (Muir and Whishaw, 2000, Whishaw et al., 1990) and as this is better elucidated, it the interpretation of such studies will become easier.

3. The cell body infusion of BDNF is highly invasive and clearly invokes some damage to surrounding parenchyma – is there a more sophisticated way to invoke the same effects?

Clearly, while the findings of rubrospinal cell survival and axonal regeneration with cell body application of BDNF are promising, this really represents a proof of concept experiment. Our laboratory has performed pilot studies with a BDNF encoding adeno-associated virus and has found that the intracranial injection into the vicinity of the red nucleus appeared to effect a trophic response in cervically axotomized rubrospinal neurons with less of an inflammatory response in the surrounding brainstem parenchyma. Nevertheless, it would obviously be more desirable to achieve the same responses by intervening at the spinal cord injury site. As stated earlier, the application of a BDNF-encoding adenovirus to the spinal cord injury site after acute injury achieved some transfection of rubrospinal neurons; it is anticipated that these investigators are currently looking into the applicability of such technology at a chronic time point.

7.6. CONCLUSIONS

Much remains to be answered about the neurobiology of chronic spinal cord injury and the development of axonal regeneration strategies to effect functional recovery. The need is compelling nonetheless, as the population of individuals with chronic spinal cord injuries only increases each year. The survival of chronically injured rubrospinal neurons and their potential to mount a regenerative response long after injury given the appropriate stimulus is encouraging nonetheless, and such findings will hopefully stimulate further work in this challenging but important line of research.

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