SIGNALS REGULATING NEURONAL
CELL BODY RESPONSES TO AXOTOMY

by

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ABSTRACT

Spinal cord injury in higher vertebrates causes paralysis because injured neurons in the adult central nervous system (CNS) often atrophy or die, and fail to regenerate their severed processes ("axons"). In contrast, injured peripheral nervous system (PNS) neurons usually survive and regenerate their axons. One reason for successful PNS responses to axon injury ("axotomy") is that they undergo numerous changes in gene expression that enhance their intrinsic growth state. These changes include the up-regulation of proteins required for axon growth, and down-regulation of non-essential proteins, such as those used mainly for neurotransmission. Similar changes in gene expression are generally only weak and transient in axotomized CNS neurons, which most likely contributes to their poor responses to injury. This thesis investigates the potential regulation of injury-induced changes in gene expression by neurotrophic factors (NTFs), small proteins important for development and maintenance of the nervous system. Three principal findings are presented here.

Firstly, in axotomized PNS motoneurons, the down-regulation of non-essential proteins associated with neuronal maturation, such as neurotransmitter enzymes and neurofilaments appears to be due to the interrupted supply of target-derived neurotrophic factors.

Secondly, in axotomized PNS motoneurons, the robust up-regulation of regeneration-associated genes and cell body hypertrophy is enhanced by non-target-derived neurotrophic factors, most likely those released by Schwann cells and immune cells at the actual site of injury. In fact, a second axon injury (to stimulate release of these endogenous neurotrophic factors) was sufficient to actually reverse the severe cell
body atrophy and up-regulate the regeneration-associated gene expression of chronically axotomized motoneurons.

Thirdly, when exogenous neurotrophic factors were used to supplement a CNS spinal cord lesion site, which does not contain Schwann cells and has only limited invasion of immune cells, the regenerative cell body responses of axotomized CNS rubrospinal neurons were greatly enhanced.

These findings provide insights into the signals regulating neuronal gene expression after injury, and identify neurotrophic factors at the site of axotomy as an important determinant of a neuron's regenerative response to injury.
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List of Abbreviations

AChE - acetylcholinesterase
BDNF - brain-derived neurotrophic factor
ChAT - choline acetyltransferase
CNS - central nervous system
CNTF - ciliary neurotrophic factor
cpm - counts per minute
d - day
DRG - dorsal root ganglion
EDTA - ethylene-diaminetetra-acetic acid
FGF - fibroblast growth factor
FMN - facial motoneuron
GAP-43 - growth-associated protein-43
IGFs - insulin-like growth factors
IL-6 - interleukin-6
ISH - in situ hybridization
LIF - leukemia inhibitory factor
NFM - neurofilament-M (medium)
NGF - nerve growth factor
NT-3 - neurotrophin-3
NT-4/5 - neurotrophin-4/5
NTF - neurotrophic factor
PCR - polymerase chain reaction
PNS - peripheral nervous system
RAG - regeneration-associated gene
RS - rubrospinal
RT-PCR - reverse transcription-polymerase chain reaction
SD - standard deviation
SEM - standard error of the mean
SSC - sodium chloride/sodium citrate
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CHAPTER 1

BACKGROUND

1.1 OVERVIEW

One of the most striking morphological features of a neuron, which makes it different from all other cells in the body, is its axonal process. For example, the axons of spinal cord motoneurons innervating the distal muscles of our feet, or of brainstem neurons innervating the lumbar levels of the spinal cord, can easily reach one meter in length. Despite this length, the axon is typically smaller than 10 micrometers (μm) in diameter, and the neuronal cell bodies rarely exceed 50 μm in diameter. As a consequence of this highly assymmetrical morphology, recovery from axon injury ("axonotomy") imposes unique challenges. Since the disconnected axon degenerates, the axon must re-grow from its proximal stump to its target tissues, a process requiring materials provided from the cell body ("axon regeneration").

When axon injury occurs within the brain or spinal cord of higher vertebrates, the cell bodies of the axotomized central nervous system (CNS) neurons often undergo atrophy and/or death, and typically fail to regenerate their axons (Ramon y Cajal, 1991). The failure of axotomized CNS neurons to regenerate their axons following injury to the spinal cord results in a permanent disruption of motor pathways from the
brain to the periphery, which is manifest as paralysis. In sharp contrast, when axon injury occurs outside the brain and spinal cord, the cell bodies of axotomized peripheral nervous system (PNS) neurons survive, and their axons regenerate from the injury site to their targets (reviewed in Fu and Gordon, 1997).

The cell bodies of axotomized PNS neurons undergo a wide spectrum of changes in gene expression, which enable them to (i) survive the injury (reviewed in section 1.2), and (ii) produce high levels of proteins required for the structure and function of a growing axon (reviewed in section 1.3). An intriguing and important question is how axon injuries trigger regenerative changes in the relatively distantly located neuronal cell bodies. Furthermore, why does axon injury fail to induce these regenerative cell body responses from axotomized CNS neurons? Although a number of mechanisms have been postulated by which axon injury might trigger changes in the neuronal cell body (reviewed in section 1.4), relatively little is known. Thus, the overall objective of this thesis (reviewed in section 1.5) is to investigate the signals regulating neuronal cell body responses to injury. As experimental models for this work, PNS motoneurons and CNS rubrospinal neurons of adult rats and mice were used (reviewed in section 1.6).
1.2 SURVIVAL OF AXOTOMIZED NEURONS – NEUROTROPHIC FACTORS (NTFs)

1.2.1 PNS neurons require neurotrophic factors for survival

An unusual feature of neurons is their constant requirement for specific protein survival factors, known as neurotrophic factors (NTFs). The mechanisms by which NTFs promote neuronal survival have not been fully elucidated, so the reasons for this dependence are not entirely understood. Since neurons are post-mitotic cells (i.e. are no longer capable of proliferation), one possibility is that NTFs maintain neuronal survival by preventing inappropriate re-entry into the cell cycle (Farinelli and Greene, 1996; Park et al., 1996; Park et al., 1997).

During development, the main source of NTFs for PNS neurons appears to be neuronal target tissues (reviewed in Davies et al., 1994). However, when axotomy deprives mature PNS neurons of their target-derived neurotrophic factors, their survival is maintained by abundant supplies of non-target-derived NTFs (Korsching, 1993, Oppenheim, 1996). The sources of these NTFs include the Schwann cells and invading macrophages of the distal nerve segment (BDNF, NT-3, NT-4/5, CNTF, LIF, GDNF, IGFs, interleukins), the axotomized neurons themselves (BDNF, FGF-2, IL-6) and their perineuronal glial cells (LIF, CNTF, IL-6, and TGF-β), as well as afferents which terminate on the cell bodies of the axotomized neurons (BDNF) (references given below).

Two families of NTFs appear to be particularly important for survival of axotomized neurons: the neurotrophins (i.e. NGF, BDNF, NT-3, and NT-4/5) and the gp130-dependent cytokines (i.e. CNTF, LIF, and IL-6). Direct evidence for the
involvement of these factors in maintaining survival of axotomized PNS neurons has been provided by gene knockout studies, and is summarized below. Taken together, the studies described below indicate that PNS neurons are exposed to a sufficient quantity of trophic support to maintain survival of the majority of the population after disconnection from their targets. This is correlated with increased retrograde transport of neurotrophins and cytokines after peripheral axotomy (Curtis et al., 1993, Curtis et al., 1994, Curtis et al., 1998). Although many of the neurotrophic factor signalling pathways converge, their actions can be synergistic (Mitsumoto et al., 1994, Rajan et al., 1998, Wu and Bradshaw, 1996), and in some cases, they may have differential effects on health of the neuronal cell body versus axon (Haase et al., 1997, Sagot et al., 1998).

1.2.2 The trkB ligands, BDNF and NT-4/5

Axotomized sensory neurons and motoneurons up-regulate their expression of the trkB high affinity receptor for the neurotrophins BDNF and NT-4/5, suggesting that these ligands may be important factors contributing to neuronal survival after injury (Ernfors et al., 1993, Kobayashi et al., 1996a, Piehl et al., 1994, Tonra et al., 1998). In accord with this idea, in trkB knockout mice there are 50% fewer countable facial motoneurons than in wildtype mice five days after transection of the postnatal day 5 mouse facial nerves (Alcantara et al., 1997). There appear to be multiple non-target-derived sources of the trkB ligands following axotomy. BDNF and NT-4/5 are produced by reactive Schwann cells in the vicinity of an axon injury site (Funakoshi et al., 1993). BDNF is also expressed by sensory and motoneurons themselves, which up-regulate its expression after axotomy (Kobayashi et al., 1996a, Tonra et al., 1998). In
addition, BDNF is anterogradely transported in the CNS (Altar et al., 1997, Fawcett et al., 1998), and over-expression of BDNF in catecholaminergic neurons that synapse onto facial motoneurons rescues the facial motoneurons from neonatal axotomy-induced death (Fawcett et al., 1998); thus, afferent inputs may constitute an additional non-target-derived source of trophic support.

1.2.3 The gp130 activators, CNTF, LIF, and IL-6

Axotomized motoneurons also up-regulate gp130 (Yao et al., 1997), the common signal transducing component for the receptor complexes of the CNTF/LIF/IL-6 family of cytokines (Heinrich et al., 1998, Murphy et al., 1997). CNTF is synthesized by myelinating Schwann cells and perineuronal astrocytes (Friedman et al., 1992, Sendtner et al., 1994, Sendtner et al., 1992). Although it is not known to be actively secreted, CNTF appears to be released from injured Schwann cells (Sendtner et al., 1992), and its retrograde transport is enhanced following axotomy (Curtis et al., 1993). Studies of CNTF knockout mice have demonstrated that it is an endogenous survival factor for both normal (Masu et al., 1993) and axotomized (Sendtner et al., 1997) motoneurons. Unlike CNTF, whose expression in the distal stump decreases after axotomy (Sendtner et al., 1992), LIF production increases in the injured nerve (Curtis et al., 1994). LIF/CNTF double knockout mice have a 35% reduction in countable facial motoneurons 14 days after facial nerve axotomy of 4 week old mice (Sendtner et al., 1996a). Recent studies have also demonstrated a role for a third gp130-dependent member of this cytokine family. IL-6 is normally up-regulated in axotomized sensory and motoneurons (Klein et al., 1997, Murphy et al., 1999, Murphy et al., 1995). It may
act in an autocrine/paracrine fashion, but it also appears to be involved in the activation of perineuronal glial cells (Klein et al., 1997). IL-6 knockout mice have a 20% reduction in survival of axotomized adult sensory neurons after two weeks, as well as impaired regeneration of sensory axons (Murphy et al., 1999, Zhong et al., 1999).

**1.2.4 Survival of CNS neurons**

Like PNS neurons, axotomized CNS neurons also rely on NTFs for their survival. However, endogenous NTF levels appear inadequate to maintain their survival. For example, BDNF function-blocking antibodies reduce the survival of axotomized corticospinal neurons from 50% to 30% (Giehl et al., 1998), while exogenous BDNF infused into the cortex rescues the entire population (Giehl and Tetzlaff, 1996). Similarly, cervically axotomized rubrospinal neurons undergo considerable atrophy, which can be prevented by infusion of BDNF into the vicinity of the red nucleus (Kobayashi et al., 1997). The lack of adequate trophic support may be due to the fact that, unlike peripheral Schwann cells of the distal nerve stump, oligodendrocytes are not known to produce significant levels of neurotrophins or gp130-dependent cytokines after axonal injury. Furthermore, CNS neurons may lack autocrine/paracrine support, as comparatively little trophic factor expression has been detected in CNS neurons after injury. For example, although both motoneurons and rubrospinal neurons express the NTF receptors trkB and FGFR-1 (fibroblast growth factor receptor-1), axotomized motoneurons up-regulate these receptors and their ligands BDNF and FGF-2, while axotomized rubrospinal neurons decrease their expression of these receptors and ligands (Kobayashi et al., 1996b, Stilwell et al., 1997).
Together, these studies demonstrate that neurons denied access to their target-derived supplies of NTFs by axotomy must acquire at least a minimal level of NTFs from alternative sources. These alternative sources may be from their environment or in an autocrine fashion.
1.3 GROWTH OF INJURED AXONS - REGENERATION-ASSOCIATED GENES (RAGs)

The likelihood that a neuron surviving axotomy will regenerate its axon is strongly influenced by both the axonal environment and the intrinsic growth state of the neuron. For example, the success of PNS regeneration is facilitated by a favourable environment for the growing PNS axons. Macrophages rapidly clear the severed axons and their myelin debris, while reactive Schwann cells express cell adhesion molecules and align themselves into Bands of Büngner to guide the growing axons back to their targets ("Wallerian Degeneration") (reviewed in Fu and Gordon, 1997). In contrast, mutant mice having a defect in Wallerian Degeneration exhibit markedly slowed PNS axon regeneration (Brown et al., 1994; Bisby et al., 1995). The axonal environment also exerts a powerful influence on axonal growth in the CNS: since macrophage invasion is relatively limited, the highly growth-inhibitory components of CNS myelin fail to be efficiently cleared, and regeneration typically does not occur. However, CNS axon growth is improved by transplanting activated macrophages into the CNS (reviewed in Schwartz et al., 1999), by using antibodies to mask inhibitory myelin epitopes (Schwab and Brosamle, 1997), by vaccinating against myelin components (Huang et al., 1999), or by experimentally removing myelin with a complement-mediated lysis approach (Keirstead et al., 1995; Dyer et al., 1998).

The preceding examples illustrate the powerful influence of the axon environment on axon growth. However, how an axon reacts when confronted with a particular environment is determined by the intrinsic growth state of the axotomized
neuron. For example, axotomized CNS neurons having a weak intrinsic growth state fail to grow even when a permissive growth environment is offered (Fernandes et al., 1999; see figure 1 below). Conversely, axotomized PNS sensory neurons have a strong intrinsic growth state, which can allow them to over-ride the growth-inhibitory influences associated with the CNS microenvironment (Davies et al., 1994, Davies et al., 1997; Neumann and Woolf, 1999). For this reason, identifying how the intrinsic growth state is regulated is an important objective for CNS regeneration research. The intrinsic determinants of the growth state are described in the remainder of section 1.3.

1.3.1 Evidence that intrinsic neuronal properties regulate the vigour of axonal growth

The capacity for axonal growth varies between neurons, and is highly affected by properties such as the neuron’s age, type/localization in the nervous system, and the distance of the axotomy from the neuronal cell body (Lieberman, 1974, Ramon y Cajal, 1991). Immature neurons generally have a greater intrinsic growth propensity than adult neurons (Chen et al., 1995, Davies et al., 1994, Dusart et al., 1997, Li et al., 1995a, Wictorin et al., 1990). This concept has been illustrated using cross age cocultures of retinal ganglion cells with tectum explants (Chen et al., 1995) or entorhinal cortex neurons with dentate gyrus explants (Li et al., 1995a). Within the mature nervous system, various neuron types respond to injuries differently; for instance, while many CNS neuron types readily extend axons into peripheral nerve or embryonic tissue transplants, cerebellar Purkinje cells are notoriously reluctant to regenerate in these paradigms (Dusart and Sotelo, 1994, Rossi et al., 1997, Rossi et al.,
This variation in neuronal growth capacity is further complicated by the demonstration that individual CNS neurons exhibit different growth capabilities depending on whether they are axotomized relatively close to or distant from their cell bodies. Figure 1. Rubrospinal neurons regenerate into peripheral nerve transplants after cervical (proximal) but not thoracic (distal) axotomy. (A) The rubrospinal tract was severed at either the C3 or T10 level of the rat spinal cord, and an autologous sciatic nerve graft was implanted into the lesion site. 2 months later the free end of the graft was exposed to 5% FluoroGold, and 14d later the animals were sacrificed. (B) Numerous retrogradely labelled rubrospinal neurons were found in the axotomized red nucleus of cervically axotomized rats. (C) No FluoroGold-positive neurons were identified in the red nucleus after thoracic axotomy. (D) Numbers of regenerating rubrospinal neurons after C3 or T10 procedures. Modified from Fernandes et al., 1999.
bodies (see Figure 1) (Doster et al., 1991, Fernandes et al., 1999, Richardson et al., 1984, Tetzlaff et al., 1991).

Appropriate neuronal gene expression is required for successful neuronal responses to axotomy (ie. neuronal survival and rapid axonal regeneration). For example, in vitro experiments (Smith and Skene, 1997) demonstrated that DRG neurons grow long extended axons if they receive a peripheral nerve axotomy prior to explantation, but only short and highly branched axons if explanted without a prior axotomy. Peripheral axotomy is believed to induce the transcription of new gene products required for axon regeneration, as the "regeneration" growth mode was lost in the presence of RNA synthesis inhibitors.

What are the regeneration-associated genes (i.e. "RAGs") induced by peripheral nerve injury? Axotomized neurons undergo a wide spectrum of changes in gene expression, which affect levels of transcription factors, cytoskeletal proteins, growth cone proteins, cell adhesion and guidance proteins, secreted proteins, ion channels, signaling molecules, and a variety of other stress response/homeostatic/housekeeping proteins (table 1 at the end of chapter 1). The precise significance of many of the changes in Table 1 is uncertain. However, of the vast array of changes in gene expression listed in table 1, there are certain genes that are inevitably expressed during axonal regeneration of PNS neurons. These genes include up-regulated expression of (i) cytoskeletal proteins, such as tubulins and actin, (ii) cytoplasmic growth cone proteins, most prominently GAP-43, and (iii) the transcription factor c-jun. At the same time, axotomized PNS neurons consistently down-regulate (i) neurofilaments, and (ii) neurotransmitter synthesizing enzymes. These changes in gene expression reflect an
enhanced intrinsic growth state, as they are expressed in neurons capable of rapid axonal regeneration, such as PNS neurons or CNS neurons regenerating into peripheral nerve transplants. In this thesis, I have used the expression of tubulins, neurofilaments, GAP-43, c-jun, and the cholinergic enzyme acetylcholinesterase as markers of the pattern of genes expressed by axotomized neurons. The functions and importance of these and other RAGs for axonal regeneration, at least in the PNS, is reviewed below.

1.3.2 Cytoskeletal proteins – tubulins and neurofilaments

Axotomized PNS neurons exhibit a variety of changes in expression for cytoskeletal proteins (Bisby and Tetzlaff, 1992). For instance, tubulins and actin are strongly up-regulated, while the intermediately sized neurofilaments are sharply down-regulated. The cytoskeletal proteins examined in this thesis are tubulin and neurofilaments.

Microtubules and their constituent tubulin molecules are of central importance for cell motility and are critically involved in the process of axonal transport and elongation of the axonal shaft (comprehensively reviewed in (Kobayashi and Mundel, 1998, Laferriere et al., 1997). Several tubulin isotypes (in mouse, 7 α and 6 β isotypes) exist, but the high tubulin expression during developmental and regenerative axonal elongation is due to the selective up-regulation of the Tα1 α-tubulin isotype and the βII and βIII β-tubulin isotypes. The exact chemical significance of these specific isotypes is unclear, as in many studies the various α or β isotypes appear to be functionally redundant. However, the strong up-regulation of tubulins is most likely required as
substrate for elongation of the microtubule-based cytoskeleton. Axotomized PNS neurons maintain their high expression of tubulins throughout the process of axonal regeneration, and indefinitely if target reconnection is prevented (Jiang et al., 1994).

The intermediate filaments consist of the type IV neurofilaments, α-internexin, and nestin, and the type III peripherin (Lee and Cleveland, 1996). The type IV intermediate filaments are down-regulated by axotomy, and are therefore likely not critical to axonal regeneration per se. For example, motoneurons in mice lacking neurofilaments are capable of axonal regeneration, but have hampered axonal maturation (Zhu et al., 1997). Down-regulation of the neurofilament network may, in fact, facilitate the axonal transport of actin and tubulins (Tetzlaff et al., 1996, Zhu et al., 1998), as neurofilaments interact with both tubulin and actin, and are as much as 10 fold more abundant within the axon than microtubules (Hirokawa et al., 1988). Supporting this idea, the rate of tubulin transport is dramatically increased in mice lacking the neurofilament light chain, and reduced in mice over-expressing the neurofilament heavy chain (Collard et al., 1995). Interestingly, in some non-mammalian vertebrates whose CNS neurons do regenerate, high levels of neurofilaments are observed (Asch et al., 1998, Jacobs et al., 1997, Zhao and Szaro, 1995). This may be due to differences in the chemical properties of neurofilaments between mammals and various other vertebrates (Pleasure et al., 1989).

In comparison to the PNS, changes in cytoskeletal protein expression is significantly less robust in non-regenerating axotomized CNS neurons, such as corticospinal neurons (Kost and Oblinger, 1993), transcollosal cortical neurons (Elliott et al., 1999), retinal ganglion cells (Fournier and McKerracher, 1997), or rubrospinal
neurons (Tetzlaff et al., 1991). However, if provided with a growth-permissive peripheral nerve environment, those CNS neurons regenerating into the transplants exhibit high expression of tubulins (Fournier and McKerracher, 1997, Kobayashi et al., 1997). This observation may indicate the presence of trophic molecules within the peripheral nerve transplants capable of enhancing tubulin gene expression.

1.3.3 Growth cone proteins mediating plasticity of the terminal axon – GAP-43

The major functions of the axonal growth cone are to sense the axonal environment and direct the growth of the axon in response to appropriate cues. Classical axonal transport studies by Skene and co-workers identified substantial increases in the levels of specific proteins within the fast axonal transport fraction of injured peripheral nerves, that are virtually undetectable in normal nerves (reviewed in Skene, 1989). Of these Growth Associated Proteins (GAPs), the most intensely studied has been the 27.6 kD protein GAP-43 (F1, B-50, neuromodulin).

GAP-43 protein has been demonstrated to interact with several other growth cone proteins, including cytoskeletal proteins (actin, spectrin, fodrin), calmodulin, and the α subunits of the G proteins (reviewed in Benowitz and Routtenberg, 1997). It is believed to be involved in the transduction of extracellular signals to the actin based sub-membrane cytoskeleton. In support of this belief, gene deletion shows that GAP-43 is necessary for FGF receptor-mediated increases in growth cone motility in response to cell adhesion molecules N-CAM and L1 (Meiri et al., 1998). In the context of CNS regeneration, it is interesting to note that GAP-43 over-expression in vitro renders DRG growth cones more resistant to myelin-associated growth cone inhibitors (Aigner and
Other functionally-related proteins, such as CAP-23, MARKS and paralemmin have many similar properties to GAP-43 (Kutzleb et al., 1998, Wiederkehr et al., 1997) and unpublished data from our laboratory show that the expression of at least CAP-23 (others not studied) is also increased after axonal injury.

Neuronal expression of GAP-43 has been a useful and reliable marker of an enhanced intrinsic growth state. Ample evidence correlates the induction of GAP-43 expression in axotomized neurons with their subsequent growth propensity. For example, the expression of GAP-43 is virtually undetectable in most parts of the mature nervous system, but it is highly expressed in developing and regenerating PNS neurons (Fernandes et al., 1999, Schreyer and Skene, 1993, Skene, 1989, Tetzlaff et al., 1991). Within the mature CNS, basal levels of GAP-43 expression are typically very low in most areas, though it remains highly expressed in some regions such as the neocortex. Induction of GAP-43 following axotomy in the CNS is generally extremely limited, and as in the PNS, rapid axonal regeneration into peripheral nerve transplants only occurs in those situations where CNS neurons express GAP-43, i.e. with more proximal (GAP-43-inducing) injuries (Doster et al., 1991, Fernandes et al., 1999, Richardson et al., 1984). It is important to note that GAP-43 expression is not, by itself, sufficient for axonal regeneration. Specific transgenic over-expression of GAP-43 did not stimulate axonal regeneration by axotomized Purkinje cells, but did increase axonal sprouting (Buffo et al., 1997). Neither is GAP-43 absolutely necessary for axonal growth per se. GAP-43 knockout mice appear to have a grossly normal nervous system, but exhibit defects in axonal pathfinding (Kruger et al., 1998, Sretavan and Kruger, 1998, Zhu and Julien, 1999). However, GAP-43 is presumably regulated in a similar fashion.
as a full complement of RAGs involved in axonal growth, which when expressed together, enhance the intrinsic ability of axons to grow. For example, neurons over-expressing GAP-43 and a closely related growth-associated protein, CAP-23, have greater growth properties than those over-expressing either protein alone (Caroni, 1997a, Caroni, 1997b). Taken together, these results suggest that expression of certain growth cone proteins increases the resistance of growth cones to inhibitory growth signals.

1.3.4 The c-jun transcription factor

Extensive research has investigated axotomy-induced changes in the activation state of constitutive transcription factors (i.e. CREB, CREM, ICER, ATF-1, ATF-2, SRF, NFkB) and the expression level of inducible transcription factors (i.e. c-jun, junB, junD, c-fos, fra-1, fra-2, krox-20, krox-24), (Herdegen and Leah, 1998). Of all these transcription factors, c-jun is the most highly correlated with regeneration, as it is strongly up-regulated in axotomized PNS neurons, and in CNS neurons regenerating into peripheral nerve transplants (Anderson et al., 1998, Broude et al., 1999, Hull and Bahr, 1994, Robinson, 1995).

Despite thorough investigation, the actual function of c-jun after axotomy has nevertheless remained enigmatic, as its expression is paradoxically associated with both neuronal survival/regeneration, and neuronal degeneration (Herdegen et al., 1997b). In support of a function in neuronal survival/regeneration, c-jun is induced and maintained in DRG neurons after peripheral axotomy but is only transiently up-regulated in a small proportion of them after dorsal rhizotomy (Broude et al., 1997,
Kenney and Kocsis, 1997a); allowing access of the injured dorsal roots to fetal transplant tissue promotes their growth and up-regulates c-jun expression (Broude et al., 1997). Similarly, when axotomized CNS neurons are given access to peripheral nerve transplants, expression of c-jun (like GAP-43) is selectively maintained in those neurons that regenerate into the transplants (Anderson et al., 1998, Broude et al., 1999, Hull and Bahr, 1994, Robinson, 1995). A close association of c-jun and GAP-43 expression has been widely reported (reviewed in Herdegen et al., 1997b), but their expression can also be dissociated in axotomized corticospinal neurons (K. Giehl and W. Tetzlaff, personal communication). Contrasting these findings are studies implicating c-jun in neuronal death. For instance, direct over-expression of c-jun in cultured sympathetic neurons provokes their death, while inhibiting c-jun prevents their sympathetic neuron death following NGF-deprivation (Estus et al., 1994, Ham et al., 1995). Similarly, Jun-N-terminal Kinase (JNK) inhibitors rescue neurons from apoptosis in several models of injury (Glicksman et al., 1998).

Inhibition of c-jun expression is likely to provide some indication of its functions. Although c-jun knockout mice die developmentally, retinal ganglion cells of c-jun -/- mice extended axons normally when transplanted into wildtype mice, demonstrating that c-jun is dispensable for developmental axonal growth (Herzog et al., 1999). However, c-jun may be involved in the regulation of neuropeptide expression after axotomy, as anti-sense knockdown of c-jun expression specifically inhibited expression of vasoactive intestinal polypeptide and neuropeptide Y in cultures of sensory neurons (Mulderry and Dobson, 1996).
1.3.5 Neurotransmitter synthesizing enzymes

A general pattern that is observed in axotomized PNS neurons is a shift in the cellular targets of proteins secreted from the axon. These changes may be grouped into (i) a down-regulation of proteins involved in modifying the post-synaptic cell, and (ii) an up-regulation of proteins involved in remodelling the extracellular matrix (ECM) and Schwann cell micro-environment. In the case of (i), among the down-regulated proteins are agrins and neuregulins (Bermingham-McDonogh et al., 1997, Thomas et al., 1995), which are involved in assembling and maintaining the post-synaptic structure, and neurotransmitter synthesizing enzymes, such as the cholinergic enzyme choline acetyltransferase in motoneurons. These reductions in features of the differentiated phenotype of neurons are likely passive in nature, resulting from interruption of target contact, and may contribute to axonal regeneration only by reducing non-essential metabolic costs.

1.3.6 Can axonal regeneration occur without RAG expression?

As mentioned above, expression of RAGs (especially GAP-43 and tubulins) is reliably observed in rapidly regenerating neurons, such as PNS neurons and CNS neurons that have been provided with a peripheral nerve transplant. However, it has become apparent that intermediate phenotypes of axonal growth exist, between the extremes of local sprouting and rapid long distance regeneration. As discussed below, while axonal growth per se does not require RAG expression, the mode of axonal
growth ultimately achieved within a particular environment depends on both the level/types of RAGs expressed and the growth permissiveness of the environment.

**Limited axonal growth can occur without up-regulation of RAGs**

In the growth permissive environment of the PNS, motoneurons expressing only low levels of RAGs can terminally sprout into adjacent denervated muscle fibres without up-regulating markers of RAG expression such as GAP-43 (Bisby et al., 1996). However, low levels of RAG expression allow only slow axonal elongation, even in a growth permissive environment. This is illustrated following central axotomy of DRG neurons, which have a heterogeneous baseline expression of RAGs. After injury of their centrally projecting axons, DRG neurons show little change in RAG expression - most notably no increase in GAP-43 (Schreyer and Skene, 1993). While the sub-population of DRG neurons that expresses high baseline levels of GAP-43 can still regrow at several mm/d within the permissive dorsal roots, the majority of DRG neurons have only a low baseline expression of GAP-43 and these re-grow with a markedly reduced rate of axonal elongation (Andersen and Schreyer, 1999). Thus, in the absence of a cell body response, differences in the baseline levels of RAGs result in different growth abilities.

**Rapid long distance axonal regeneration is associated with pronounced changes in RAG expression**

Rapid axonal growth (3-4 mm/d in the rodent) in the presence of pronounced changes in RAG expression consistently occurs in peripherally axotomized PNS neurons.
The strong up-regulation of RAGs that is observed in PNS neurons is contrasted by the incomplete induction of, and failure to sustain, RAG expression in axotomized CNS neurons. For example, different populations of brainstem-spinal cord neurons of the fish show different regenerative propensities after spinal cord injury. Those neurons which express a fuller complement of regeneration-associated genes, i.e. GAP-43 + L1, show greater axonal regeneration than those which increase only GAP-43, which is greater than those that show no changes at all (Becker et al., 1998). Similarly, neurons that over-express multiple growth-associated proteins, i.e. GAP-43 and CAP-23, have greater axonal growth than those that express either protein individually (Caroni, 1997a).

It is important to note that in most cases of axon growth elicited through experimental manipulations of the CNS lesion site, it remains to be determined whether these manipulations have stimulated (i) rapid axon growth via enhanced expression of RAGs (peripheral-type “regeneration”), or (ii) intermediate forms of slower/limited axonal growth, resulting from decreased environmental growth inhibition, and driven only by the pre-existing baseline levels of RAG expression. Strategies for stimulating regenerative cell body responses including RAG expression in axotomized CNS neurons is presumably a pre-requisite to enabling them to undergo rapid axonal regeneration, and should ultimately result in more vigorous and effective regeneration within the CNS.
The molecular signals triggering regenerative cell body responses following an axon injury are not clearly identified. Some thirty years ago, Cragg summarized over 10 conceivable scenarios by which the neuronal cell body might be informed about an axonal injury to trigger those changes important for axonal regeneration (Cragg, 1970). Despite this early conceptualization of the possible signals of axotomy, our understanding of their molecular nature is still very incomplete.

The temporal sequence of events following axotomy suggests that both rapid and slower retrograde signals are involved in triggering the multitude of changes observed after axotomy. For instance, unmasking of a connexin-43 epitope is seen in perineuronal glial cells that surround axotomized rat facial motoneurons within 45 minutes of axonal injury (Rohlmann et al., 1994), lending support to the notion that some injury signals are electrically propagated (Ambron and Walters, 1996) and might act via Ca++ and cAMP to regulate gene expression. This first electrical phase may be followed by a wave of rapid molecular signals, e.g. the loss of “NF-kB binding” which somehow spreads from the site of axonal injury as fast as 2.5 cm within 15 min (Povelones et al., 1997). This reflects a velocity of 2400 mm/day and would by far exceed the reported speed of retrograde transport at 50-200 mm/day.

These first two rapid phases of changes appear to be followed by signals travelling at the velocity of conventional retrograde transport. A retrogradely transported signal appears to be involved in the increase in c-Jun-kinase, which occurs within 30 min after axonal injury close to the cell body but takes 3 hours when the axon is injured further distally (Kenney and Kocsis, 1997b). Retrogradely transported signals
may include cytoplasmic neuronal molecules modified/activated at the site of injury (Bisby, 1982, Cragg, 1970), e.g. MAP-kinases (Ambron and Walters, 1996), or extrinsic molecules obtained from the injury site or axonal targets. Such retrogradely transported signals may be "positive" (appearing after the injury) or "negative" (disappearing after the injury) in nature (Skene, 1989, Woolf et al., 1990).

The concept of a "positive" signal is exemplified by the actions of Leukemia Inhibitory Factor (LIF). LIF is produced within hours after injury by non-neuronal cells, most likely Schwann cells and satellite cells (Matsuoka et al., 1997). A variety of axotomy-induced neuropeptide changes, most prominently galanin expression, are not observed in mice with gene deletions for LIF (Sun and Zigmond, 1996a, Zigmond et al., 1996). In addition to deficiencies in neuropeptide expression, peripheral neurons of LIF knockout mice also fail to up-regulate the Schwann cell mitogen Reg-2 (Livesey et al., 1997). The effects of LIF are likely to be mediated via gp130-JAK-STAT signalling, as LIF knockout mice also fail to activate STAT signalling after injury (Rajan et al., 1995).

In addition to lesion site-derived positive signals, other positive signals seem to come from the denervated distal nerve and are taken up as the regenerating axons grow through it. In hypoglossal motoneurons, for example, the re-expression of the low affinity p75-neurotrophin receptor was only observed if the crushed axons were allowed to regenerate into the distal nerve stump; nerve ligation or blockade of axonal transport from the nerve stump inhibited p75 up-regulation (Bussmann and Sofroniew, 1999).

"Negative" signals (i.e. loss of a constitutive signal from the axonal target or nerve sheath) also appear to trigger many neuronal cell body responses. For example,
blockade of axonal transport (without axotomy) stimulates the up-regulation of GAP-43 and Tα1 α-tubulin, suggesting that retrogradely transported repressors normally inhibit their expression (Skene, 1989, Wu et al., 1993). The GAP-43 repressor is likely derived from the terminal or target cells rather than the sheath cells of the nerve, as no difference in GAP-43 expression was observed after proximal versus distal axotomies in spinal motoneurons and DRG neurons (Fernandes et al., 1999, Liabotis and Schreyer, 1995). As described below, this thesis work begins by investigating the possible role of a negative signal, axotomy-induced loss of target-derived trophic molecules, in triggering changes in neuronal gene expression.
1.5 SUMMARY OF HYPOTHESES AND OBJECTIVES

The overall hypothesis is that if the genes expressed by regenerating PNS neurons are selectively modulated in injured CNS neurons, CNS regeneration will be facilitated/improved.

The overall objective of these experiments was to investigate the regulation of axotomy-induced changes in neuronal gene expression. The specific objectives were as follows. Using facial motoneurons as a successfully regenerating PNS model:

(1) Test the hypothesis that loss of target-derived trophic support is a negative signal triggering the down-regulation of the neurotransmitter enzyme acetylcholinesterase (AChE) in axotomized motoneurons (Chapter 3).

(2) Test the hypothesis that loss of target-derived trophic support is a negative signal triggering the up-regulation of regeneration-associated genes such as GAP-43, Tα1-tubulin, and c-jun (Chapter 4).

Based on findings from these studies, I moved to the mouse rubrospinal system as a non-regenerating CNS model, in order to

(3) Characterize the cell body responses of rubrospinal neurons to axotomy in adult mice (Chapter 5).

(4) Test the hypothesis that neurotrophins applied to a spinal cord injury site can act as positive signals to enhance regenerative cell body responses of axotomized rubrospinal neurons (Chapter 5).
1.6 RATIONALE FOR EXPERIMENTAL MODELS

The general approach taken in this thesis is to investigate the signals regulating axotomy-induced gene expression by successfully regenerating peripheral neurons, and then to apply this information to enhance the intrinsic growth capacity of non-regenerating CNS neurons.

1.6.1 Facial motoneurons as a successfully regenerating PNS model

Motoneurons offer several advantages as a successfully regenerating PNS model for the present studies. Importantly, motoneuron cell bodies are located within the CNS environment itself, rather than in a ganglion. The facial motoneuron (FMN) model is preferable to that of spinal cord motoneurons, as the neurons of the facial nucleus (i) lie within a well-defined region that can be easily identified histologically or for tissue micro-dissection, (ii) are not intermingled with interneurons, and (iii) are in the same region of the brainstem as the non-regenerating model I will use, rubrospinal neurons (below). Furthermore, the axons of FMNs are easily accessible for surgical manipulations as they exit the skull from the stylomastoid foramen. Of relevance to this thesis, the baseline injury-responses of the FMN model have been previously published, and serve as a control to ensure the accuracy/reproducibility of the techniques I use (Tetzlaff et al., 1988).

1.6.2 Rubrospinal neurons as a non-regenerating CNS spinal cord injury model

Rubrospinal (RS) neuron cell bodies are located in the midbrain. All magnocellular (larger and located more caudally), and the majority of parvocellular
(smaller and more rostral) RS neurons innervate motoneurons and interneurons within the spinal cord. Rubrospinal axons decussate within the midbrain itself, and then project to the spinal cord via the contralateral rubrospinal tracts, situated in the dorsolateral funiculus of the spinal cord. The tract can therefore be reliably transected by a lateral hemi-section of the spinal cord, sparing the contralateral fibres.

The injury responses of RS neurons are highly affected by the distance of the axotomy from the RS cell bodies (reviewed in Tetzlaff et al., 1994). Transection of the RS tract in the cervical spinal cord of the rat results in a severe atrophy of the RS cell bodies during the second week after injury. However, transection of the RS tract in the rat thoracic spinal cord results in little atrophy or morphological changes of the cell body. Similarly, expression of the RAGs GAP-43 and Tα1-tubulin is stimulated in cervically but not thoracically axotomized RS neurons (Fernandes et al., 1999). RAG expression in cervically axotomized rat rubrospinal neurons is aborted concommitant to the rubrospinal atrophy during the second week (Tetzlaff et al., 1991). The reasons for distance-dependent changes in gene expression are unknown.

### 1.6.3 Rat and mouse models

In this thesis, I have used both rat and mouse rodent models. Rats were initially used in order to facilitate surgical and microdissection protocols. However, mouse models were preferred during the later studies, to take advantage of transgenic mouse models in future studies. The neuronal injury responses in mice and rats have not been directly compared. However, it has been observed that mouse neurons are less
resistant to axotomy-induced degeneration than those of the rat, a feature that can reduce the experimental time required for studies of chronically injured neurons.

<table>
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(M = motor, S = sensory, A = autonomic)
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- CHAPTER 2 -

MATERIALS AND METHODS
2.1 SURGICAL PROCEDURES

2.1.1 Anaesthesia

Rats

Studies using rats (chapters 3 and 4) were mainly performed at the University of Ottawa. Adult male Sprague-Dawley rats (250-400g) were obtained from Charles River Laboratory (Quebec, Canada) and housed in a 12h:12h light:dark cycle with access to a standard diet and water *ad libitum*. The rats were anaesthetized with a mixture of sodium pentobarbitol (32 mg/kg) and chloral hydrate (150 mg/kg), according to the guidelines of the University of Ottawa Animal Care Committee.

Mice

Studies using mice (chapter 4 and 5) were performed at the University of British Columbia. C3H and CD1 mice were used at ages of 8-12 weeks old. Mice were maintained in a 12 hr light/dark cycle with access to standard rodent diet and water *ad libitum*. Each mouse was anaesthetized for surgery with a sterile solution of 100 mg/ml ketamine hydrochloride and 10 mg/ml of xylazine, in accordance with the guidelines of the Canadian Council for Animal Care and the local animal care committee.

2.1.2 Facial nerve axotomy

Using deeply anaesthetized rodents, the facial nerve was exposed at its exit from the stylomastoid foramen. The buccal branch of the facial nerve was transected approximately 2 mm distal to the foramen. To prevent regeneration of the axons to
their targets, a 4-5 mm (rats) or 2-3 mm (mice) segment of nerve was removed distal to the foramen (i.e. nerve resection). Care was taken to avoid injuring the smaller auricular branch of the facial nerve, which served as an internal control.

2.1.3 Osmotic pump implantation onto the axotomized facial nerve

In chapters 3 and 4, osmotic pumps (1 μl/h flow rate, model 2001, Alzet) were used to infuse neurotrophin-containing solutions onto the proximal nerve stumps of the axotomized facial nerve. The rats were divided into the following treatment groups, as shown schematically in figure 2. GROUP 1: Rats received a facial nerve resection involving removal of a 5 mm nerve segment to prevent reconnection to the distal stump, allowing us to determine the effects of axotomy on motoneuron gene expression. Gene expression in the axotomized FMNs was compared to the contralateral uninjured FMNs. GROUP 2: One facial nerve was axotomized as for Group 1. The contralateral facial nerve was likewise axotomized and an osmotic pump containing vehicle implanted onto the proximal nerve stump. This allowed us to measure any effect of vehicle compared to axotomy alone. GROUP 3: One facial nerve was axotomized and and an osmotic pump containing vehicle solution was implanted onto the proximal nerve stump. The contralateral facial nerve was also axotomized and received an osmotic pump containing one of the neurotrophin solutions. This permitted measurement of neurotrophin effects relative to contralateral vehicle treatment. GROUP 4: One facial nerve was axotomized and received a neurotrophin pump. The contralateral facial nerve was left unoperated. This allowed comparison of
Figure 2. Experimental paradigms to assess influences of axotomy and exogenously applied neurotrophins on facial motoneurons. Group 1 underwent a single facial nerve axotomy ("AX") to measure the relative change in FMN gene expression compared to the uninjured contralateral ("C") FMNs. Group 2 underwent axotomy of both facial nerves, with one side left untreated ("AX") and the proximal stump of the opposite side receiving an osmotic pump containing vehicle solution ("AX + vehicle pump"); this allowed assessment of possible vehicle pump effects. Group 3 underwent axotomy of both facial nerves, with one side receiving a vehicle pump ("AX + vehicle pump") and the opposite side receiving a neurotrophin pump ("AX + NT pump"); this paradigm allowed assessment of neurotrophin effects. Group 4 received a single facial nerve axotomy followed by implantation of a neurotrophin pump ("AX + NT pump") in order to compare gene expression following neurotrophin treatment to that in non-axotomized contralateral ("C") FMNs.
gene expression in neurotrophin-treated axotomized facial motoneurons to contralateral uninjured motoneurons. Thus, in Groups 2 and 3 both facial nerves were axotomized, which the animals tolerated without weight loss. In addition, in order to avoid bias towards one particular side (left vs. right) the left or the right side was randomly selected for neurotrophin treatment in different animals.

The experimental arrangements described for Groups 1, 2, 3, and 4 involve comparisons between the left and right facial nuclei within individual animals. This intra-animal comparison was selected to preclude inter-animal variabilities in gene expression, as well as to ensure that the populations of neurons compared undergo identical tissue fixation and hybridization conditions. Although it is possible that levels of AChE expression in motoneurons contralateral to axotomy may not be identical to those in motoneurons of completely uninjured rats, the magnitude of our findings are likely to err, if at all, on the slightly conservative side. This is because (i) our results are normalized against the contralateral nucleus, so that any contralateral effects of axotomy/neurotrophin treatment resulting from release of diffusible factors would tend to reduce rather than increase the statistical significance of neurotrophin effects, and (ii) both facial nerves were axotomized in Groups 2 and 3, so that contralateral effects are internally controlled for.

In neurotrophin-treated rats, an osmotic mini-pump containing any one of the four neurotrophins (NGF, BDNF, NT-3 or NT-4/5) or vehicle was connected to a Silastic tubing (0.025 inches I.D.x 0.047 inches O.D.; Dow Corning) which was placed over the nerve stump and held in place with a 6.0 Prolene suture (Ethicon). Silastic Silicone-like Medical Adhesive (Dow Corning) prevented leakage from the tubing.
Figure 3. Schematic and photo of pump implantation procedure
The pumps were inserted under the skin of the back, Penicillin-Streptomycin solution (750 µl at 5000 U/ml) was instilled into the wound area, and the skin closed with wound clips. A photo during osmotic pump implantation is shown in Figure 3.

**Mini-pump preparation and Neurotrophin solutions**

Total volume required to fill each mini-pump (Alzet, model 2001, 1 µl/hr flow rate) and 6-8 cm of Silastic tubing was approximately 250 µl. Vehicle solution consisted of 0.8% saline in 20mM sodium phosphate buffer (pH 7.2) containing 0.5% rat serum albumin as a protein carrier and 100 U/ml each of Penicillin and Streptomycin antibiotics. The neurotrophins (kindly provided by Regeneron Pharmaceuticals Inc.) were supplied as concentrates in acetate buffer and were then diluted in the vehicle solution to a final concentration of 5, 50, or 500 ng/µl. The BDNF concentration range chosen was based on a previous study by Friedman et al. (1995). All pumps were pre-incubated in 20 mM PBS, pH 7.4, for 12 hours at 37°C prior to surgery to initiate an even flow rate. Post-mortem analysis confirmed that the free end of the silastic tubings had been maintained over the proximal nerve stump.

2.1.4 Re-injury of chronically axotomized facial motoneurons

In mice to be used for the chronic facial nerve axotomy study, the left facial nerve was resected (ie. axotomized and a 3-4mm segment removed) under sterile conditions. Nerve resection prevents the growing axons from regaining access to the distal nerve stump and finding their targets. The skin incision was closed using metal suture clips. Ten weeks later, the mice were re-anaesthetized, the sutures removed, and
the injured nerves examined. In each case, the proximal nerve stump was found to have grown into large neuroma. In chronically axotomized control animals, the wound was closed again following the visual inspection. In mice receiving a second injury to the nerve, the neuroma was cut out. One week later (i.e. 11 weeks since initial injury), control and re-injured mice were killed by perfusion with 4% paraformaldehyde.

2.1.5 Retrograde labelling of rubrospinal neurons- choice of tracers

Retrograde labelling is a widely used technique for tracing the projection pathways of neuronal populations. Current techniques involve the use of fluorescent dyes that are retrogradely transported to and accumulate in neuronal cell bodies. Importantly, a variety of fluorescent tracers are available, which differ in their properties based on their molecular nature. In the present experiments, retrograde tracing was performed to identify the location of mouse rubrospinal neurons projecting to the cervical versus lumbar spinal cord.

The retrograde neuronal tracer Fast Blue (Sigma-Aldrich Canada, Oakville, ON) was predominantly used in these experiments. Concentrations ranging from 1% to 5% were initially used, and showed no difference in retrograde labelling characteristics. Subsequent experiments were therefore performed using 1% Fast Blue. Retrograde labelling also appeared unchanged regardless of whether Fast Blue was dissolved in sterile water or dilute dimethylsulfoxide (0.2% DMSO).

In some experiments, Fluoro-Ruby (cat.# D-1817, Molecular Probes, Eugene, OR) was used in combination with Fast Blue. Fluoro-Ruby is a 10,000 MW dextran amine molecule that has been labelled with tetramethylrhodamine (a fluorescent dye)
and lysine (a fixable residue that is cross-linked in place by fixation with aldehydes).

Fluoro-Ruby generally does not retrogradely label neurons in the absence of axonal injury (Richmond et al., 1995), probably due to its large size.

### 2.1.6 Cervical/thoracic double retrograde labelling of rubrospinal neurons

Since the rubrospinal system had not yet been characterized in mice, we used a double labelling paradigm to map the location of rubrospinal neurons cell bodies projecting to the cervical versus lumbar levels of the spinal cord. To retrogradely label lumbar-projecting rubrospinal neurons, the T10 spinal cord was laminectomized and the dura of the spinal cord was opened. Iris scissors were used to perform a lateral hemi-section of the T10 spinal cord, and a left lateral hemi-section performed. A small piece of Gelfoam (Upjohn, Kalamazoo, MI) soaked in 0.5-1.0 μl of 16% Fluoro-Ruby (Molecular Probes, Eugene, OR) was inserted into the injury site, and the wound closed. To label the cervical-projecting rubrospinal neurons in the same animals, the neck musculature was split along the midline and a C3/C4 left laminectomy performed to reveal the dorsal spinal cord. Using iris scissors, a window in the dura was opened, and a stereotaxic injection of 400 nl of 5% Fast Blue (Sigma-Aldrich Canada, Oakville, ON) was made into the left half of the spinal cord. The neck muscles were then sutured together using 6-0 Prolene (Ethicon, Somerville, NJ) and the skin closed with mouse wound clips.
2.1.7 Cervical axotomy and Fast Blue retrograde labelling of rubrospinal neurons

For examination of rubrospinal cell body responses to injury, the left dorsal spinal cord was isolated at the C3 level and the dura opened as described above. A left hemi-section of the C3 spinal cord was then performed using iris scissors. Completeness of the lesion was confirmed by passing fine Dumont #5 forceps several times through the injury site. A small piece of Gelfoam (Upjohn, Kalamazoo, MI) was soaked in 2.5 μl of either 2.5% Fast Blue, 2.5% Fast Blue + 16% Fluoro-Ruby, or 2.5% Fast Blue + 25 μg of BDNF, and was placed completely into the lesion site. The neck muscles were then sutured together using 6-0 Prolene (Ethicon, Somerville, NJ) and the skin closed with mouse wound clips.

In previous experiments, continuous infusion of BDNF at a concentration of 500 ng/μl directly into the red nucleus of rats stimulated rubrospinal cell body responses (Kobayashi et al., 1997). In pilot experiments, we found that infusion of 500 ng/μl of BDNF into the cervical spinal cord injury site of rats had no effects on the atrophy of the axotomized rubrospinal neurons. Recent studies have shown that neurons are about one magnitude of order less responsive to neurotrophins at their distal axons than at their cell bodies (Toma et al., 1997). In the case of BDNF, this is compounded by the expression of truncated trkB receptors by predominantly non-neuronal cells within the spinal cord, which appear to act as a “sponge” to sequester BDNF (Fryer et al., 1997). In the present study, we therefore used a 20x higher concentration of BDNF than in the pilot experiments.
2.2 HISTOLOGICAL TECHNIQUES

2.2.1 Tissue collection

Animals were given a lethal dose of chloral hydrate (approximately 1 g/kg). Tissue to be used for RT-PCR or \textit{in situ} hybridization were rapidly dissected from freshly killed animals, placed onto gel blotting paper, and frozen on dry ice. Overdosed animals whose tissues were to be used for \textit{in situ} hybridization, histochemical stainings, or histological analyses were transcardially perfused with phosphate-buffered saline (PBS) followed by ice cold freshly hydrolyzed 4\% paraformaldehyde (rats: 200 ml PBS, 300 ml paraformaldehyde; mice: 10 ml PBS, 19 ml paraformaldehyde) prior to dissection of tissues. Perfusion-fixed tissue was post-fixed overnight (8-12 hours) in 4\% paraformaldehyde, cryoprotected in 14\% and 22\% solutions of sucrose in PBS (at least 12 hours each), and then slowly frozen from base upwards in a petri dish of dry ice-cooled isopentane.

2.2.2 Cryostat cutting

Coronal sections through the facial nucleus or red nucleus were collected onto Superfrost Plus slides (Fisher Scientific). Brains were sectioned at thicknesses of 12 to 20 \textmu m, at temperatures ranging from -16\degree C to -25\degree C. Brains were sectioned in a caudal to rostral direction. Sections mounted onto slides were stored at -80\degree C until use. Correct left/right alignment of the tissue during cutting was achieved by adjusting the position of the tissue block prior to reaching the region of interest, i.e. at the nucleus
ambiguus for the facial nucleus, or at the facial nucleus for the red nucleus. Correct left/right alignment was of particular importance for analysis of the mouse red nucleus, as rubrospinal neurons become progressively smaller when progressing from the caudal pole (magnocellular) to the rostral pole (parvocellular). Additional markers for balanced tissue alignment prior to arriving at the red nucleus were the trochlear nucleus, oculomotor nucleus, and the decussation of the superior cerebral peduncle.

Sections compared using in situ hybridization were always mounted adjacent to each other on the same microscope slide, to ensure identical hybridization conditions and autoradiographic exposure. Different treatment groups were randomly arranged on the microscope slides, to eliminate any systematic treatment differences of the slides.

2.2.3 Nissl staining

Cellular morphology was examined on sections that were Nissl stained using 0.2% cresyl violet. Slides containing tissue sections were first placed in 4% paraformaldehyde (15-30 minutes), to ensure adherence of tissue to the slides. Slides were then rinsed in distilled water (about 2 minutes). Staining in 0.2% cresyl violet typically required 3-5 minutes, but tissues which had already been processed for in situ hybridization required longer periods. To coverslip, stained slides were dehydrated in ascending concentrations of ethanol, chloroform, and coverslipped with Entellan (BDH, Toronto). In some cases, strong Nissl staining appeared to quench the intensity of the in situ hybridization signals. Thus, for quantification of ISH signals, fluorescent staining of cell bodies with ethidium bromide was done instead. Slides were stained in
a 0.01% solution of ethidium bromide for 10 minutes to 1 hour, rinsed under cold running tap water for 20-30 minutes, and finally washed in 3 washes (10 minutes each) of double distilled water with gentle shaking. Staining with ethidium bromide was more successful with perfusion-fixed than fresh frozen tissues, and yielded good resolution of the neuronal cell body profiles, with particularly intense staining of perineuronal glial cells.

2.3 ANALYSIS OF TISSUES

2.3.1 In situ hybridization (ISH)

In situ hybridization (ISH) is a technique used to detect changes in the levels of specific mRNA sequences. Importantly, because it is performed directly on tissue sections, it allows visualization of the cellular localization of the mRNA. ISH was performed using radioactively labelled synthetic oligonucleotides complementary to specific mRNAs of interest. Oligonucleotide sequence data are given in figure 4. Oligonucleotides were end-labelled with $^{35}$S-α-dATP to an activity of at least 600,000 cpm/μl using the enzyme terminal transferase, which adds a 3’ tail of radioactive dATP molecules

Hybridization of radioactive oligonucleotides to tissue sections was performed using a modified protocol of Verge et al. (1992). Pretreatment of slides involved fixation in paraformaldehyde, dehydration/delipidization in ethanol and chloroform, with an intermediate permeabilization step (for perfusion-fixed slides only), as described below. Slides stored at -80°C were post-fixed for 20 minutes in 4% paraformaldehyde
at 4°C, and then rinsed in two washes of PBS (3 minutes each). Perfusion-fixed tissue
then underwent a permeabilization step in a solution of proteinase K (20 μg/ml in 50
mM Tris and 5 mM EDTA), one rinse in PBS (3 min), a second fixation in 4%
paraformaldehyde (5 min), and two more rinses in PBS (3 min each). The treatment
with proteinase K is to improve the access of the oligonucleotide probe to target
mRNAs within the cells, while the second fixation is to stabilize the tissue again
following the permeabilization. Finally, both fresh frozen and perfusion-fixed tissue
were dehydrated/delipidized in 60%, 80%, 95%, and 100% ethanol (1 minute each; the
60% and 80% solutions were diluted in 0.3 M sodium acetate, pH 5.2), chloroform (5
minutes), 100% and 95% ethanol (1 minute each). Sections were air-dried for 10
minutes, and then each slide received 100 μl of the hybridization solution, was
coverslipped, and was incubated for 16 hours at 43°C. Each ml of hybridization
solution contained 1.2 x 10^7 cpm of labelled oligonucleotide, 200 μg of salmon sperm
dNA, and 200 μg tRNA, in a solution of 50% deionized formamide, 10% dextran
sulfate, 0.5% SDS, 5x SSPE (0.9 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA), 5x Denhardt’s
solution, and 200 mM dithiothreitol. In earlier experiments, slides were pre-hybridized
for 45 minutes with an identical solution lacking the radioactive probe. This step was
omitted in later experiments with little apparent increase in background signal.

Following the 16 hours of hybridization, unbound and non-specifically bound
probe was washed from the slides with increasingly stringent washes in sodium
chloride/saline citrate (SSC) buffer. All SSC wash solutions contained 2-
mercaptoethanol (200 μl/100 ml) except for the final two washes in 0.25x and 0.1x SSC.
Coverslips were removed in 4x SSC at room temperature. Subsequent washes were for
15 minutes each at 48-50°C in 2x SSC, 1x SSC (three times), and 0.5x SSC. The two final SSC 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 (2 x 5 minutes). Finally, the sections were dehydrated by 30s dips into 60% and 95% EtOH.

Autoradiography was performed using Kodak NTB2 photographic emulsion diluted 1:1 with double-distilled H2O. The exposure times varied according to the probe characteristics and amount of target mRNA. Exposure times were approximately 1 day (α-tubulins), 5 days (GAP-43), 7 days (c-jun and NF-M). Slides were developed using Kodak D-19 developer and fixed with Kodak Fixer.

ISH yields silver grains over cells in which radioactive probes had bound to target mRNAs. Changes in the amount of silver grains is generally assumed to be roughly proportional to changes in the levels of the target mRNA, although it has not been established how directly proportional this relation is. As previously described (Kobayashi et al., 1997), a computerized image analysis system (Northern Exposure, Empix) was used to quantify the ISH signals. Slides were number coded to eliminate any bias during quantification (single blind analysis). Silver grains resulting from the ISH process were visualized using darkfield optics. Cell profiles were visualized under brightfield illumination (cresyl violet staining), fluorescence illumination (ethidium bromide staining), or phase contrast. Cell profiles were traced, and the area fraction occupied by grains (ie. grain density) was then automatically measured from the corresponding darkfield image. Background autoradiographic signal was subtracted to obtain a corrected grain density. Since axotomy and neurotrophin treatments induce significant changes in neuronal size, changes in the density of ISH signals can occur in
the absence of any change in mRNA gene expression. Therefore, rather than comparing signal density (ie. ISH signal per unit area), the signal densities were converted to ISH signal per neuron. This was accomplished by multiplying the corrected grain density by the approximate cell volume. The cell volume was calculated from the cross-sectional area (ie. the cross-sectional area “A” is measured and \( A = \pi r^2 \), so \( r = (A/\pi)^{1/2} \); since the volume of a sphere is \( V = (4/3)\pi r^3 \), therefore \( V \) can be calculated from the equation \( V = 4/3\pi (A/\pi)^{3/2} \)). In this calculation, the simplifying assumption is made that the neuronal soma is a sphere; although this is not likely to be the case, this assumption is made for all cells and therefore should not affect comparisons of relative ISH signal/neuron. The quantification was limited to those cells cut through the nucleus, resulting in data collection from about 25-30 (facial) or 15-25 (rubrospinal) cells per tissue section. For each section, the average ISH signal/neuron/nucleus was expressed as percentage of the average signal from the vehicle-treated motoneurons in the contralateral nucleus. At least three sections, more than 100 µm apart, were quantified per facial nucleus and the mean percentage calculated for each animal.

Using northern blots, comparable changes in gene expression were observed as with use of ISH. Additional controls to confirm the specificity of the technique included competition experiments with 100-fold excess of unlabelled probe and pre-treatment of tissues with RNAse, both of which eliminated hybridization signals.
**acetylcholinesterase (AChE) (50mer)**
(5'-CAAGTCAATGGAGGCCGTTCAAGATGTCAGCATAGACCCGAGC-3')
Complementary to rat AChE mRNA, bases 1360-1409 in Legay et al., 1993 (accession# S50879).

c-jun (60mer):
(5'-
GCAACTGCTGCGTTAGCATGAGTGCCACCCACTGTAACGTGGTTATGACTT
TCTGTT-3')
Complementary to rat c-jun mRNA, bases 1346-1287 in Sakai et al., 1989 (accession# X17163).

**neurofilament-M (NFM) (49mer)**
(5'-CCCAGTGATGCTTCTGAAATGTGCTAAATCTGCTCACCCTCC-3')
Complementary to rat NFM mRNA, bases 1222-1270 in Napolitano et al., 1987 (accession# M18628).

**GAP-43 (52mer):**
(5'-
GCATCGGTAGTAGCAGAGCCATCTCCCTTCTCTCTCCACACCACACGCAA-3')
Complementary to GAP-43 mRNAs in rat (100%; bases 273-324 in Basi et al., 1987; accession# M16736) and mouse (100%; bases 364-415 in Cimler et al., 1987; accession# J02809).

**Tα1 α-tubulin (47mer):**
(5'-AAACCCATCAGTGAAATGGACGGCTCGGTCTTCACCATAATCATCA-3')
Complementary to 3' untranslated end of the rat Tα1 α-tubulin mRNA, bases 1548-1594 of Lemischka et al., 1981, acc# V01227; 100% complementary

**Total α-tubulins (45mer):**
(5'-ATGCTGCGCTTTGTGCTGGTCTGACAAATATCCATCT-3')
Complementary to mouse α-tubulin coding sequences:
- mα2 - bases 1057-1101 of Lewis et al., 1985, acc# M28727; 100% complementary
- mα6 - bases 1021-1066 of Villasante et al., 1986, acc# M13441; 2 mismatches (bases 3 and 6 of oligonucleotide).
- mα1 - bases 1026-1071 of Lewis et al., 1987, acc# M28729; 2 mismatches (bases 3 and 6 of oligonucleotide).
- mα7 - bases 1017-1062 of Villasante et al., 1986, acc# M13443; 3 mismatches (bases 3, 30, and 33 of oligonucleotide).

**trkC (47mer)**
(5'-GGAGCATGGTGTCTCACCACCTAGTACGACTGTAGACG-3')
Complementary to bases 2109-2272 (excluding 2134-2250) of Valenzuela et al., 1993 (accession# L14447), thereby bridging the potential insertion site in the tyrosine kinase domain.

*Figure 4. Sequences of oligonucleotide probes used for ISH.*
2.3.2 Reverse transcription-polymerase chain reaction (RT-PCR)

Another method of measuring changes in mRNA levels is reverse transcription-polymerase chain reaction (RT-PCR). This technique is particularly suited for target mRNAs that are in low abundance, or for measuring changes in mRNA levels from extremely small amounts of tissue (ie. the micro-dissected facial nucleus).

Total RNA was isolated from the facial nucleus and reverse transcribed into cDNA using as described by Kobayashi et al. (1996), with slight modifications as described below. Full length trkC mRNAs were selectively amplified by polymerase chain reaction (PCR) using previously described primers (Offenhauser et al., 1995) that bracket the potential insertion site within the trkC kinase domain. The amplified cDNA sequences corresponded to both non-inserted and inserted full length trkC mRNA species. Hence the PCR products had sizes of 299, 341, 374, and 416 bp, corresponding to the non-inserted and 14, 25, and 39 amino acid inserted trkC isoforms, respectively. PCR amplification of trkC species was initially performed between 18 and 32 cycles (45s at 94°C, 1 min at 55°C, 1 min 30s at 72°C), and found to be within the linear range of amplification at 30 cycles. Serial dilutions from 12 to 0.75 ng of total RNA were amplified in 50 μl PCR reactions. Amplication of cyclophilin mRNA was used to ensure equivalent amounts of input cDNA (Mearow et al., 1993). PCR products were visualized on 5% acrylamide gels.

2.3.3 AChE biochemistry

AChE enzymatic activity was measured using a spectrophotometrically quantifiable biochemical reaction. Facial nuclei were punched from fresh frozen brain
stems using a blunted cannula. Each nucleus was homogenized on ice (2x15s) in 1 ml of a high salt/detergent buffer containing protease inhibitors (bacitracin 1.0 mg/ml, aprotinin 25 U/ml). Homogenates were then centrifuged at 20,000g for 20 min at 4°C and the supernatants used immediately for reaction with acetylthiocholine as substrate for acetylcholinesterase (Ellman et al., 1961). 200 µl of homogenate was incubated with 1 ml of reaction mixture containing 0.22 mg/ml acetylthiocholine and 0.2 mg/ml Ellman's Reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) in phosphate buffer. Spectrophotometric measurement of AChE activity was made at 412nm, and was performed in the presence of 10^{-5}M tetraisopropylpyrophosphoramide (iso-OMPA), a non-specific cholinesterase inhibitor (previously described in Gisiger and Stephens, 1988; Jasmin and Gisiger, 1990). Non-specific hydrolysis was determined by incubating the samples in the presence of 5-bis(4-allydimethylammonium phenyl)pentanone dibromide (BW284c51), a specific acetylcholinesterase inhibitor. AChE activity per sample was normalized according to the amount of soluble protein measured using a BCA protein assay reagent kit (Pierce Chemical Company, Rockford, IL). In each case, results were expressed as a percentage of the acetylcholinesterase activity measured within the contralateral intact facial nucleus.

2.3.4 AChE histochemical staining

To demonstrate the cellular localization of the AChE activity, a histochemical reaction for AChE activity was performed on tissue sections. Coronal sections (12 µm) through the facial nucleus of perfused brainstems were mounted onto Superfrost Plus slides (Fisher Scientific Co.) and stored at -80°C. Sections were stained using the
histochemical technique of Karnovsky and Roots (1964). Reaction mixture contained 0.5 mg/ml acetylthiocholine, 10 mM sodium citrate, 3 mM CuSO$_4$, and 0.5 mM potassium ferricyanide in 0.1M sodium acetate buffer, pH5.2. Acetylthiocholine serves as an artificial substrate for tissue acetylcholinesterase. The resultant liberation of thiocholine reduces the ferricyanide to ferrocyanide, which reacts with the Cu$^{2+}$ to yield a brown insoluble precipitate. Iso-OMPA was used as in 2.3.3 above to block activity of non-specific cholinesterases. Specificity of the staining procedure was confirmed by including the acetylcholinesterase inhibitor BW284c51 in the reaction mixture, which eliminated all staining (not shown).

2.3.5 Quantification of cell sizes

Cell sizes were measured using a computerized image analysis program (Northern Eclipse; Empix, Mississauga, ON).

In chapter 4, the cross-sectional areas of normal, axotomized, and neurotrophin-treated facial motoneurons were measured. Sizes were measured for 25-40 facial motoneurons per nucleus, from 4-6 animals per treatment. In chapter 5, the cross-sectional areas of normal, axotomized, and BDNF-treated lumbar-projecting rubrospinal neurons were measured. Size measurements of rubrospinal neurons were always done at equal levels of the magnocellular red nucleus, between 100 and 200 μm from the caudal pole. The cross-sectional areas of 50-100 rubrospinal neurons were measured in each category, pooled from 3 animals in each case. Data were expressed as indicated in 2.3.7 below.
2.3.6 Photomicrographs

Since Fluoro-Ruby, and to a lesser extent, Fast Blue, are easily washed out of tissue sections during alcohol dehydration, colour fluorescent images were captured prior to staining or *in situ* hybridization. Images were captured using a Zeiss Axioskop microscope equipped with a SPOT digital colour camera (Diagnostic Instruments Inc., MI) and standard fluorescent/darkfield/brightfield settings. Analysis of cell sizes and ISH signals were made using Northern Eclipse image analysis software (Empix, Mississauga, Ontario, Canada). Montages of images were generated into figure plates using Adobe PhotoShop (Adobe Systems Incorporated, San Jose, CA), with only minor adjustments to contrast and brightness which were always performed equally for control and lesioned tissues.

2.3.7 Data analysis and statistics

Unless otherwise stated, the data obtained from individual treatment groups are graphed in terms of their mean (μ) and standard deviation (σ), as measures of central tendency and variability of the data, respectively. Within the results section, the results from each treatment group are further described in terms of their median value and the 25th-75th percentile values.

In chapters 3 and 4, in situ hybridization data are typically collected from 5 different treatment groups, i.e. no pump, NGF-treated, BDNF-treated, NT-3-treated, and NT-4/5-treated. Within each of these treatment groups, there are data points from 3-7 separate animals. To control for inter-animal variabilities, the data from each of these animals is expressed as a percentage of that from its contralateral side, which was
vehicle-treated. To accommodate this data structure, an analysis of variance (ANOVA) for the five groups was performed. Since the data was calculated as percentages (of contralateral), the non-parametric Kruskal-Wallis ANOVA was performed to test for difference between groups. When the ANOVA indicated rejection of the null hypothesis (i.e., that all groups are drawn from the same population), Dunn's test was used to locate the groups that differed significantly from each other. Significance levels are indicated in the results section of each chapter.

In chapter 5, the sizes of 60-90 rubrospinal neurons were measured from each of several different treatment groups (control, vehicle-treated after 7, 14, or 21d, and BDNF-treated after 14d. For comparison of the 14d vehicle treatment to the 14d BDNF treatment, these two samples were first tested for a normal distribution. Since they were not normally distributed, a non-parametric Mann-Whitney U test was used to test for significant difference.
TARGET-DERIVED NEUROTROPHINS AND THE REGULATION OF MOTONEURON ACETYLCHOLINESTERASE EXPRESSION

3.1 SUMMARY

One of the consistent changes in gene expression observed following axon injury is a decreased expression of neurotransmitter enzymes. Understanding the signals regulating this change may provide clues to the regulation of regeneration-associated gene expression after injury. Since a variety of techniques for measuring levels of the cholinergic enzyme acetylcholinesterase (AChE) have been established, in this chapter the possibility was tested that the injury-induced decrease in AChE is due to loss of target-derived trophic support. Seven days after facial nerve transection, AChE mRNA and enzyme activity levels were both markedly reduced in untreated and vehicle-treated facial motoneurons, suggesting stimulation of motoneuron AChE expression by muscle-derived factors. Since skeletal muscle is a source of NT-3, NT-4/5, and BDNF, these neurotrophins were individually infused onto the proximal nerve stump for 7
days beginning at the time of axotomy. In situ hybridization, biochemical assay and histochemical staining revealed that the downregulation of AChE mRNA and enzymatic activity was prevented by the trkB ligands, NT-4/5 and BDNF. In contrast, NT-3 had limited effects, and NGF was without effect. Since motoneurons normally express both trkB and trkC receptors, and the trkC ligand NT-3 is the most abundant muscle-derived neurotrophin, we investigated possible reasons for the limited effects of NT-3. In situ hybridization and reverse transcription PCR both revealed a downregulation of trkC mRNA in axotomized motoneurons, which contrasted the upregulation of trkB expression. Furthermore, isoforms of trkC were detected carrying insertions within their kinase domains, known to limit trkC-mediated signal transduction. These results support the concept that (i) muscle-derived neurotrophins broadly modulate the cholinergic phenotype of their innervating motoneurons, and (ii) that the decrease in cholinergic enzyme expression after axotomy is due to interruption of motoneuron access to these muscle-derived neurotrophic factors. These results have been previously published (Fernandes et al., 1998).
3.2 INTRODUCTION

Neurotrophic factors (NTFs) derived from axonal targets profoundly influence neuronal survival, differentiation, and synaptic function in the developing and mature nervous system (Munson et al., 1997, Schotzinger et al., 1994; for reviews, see Davies, 1994, Greensmith and Vrbova, 1996, Landis, 1990, Lowrie and Vrbova, 1992, Thoenen, 1995). After axonal injury, which interrupts the bi-directional flow of neuron-target interactions, the majority of mature cholinergic neurons survive but rapidly decrease their expression of the cholinergic neurotransmitter synthesizing enzyme, choline acetyltransferase (ChAT) (eg. Kou et al., 1995, Lams et al., 1988, Peterson et al., 1990). Consistent with a model of target regulation of neurotransmitter enzymes, infusion of pharmacological doses of putative target-derived NTFs restores ChAT immunoreactivity to axotomized cholinergic neurons (Friedman et al., 1995, Hagg et al., 1989, Yan et al., 1994).

A second enzyme associated with cholinergic neurotransmission is acetylcholinesterase (AChE). Although AChE is found to be co-expressed with ChAT in mature cholinergic neurons, it is also expressed in the absence of ChAT in certain non-cholinergic, non-cholinoceptive regions of the adult nervous system (Bernard et al., 1995, Greenfield, 1984, Hammond et al., 1994, Landwehrmeyer et al., 1993, Legay et al., 1993, Levey et al., 1983). Furthermore, expression of AChE mRNA, but not ChAT, is an early event during neuronal differentiation (Coleman and Taylor, 1996). These observations may be accounted for by a variety of non-acetylcholine-related functions for AChE (Dupree and Bigbee, 1994, Dupree and Bigbee, 1996, Krejci et al.,
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1991, Layer et al., 1993, Srivatsan and Peretz, 1997, Webb et al., 1996; reviewed in Appleyard, 1992, Layer and Willbold, 1995, Massoulie et al., 1993). Thus, co-regulation of ChAT and AChE cannot be assumed per se, as these enzymes may be differentially regulated following injury or in response to different NTFs.

Previously, it has been shown that adult rat facial motoneurons undergo a reduction in AChE enzymatic activity after axotomy (Tetzlaff and Kreutzberg, 1984). Since mature motoneurons express trkB and trkC high affinity tyrosine kinase receptors (Henderson et al., 1993), muscle-derived members of the neurotrophin family of NTFs may play a role in AChE regulation in motoneurons. Adult muscle produces the trkC ligand, NT-3, and the trkB ligands NT-4/5 and possibly low levels of BDNF (Funakoshi et al., 1993, Griesbeck et al., 1995, Koliatsos et al., 1994, Timmusk et al., 1993). Several studies have demonstrated that exogenous NT-4/5 and BDNF can prevent the decrease in ChAT immunoreactivity after axotomy of adult motoneurons (Chiu et al., 1994, Friedman et al., 1995, Tuszynski et al., 1996, Yan et al., 1994). Based on these effects, neurotrophins are candidate muscle-derived factors regulating AChE expression in vivo. Neurotrophin application in vitro induces an increase in total AChE enzymatic activity in various populations of embryonic neurons (Alderson et al., 1990, Greene and Rukenstein, 1981, Martinez et al., 1987, Ojika et al., 1994, Raynaud et al., 1988), including embryonic motoneurons (Wong et al., 1993). Furthermore, a recent in vivo study reported that BDNF can maintain the survival of avulsed motoneurons, which normally undergo cell death, and that these rescued motoneurons express ChAT and AChE (Kishino et al., 1997).
Neurotrophins have previously been shown to stimulate survival (reviewed in Sendtner et al., 1996b) and electrophysiological (Munson et al., 1997) responses from motoneurons. In the present study, the possibility was assessed that AChE gene expression in motoneurons may be regulated by the neurotrophin family members found in muscle, i.e. NT-3, NT-4/5, and BDNF. I investigated whether the axotomy-induced reduction in motoneuron AChE enzymatic activity occurs at the mRNA or protein activity level, tested the abilities of the individual neurotrophins to prevent the axotomy-induced changes in AChE mRNA, biochemical activity, and histochemical localization, and examined the expression of trkB and trkC neurotrophin receptors in intact, axotomized, and neurotrophin-treated motoneurons.
3.3 RESULTS

3.3.1 Effect of axotomy on AChE mRNA expression

Using rats from Group 1 (see figure 2; described under "Experimental Paradigms" in Materials and Methods), in situ hybridization for AChE mRNA followed by autoradiography was used to determine whether the decreased AChE activity found in axotomized motoneurons (Tetzlaff and Kreutzberg, 1984) was due to downregulation of mRNA levels. Darkfield micrographs, which show the autoradiographic silver grains (Fig. 5A), clearly demonstrated reduced hybridization signal over the axotomized facial nucleus when compared to the uninjured contralateral facial nucleus (Fig. 5B). In brightfield micrographs (Fig. 5C and D), the autoradiographic signal is localized to the facial motoneurons. Quantification of the ISH signal/cell determined that AChE mRNA expression by axotomized FMNs was reduced to 37 +/- 4% SEM (n=13 animals) compared to the non-injured contralateral side.

3.3.2 Dose response analysis of axotomized FMNs to BDNF

To identify the concentrations at which axotomized FMNs would respond to neurotrophins in the osmotic pump infusion paradigm, we performed a dose response analysis using 10-fold increments in BDNF concentration. Rats were treated as shown for Group 3 (Fig. 2). Treatment with 5 ng/μl BDNF did not alter AChE mRNA levels in relation to contralateral vehicle infusion as measured with in situ hybridization (Fig. 6A). However, infusion of either 50 ng/μl BDNF (Fig. 6B) or 500 ng/μl BDNF (Fig. 6C) increased AChE mRNA hybridization signal (171 +/- 17% SEM, n=4 animals, and 196
Figure 5. Reduced autoradiographic in situ hybridization (ISH) signal for AChE mRNA in the axotomized facial nucleus. Seven days following resection of the buccal branch of the facial nerves of rats from Group 1, darkfield micrographs reveal that ISH signals are reduced within the intermediate and lateral sub-groups ("i,l") of the axotomized facial nucleus (A) in comparison to the contralateral uninjured facial nucleus (B). Note that the ISH signal is not reduced in the medial sub-group ("m") which projects via the non-axotomized auricular branch of the facial nerve. Higher magnification brightfield micrographs localize the specific ISH signals to the cresyl violet stained axotomized (C) and contralateral (D) FMNs. Scale bar in C and D, 30 μm.
+/- 15% SEM, n=6 animals, respectively). We also measured significantly larger soma sizes with BDNF treatment; importantly, however, the increases in AChE mRNA levels with 50 and 500 ng/μl BDNF treatments was several fold greater than the 10-15% increase in soma volume (data not shown), indicating that the specific signal per cell volume ratio was increased. Furthermore, the stimulation of AChE mRNA levels is not likely to be a non-specific effect on all motoneuron mRNAs, as we found that sections from the same animals did not exhibit a BDNF effect on the expression of c-jun mRNA (not shown). Dose response data are summarized in Fig. 6D, illustrating the similar effects of 50 ng/μl and 500 ng/μl BDNF treatments on AChE hybridization signal.

3.3.3 Responsiveness of AChE mRNA expression to members of the neurotrophin family

Since NT-3 and NGF treatments elicited no detectable effects at 50 ng/μl, therefore we used the highest neurotrophin dose (500 ng/μl/h) in order to avoid false negative findings. High doses of neurotrophins did not elicit morphological or gene expression changes indicative of high dose toxicity.

In animals operated as shown for Group 3, low levels of AChE ISH signal were observed over vehicle-treated axotomized FMNs contralateral to all neurotrophin treated FMNs, confirming consistent hybridization conditions and the absence of contralateral effects of neurotrophins (Fig. 7A). FMNs treated with NGF (Fig. 7B) or NT-3 (Fig. 7D) were not significantly different from their contralateral vehicle-treated controls. However, FMNs treated with BDNF or NT-4/5 exhibited high levels of cytoplasmic ISH signal (Fig. 7C and E, respectively). Interestingly, although treatment with NT-3 was
Figure 6. Dose response analysis of axotomized FMNs to BDNF.
Representative scatterplots showing AChE ISH signal/neuron in the facial nuclei of rats from Group 3, receiving neurotrophin pumps for 7d applications of either 5 ng/μl/h (A), 50 ng/μl/h BDNF (B), or 500 ng/μl/h BDNF (C). Open circles represent the contralateral cells which were axotomized and treated with vehicle. Sections were processed on the same slide for equal ISH conditions. Summary of dose response data (D) demonstrates similar effects of 50 and 500 ng/μl BDNF treatments on AChE mRNA levels, in comparison to contralateral axotomized FMNs treated with vehicle (normalized to 100%). In D, each open circle represents the mean ISH signal/neuron/animal; filled circles represent the mean of each treatment group.
ineffective on the FMN population as a whole, a few FMNs appeared to express higher levels of AChE mRNA (not shown).

Quantification of the ISH signals using computerized image analysis measurements (Fig. 7 F) determined that treatment of axotomized FMNs with BDNF or NT-4/5 maintained the average ISH signal/neuron at 196 +/- 15% SEM (n=6 animals) and 202 +/- 34% SEM (n=4 animals) respectively, or approximately 2-fold higher than vehicle-treated contralateral FMNs. ISH signal in response to NGF treatment (113 +/- 26% SEM, n=4 animals) was not significantly different from vehicle control treatment, while NT-3 treatment had an average ISH signal/neuron that was 141 +/- 18% SEM (n=4 animals) of contralateral. However, only BDNF and NT-4/5 treatments yielded statistically higher ISH signals than contralateral vehicle treatment (P<0.05).

A few animals from Group 4 were also used for in situ hybridization. In comparison to the contralateral non-axotomized FMNs, AChE mRNA levels in FMNs from Group 4 rats were reduced to 38 +/- 11% (SEM) with NGF (n=2) and 27 +/- 3% (SEM) with NT-3 (n=2) (data not shown). This was not significantly different from axotomy alone (37 +/- 4%, n=13, Fig. 3). Axotomized motoneurons treated with BDNF, however, actually exhibited higher mRNA levels than the intact contralateral motoneurons (147 +/- 10%, n=2). Taking into account the approximately 15% hypertrophy, the specific signal/cell volume ratio was still approximately 30% higher in BDNF-treated FMNs than in intact motoneurons, indicating that BDNF actually restores AChE mRNA levels to supra-normal levels.
3.3.4 Histochemical localization and biochemical measurement of AChE activity

To assess whether the BDNF and NT-4/5-induced maintenance of AChE mRNA expression correlated with higher levels of AChE enzyme activity, facial nuclei were microdissected from the brainstems of axotomized Group 1 and neurotrophin-treated Group 4 rats, and the AChE biochemical activity measured (Figure 8). In normal unoperated animals, there was no significant difference in total AChE activity between left and right facial nuclei (102 +/- 9% SEM, n=5 animals), confirming a reproducible microdissection technique of the facial nucleus that was not biased to one side. At 7d following axotomy, AChE activity was reduced to 66 +/- 9% SEM (n=5 animals) of the contralateral uninjured facial motonucleus, in agreement with previous results (Tetzlaff and Kreutzberg, 1984). Treatment with 500 ng/μl of NT-3 had no significant effect (62 +/- 7% SEM, n=5 animals) on the expression of total AChE activity, but treatment with 500 ng/μl of BDNF (92 +/- 5% SEM, n=4 animals) and NT-4/5 (95 +/- 7% SEM, n=2 animals) prevented the axotomy-induced decrease in AChE activity.

Sections through the facial nucleus from Groups 1, 2 and 3 were also processed for AChE histochemistry. Histochemical localization of AChE enzyme activity identified strong reactivity in FMN cell bodies contralateral to an axotomy (Fig. 9A), which was severely depleted in untreated (Fig. 9B) and vehicle-treated (Fig. 9C) axotomized FMNs. Some heterogeneity was noted in the response to NT-3 (Fig. 9D), as many NT-3 treated FMNs displayed moderate AChE activity. More significantly, following treatment with BDNF or NT-4/5, virtually all axotomized FMNs exhibited AChE staining within their cytoplasm (Fig. 9 E, F, respectively). AChE positive nerve terminals in the immediate vicinity of the FMN cell bodies were evident in all cases.
3.3.5 Effects of axotomy and neurotrophins on trkB and trkC receptor expression

Since it was unclear how axotomy and neurotrophin treatments might have altered the responsiveness of FMNs to neurotrophins, we examined the expression of trkB and trkC receptors. Axotomy was found to induce opposing changes in expression of full length trkB and trkC receptors. Consistent with previous reports, we detected an approximately 2-3 fold increase in full length trkB mRNA within axotomized FMNs (Kobayashi et al., 1996a, Piehl et al., 1994), which was not altered by infusion of BDNF or NT-4/5 in our paradigm (not shown). In contrast, we identified an axotomy-induced downregulation of trkC mRNA (Fig. 10A and B). Since multiple isoforms of the trkC receptors exist, we used an ISH probe that spans the potential insertion site within the kinase domain, and is therefore specific for the full length non-inserted trkC isoform (trkCni). The expression of trkCni was reduced in the axotomized FMNs 7 days after axotomy, and the downregulation appeared uniform as illustrated by scatterplot analysis of ISH signals (Fig. 10C). To determine whether this downregulation was due directly to the loss of target-derived NT-3 ligand, we used the probe specific for trkCni for in situ hybridization to sections from the NT-3 treated rats. The decline in trkC hybridization signal was not prevented by NT-3 infusions at concentrations of 500 ng/μl (Fig. 10D) and 50 ng/μl (not shown).

The trkC receptor profile within the facial nucleus was further characterized using total RNA extracted from microdissected unoperated and axotomized facial
Figure 7. Effects of neurotrophins on AChE mRNA by axotomized facial motoneurons (FMNs). Cresyl violet stained sections from rats operated according to Group 3. Shown are axotomized FMNs treated with vehicle (A) or 500 ng/μl of NGF (B), BDNF (C), NT-3 (D), or NT-4/5 (E). Note that BDNF and NT-4/5 treated FMNs maintained AChE mRNA expression, while NT-3 and NGF were without obvious effect. Quantification of average ISH signal/neuron, normalized against contralateral vehicle-treated FMNs, confirms statistically significant effects (*p<0.05) of BDNF and NT-4/5, but not NT-3 or NGF (F). NT-3 had a marginal effect on AChE mRNA levels, however this effect did not reach statistical significance. Scale bar, 20 μm.
Figure 8. Biochemical analysis of AChE enzyme activity in the facial nucleus after axotomy and neurotrophin treatment. Animals from Groups 1 and 4 were used for biochemical measurement of total tissue AChE activity within the microdissected facial nuclei. Control unoperated rats had no significant difference in AChE activity between left and right sides ("Uninjured", n=5). Axotomized facial nuclei from Group 1 animals ("AX", n=5), which did not receive a pump, and axotomized facial nuclei from Group 4 animals that received an NT-3 pump ("AX + NT-3", n=5), both had significantly reduced AChE activity compared to the contralateral non-axotomized nuclei. In contrast, axotomized facial nuclei from Group 4 animals treated with BDNF ("AX + BDNF", n=4) or NT-4/5 ("AX + NT-4/5", n=2) maintained their AChE expression, showing no significant difference from the non-axotomized contralateral nuclei.
Figure 9. Histochemical localization of AChE enzyme activity in the facial nucleus. Rats were operated as shown for Groups 1, 2, and 3. In Group 1 rats, abundant histochemical reaction product clearly identifies the cell bodies of the non-axotomized FMNs contralateral to axotomy (A), while the axotomized FMNs were depleted of AChE activity (B). In Group 2 rats, axotomized FMNs that received vehicle pump implantation are also depleted of AChE activity (C). However, in Group 3 rats, virtually all FMNs that received a BDNF pump (E) or NT-4/5 pump (F) maintained strong cytoplasmic AChE staining. FMNs treated with an NT-3 pump (D) exhibited a heterogeneous response that included both moderately stained (bold arrows) and unstained (thin arrowheads) FMNs. In all groups, AChE-reactive afferent terminals were visible as punctate reaction product around the perimeter of the FMN cell bodies. Scale bar, 80 μm.
nuclei. Reverse-transcription followed by PCR, using primers bracketing the potential insertion site in the trkC kinase domain, demonstrated the presence of the trkCni mRNA, as well as of inserted full length trkC isoforms (Fig. 11). Multiple comparisons of serial dilutions containing 12, 6, and 3 ng of template indicated an approximate 2-3 fold reduction in mRNA for trkCni mRNA, as well as for the larger inserted trkC isoforms. Inserted trkC isoforms appeared to include the 14, 25, and 39 amino acid insert variants, the presumed 14 amino acid variant yielding the strongest band after PCR amplification. Bands for trkCni and the 14 amino acid inserted trkC variant appeared equally intense.
Figure 10. Expression of the NT-3 high affinity receptor, trkC, in axotomized and neurotrophin-treated facial motoneurons. Autoradiographic ISH signal for full length trkC receptor mRNA (non-inserted isoform) in non-axotomized contralateral (A) and axotomized (B) FMNs. Representative scatterplots illustrate the axotomy-induced down-regulation of non-inserted full length trkC mRNA (C), which is not significantly changed by 7-day infusion of NT-3 ligand onto the proximal nerve stump (D). Scale bar, 20 μm.
Figure 11. Profile of trkC receptor isoforms in axotomized facial motoneurons (FMNs). Non-inserted (large arrow) and inserted (small arrowheads) isoforms of full length trkC mRNAs are detected in the facial nucleus 7 days after axotomy. Note the axotomy-induced 2-3 fold reduction in levels of all full length trkC mRNAs. The apparent band sizes are consistent with the full length trkC isoform sizes of 299 bp (non-inserted), as well as 341 bp, 374 bp, and 416 bp, representing the 14, 25, and 39 amino acid insertion variants, respectively.
3.4 DISCUSSION

In the present study, it has been shown that axotomy of FMNs results in a reduction in their mRNA expression, as well as their total enzyme activity, for the neurotransmitter hydrolyzing enzyme acetylcholinesterase. Although FMNs express both trkB and trkC neurotrophin receptors, only the trkB ligands, BDNF and NT-4/5, prevented the decrease in AChE mRNA expression, biochemical enzyme activity, and somal histochemical staining, and stimulated hypertrophy of the FMN cell bodies, when infused onto the proximal nerve stump. In contrast, NT-3 and NGF did not have significant effects. The effects of BDNF and NT-4/5 on FMN gene expression and soma size were consistent with the axotomy-induced upregulation of the full length trkB receptor. In the case of NT-3, the trkC receptor profile was found to consist of comparable levels of the non-inserted and inserted isoforms of the full length receptor, particularly the 14 amino acid variant, and expression of both the non-inserted and inserted isoforms was sharply reduced after axotomy, correlating with the lack of NT-3 effects. Injury-induced changes in the neurotrophin receptor mRNA expression were not detectably altered by infusion of the respective ligands.

3.4.1 Regulation of acetylcholinesterase by neurotrophins

Our results confirm and extend the earlier report that axotomy of adult rat facial motoneurons reduces AChE activity to approximately 60% of levels in the uninjured contralateral motoneurons (Tetzlaff and Kreutzberg, 1984). We detected a concomitant decline in AChE mRNA to levels of 30-40% of contralateral. The more
pronounced reduction in mRNA levels than enzyme activity is likely explained by a slower turnover of protein than mRNA or by the presence of AChE activity in cholinergic afferent terminals (Li et al., 1995b) that were visible on the FMN cell bodies by enzyme histochemistry. However, we cannot exclude the possibility of post-translational modifications that increase enzyme specific activity.

Treatment of the axotomized FMNs with the trkB ligands, BDNF or NT-4/5, maintained AChE mRNA expression and prevented the axotomy-induced reduction of AChE activity. The mechanism underlying the neurotrophin-induced maintenance of AChE mRNA levels has not been established. In vitro studies have demonstrated that the RNA synthesis inhibitors can abolish the NGF-induced stimulation of AChE expression in PC12 cells (Greene and Rukenstein, 1981); however, this may indicate either a direct effect of NGF on AChE transcription or an indirect effect via expression of stabilizing RNA-binding proteins. A recent study in PC12 cells demonstrated that NGF can activate the transcription factor Sp1 (Yan and Ziff, 1997), whose recognition site appears essential for transcriptional activation of the AChE promoter (Getman et al., 1995). On the other hand, stabilization of existing AChE mRNA transcripts was recently shown to underlie the increased AChE mRNA expression that occurs when P19 cells undergo neuronal differentiation (Coleman and Taylor, 1996). Further studies are necessary to differentiate between these possibilities in axotomized motoneurons.

3.4.2 Comparison of neurotrophin effects on AChE and ChAT in motoneurons

Studies examining neurotrophin effects on ChAT expression in adult motoneurons have used a variety of application paradigms, including local treatment
at the lesion site (Chiu et al., 1994, Friedman et al., 1995, Wang et al., 1997, Yan et al., 1994), subcutaneous injections (Yan et al., 1994; Friedman et al., 1995), intravenous injection (Yan et al., 1994) and intracerebroventricular infusion (Tuszynski et al., 1996, Yan et al., 1994). With some exceptions (see Clatterbuck et al., 1994, Piehl et al., 1995a, Wang et al., 1997) which may be due to variations in experimental protocols, the majority of these results have demonstrated that pharmacological doses of BDNF or NT-4/5 maintain ChAT expression in axotomized adult motoneurons. A recent quantitative RNAse protection study demonstrated that BDNF maintained ChAT mRNA levels in axotomized hypoglossal motoneurons (Wang et al., 1997); thus, the neurotrophin effects on ChAT expression are likely to be mediated by increases in mRNA transcription or stability rather than rate of translation or specific activity of the enzyme. These results parallel the present findings that BDNF and NT-4/5 maintain AChE mRNA expression and enzyme activity. Collectively, the evidence indicates that signalling in response to either of the trkB ligands, BDNF or NT-4/5, can regulate both of the enzymes associated with the cholinergic phenotype at the level of their mRNA expression in facial motoneurons.

In comparison to BDNF and NT-4/5, NT-3 treatment only stimulated a small increase in AChE mRNA, which did not reach statistical significance. NT-3-treated axotomized FMNs were heterogeneously stained by AChE histochemistry and, unlike axotomy alone or axotomy plus vehicle treatment, included a small number of clearly AChE-positive motoneurons. The reason for the heterogeneous NT-3 effect is unclear. NT-3 has been reported to be the most abundant muscle-derived neurotrophin in adult muscle, with mRNA levels of approximately 2-3X those of NT-4/5 and 20X those of
BDNF (Funakoshi et al., 1995, Griesbeck et al., 1995). Furthermore, potent effects of NT-3 on motoneurons during embryonic and neonatal stages of development have been described (Wong et al., 1993; reviewed in Sendtner et al., 1996b). However, there are few reported effects of NT-3 on mature motoneurons (Haase et al., 1997), and to our knowledge, regulation of motoneuron transmitter enzymes in adult rats by NT-3 has not been reported. The limited NT-3 effect which we detected was not due to the quality of NT-3, since the same batch was effective in parallel investigations on other neurons within the CNS (Giehl and Tetzlaff, 1996). One possibility for the minor NT-3 effects is that only sensory afferents and γ-motoneurons might be exposed to muscle-derived NT-3, as the main source of NT-3 in mature muscle appears to be intrafusal muscle spindles (Copray and Brouwer, 1994); since the facial motonucleus is known to contain only α-motoneurons, it is possible that they are not normally responsive to NT-3. However, mature α-motoneurons have been shown to express trkC receptors (Henderson et al., 1993, Johnson et al., 1996, Merlio et al., 1992) and are capable of retrogradely transporting iodinated NT-3 (Di Stefano et al., 1992). The possibility was therefore investigated that the partial NT-3 effects on axotomized motoneurons may be due to the composition of, or injury-induced changes in, the trkC receptor profile.

3.4.3 Axotomy differentially regulates trkB and trkC receptor expression

Axotomy of FMNs has been shown to lead to an upregulation of mRNA for the full length trkB isoform (Kobayashi et al., 1996a, Piehl et al., 1994), which is consistent with the responsiveness of axotomized motoneurons to treatment with BDNF or NT-4/5. We found that this 2-3 fold mRNA upregulation was not detectably altered by the
infusion of either BDNF or NT-4/5 onto the nerve stump (not shown). In other studies, BDNF infusion into the midbrain of adult rats also did not alter trkB mRNA levels, and resulted in a prolonged analgesia (Frank et al., 1997); thus, it is possible that findings of reduced trkB immunoreactivity following BDNF treatments (Dittrich et al., 1996, Frank et al., 1996, Frank et al., 1997) may be due to increased receptor turnover rather than downregulation of trkB expression (Frank et al., 1997).

In contrast to the upregulation of the full length trkB receptor, we found mRNA for the non-inserted full length trkC receptor to be downregulated after axotomy, offering one explanation for the limited NT-3 effects on AChE expression. Infusion of NT-3 did not prevent this trkC downregulation. To further characterize the trkC expression, we tested for the presence of "inserted" isoforms of full length trkC receptors, which carry insertions of 14, 25, or 39 amino acids within their kinase domain (Tsoufas et al., 1993, Valenzuela et al., 1993). The presence of such inserts within the trkC kinase domain appears to limit its signalling capabilities (Gunn-Moore et al., 1997, Tsoufas et al., 1996) by interfering with the sustained activation of the mitogen-activated protein kinase (MAPK) pathway (Gunn-Moore et al., 1997). In vitro studies have shown that PC12 cells respond to NT-3 with neurite outgrowth when transfected with the non-inserted full length trkC, but not when transfected with inserted full length trkC variants (Lamballe et al., 1993, Tsoufas et al., 1993, Valenzuela et al., 1993). The use of RT-PCR primers that bracket the potential kinase insertion site allowed us to identify the presence of both non-inserted and inserted trkC receptor isoforms within the microdissected facial nucleus, as well as demonstrate that both inserted and non-inserted full length trkC isoforms are downregulated after axotomy.
Since we used RNA extracted from the microdissected facial nucleus, we cannot exclude a non-neuronal contribution to the expression of inserted trkC isoforms. However, we believe them to be predominantly of neuronal origin, as (i) virtually all ISH signals were located over neurons when using the extracellular domain probe common to all trkC isotypes (not shown), and (ii) the inserted trkC isoforms were downregulated in a similar manner as the non-inserted trkC isoform. Since NT-3 binding to trkC is followed by receptor dimerization and mutual phosphorylation, the presence of inserted isoforms may exert a dominant negative effect on non-inserted trkC signalling pathways, contributing to the mitigated responsiveness of axotomized motoneurons to NT-3. Further studies will be required to determine whether a differential distribution of inserted and/or truncated trkC receptors accounts for the heterogeneous effects of NT-3.

3.4.4 Conclusions

The roles of endogenous muscle-derived neurotrophins for mature motoneurons have not been clearly established. NT-4/5 is expressed in an activity-dependent fashion in skeletal muscle, and NT-4/5 injection into muscle elicits motoneuron sprouting (Funakoshi et al., 1995). Recently, inhibition of endogenous trkB ligands in muscle has been shown to reduce motoneuron conduction velocity, further suggesting a dynamic regulation of motoneuron properties by endogenous neurotrophins (Munson et al., 1997). The present findings showing the potent effects of NT-4/5 and BDNF on AChE mRNA and total enzyme activity, together with their reported effects on ChAT expression, support the concept of a broad influence of trkB ligands on the cholinergic
phenotype of mature motoneurons. We also demonstrated that the most abundantly expressed neurotrophin in muscle, NT-3, appears to have only limited effects on neurotransmitter enzyme expression in adult motoneurons after axotomy, and that this may be due to the presence of inserted trkC receptor isoforms that can limit the responsiveness to NT-3. Furthermore, axotomized motoneurons downregulate their trkC expression, which most likely renders them less responsive to NT-3 after injury.
4.1 SUMMARY

Results from the previous experiments suggested that at least one subset of axotomy-induced changes in gene expression, the down-regulation of neurotransmission-related enzymes, is due to the interruption of target-derived trophic support. **In the following experiments, it was investigated whether the prominent axotomy-induced changes in regeneration-associated gene expression may also be caused by loss of target-derived trophic support.** It was predicted that treatment of axotomized motoneurons with neurotrophins, at the concentrations that prevented the decrease in acetylcholinesterase in the previous chapter, would likewise prevent the up-regulation of regeneration-associated genes. Similar to results in the previous chapter, only BDNF and NT-4/5 were found to have significant effects on any of the parameters examined. Motoneurons treated with these trkB ligands showed a partial attenuation of the axotomy-induced down-regulation of neurofilament mRNA.
However, BDNF and NT-4/5 failed to reverse the strong up-regulation of regeneration-associated genes, such as GAP-43, Tα1 α-tubulin, or c-jun. Rather, quantification of the ISH signals revealed a more pronounced up-regulation of GAP-43 and Tα1 α-tubulin mRNAs than for vehicle-treated motoneurons. Moreover, motoneurons treated with BDNF and NT-4/5 were significantly hypertrophied. These results suggested that neurotrophic factors from non-target-derived sources may enhance the regenerative state of axotomized motoneurons. Since reactive Schwann cells at axon injury sites are an endogenous source of several neurotrophic factors, it was hypothesized that a second axon injury of motoneurons that had been chronically axotomized would be sufficient to initiate a regenerative cell body response. Re-axotomizing motoneurons whose axons had been resected ten weeks earlier was indeed found to greatly reverse their atrophy and up-regulate their mRNA expression of GAP-43 and Tα1 α-tubulin. These results show that (i) loss of target-derived neurotrophins is unlikely to be the trigger for motoneuron regeneration-associated gene expression, (ii) non-target-derived neurotrophins can enhance regenerative cell body responses of axotomized motoneurons, and (iii) neurotrophic factors may even stimulate regenerative cell body responses from highly atrophic, chronically axotomized neurons.
4.2 INTRODUCTION

Axotomized peripheral neurons undergo complex changes in gene expression that are believed to facilitate the process of axonal regeneration. The more prominent of these changes include the up-regulation of regeneration-associated genes, including (i) the cytoskeletal proteins actin and tubulin, (ii) the cytoplasmic growth cone protein GAP-43, and (iii) the transcription factor c-jun. At the same time, axotomized PNS neurons consistently down-regulate expression of (iv) neurotransmitter enzymes, and (v) neurofilaments, which are considered markers of their mature differentiated phenotype. In addition to these, PNS neurons exhibit a variety of changes in (vi) neurotrophic factors and their receptors, (vii) neuropeptides, (viii) cell adhesion and guidance molecules, and many others (reviewed in Fernandes and Tetzlaff, 2000). The regulatory mechanisms presiding over the majority of these changes in gene expression are unknown. However, in theory, changes induced by axotomy can be the result of (i) a new signal originating following the axotomy (a "positive" signal) or (ii) the disappearance of a constitutive signal (a "negative" signal).

The actions of a lesion site-derived "positive" signal are best illustrated by the example of Leukemia Inhibitory Factor (LIF). LIF is normally produced in the region of the injury site by Schwann cells and immune cells (Matsuoka et al., 1997). In LIF knockout mice, certain changes normally observed after peripheral nerve axotomy are highly attenuated or absent, such as intracellular signalling by the Signal Transducers and Activators of Transcription (STATs) (Rajan et al., 1995), and up-regulation of the
neuropeptide galanin (Sun and Zigmond, 1996b; Zigmond et al., 1996) or the mitogen Reg-2 (Livesey et al., 1997).

One type of "negative" signal for axotomized peripheral neurons is the loss of target-derived neurotrophic factors. As shown in the previous chapter, axotomized motoneurons rapidly down-regulate their mRNA and protein expression for the cholinergic neurotransmitter enzymes, choline acetyltransferase and acetylcholinesterase. However, treating axotomized motoneurons with appropriate types of muscle-derived neurotrophic factors is sufficient to prevent the down-regulation of these cholinergic enzymes (Fernandes et al., 1998). Similarly, axotomized sensory neurons undergo a wide range of changes in neuropeptide expression, and many of these changes can be reversed by intrathecal administration of the neurotrophin NGF (Verge et al., 1995).

Several observations suggest that negative signals are also responsible for triggering expression of regeneration-associated genes (RAGs), such as tubulins, GAP-43, and c-jun, in axotomized motoneurons. For instance, pharmacological or cold block of axonal transport induces RAG expression without an axonal injury (Skene, 1989; Wu et al., 1993). Furthermore, while RAG expression returns to baseline levels upon target innervation, it remains high for weeks to months if target re-innervation is prevented. Recent studies using axotomized sensory neurons indicate that target-derived neurotrophic factors may normally act as repressors of RAG expression. For example, administration of the neurotrophins Nerve Growth Factor (NGF) or Neurotrophin-3 (NT-3) attenuates the increase in GAP-43 expression by axotomized sensory neurons (Mohiuddin et al., 1999).
Muscle expresses the neurotrophins NT-3, NT-4/5, and BDNF (Funakoshi et al., 1993; Griesbeck et al., 1995; Koliatsos et al., 1993; Timmusk et al., 1993), and adult motoneurons express the trkB and trkC neurotrophin receptors (Henderson et al., 1993) and can retrogradely transport iodinated neurotrophins (Curtis et al., 1998; DiStefano and Curtis, 1994). Thus, these neurotrophins are potential muscle-derived factors repressing RAG expression in motoneurons. To investigate this possibility, we studied the influences of exogenous neurotrophins on axotomy-induced gene expression for GAP-43, Tu1 α-tubulin, c-jun, and medium neurofilaments (NFM). Facial motoneurons were used as a model for this study, as their axotomy-induced changes in gene expression (Tetzlaff et al., 1988) and their dose response curve to neurotrophins (previous chapter, Fernandes et al., 1998) have been previously characterized. Contrary to our initial hypothesis, our results suggest that loss of target-derived neurotrophins is not, by itself, the trigger for RAG expression in motoneurons. Furthermore, our results indicate that neurotrophic factors released at an axonal injury site can enhance the regenerative cell body responses of axotomized motoneurons, even reversing the atrophy and stimulating the RAG expression of chronically axotomized motoneurons.
4.3 RESULTS

4.3.1 BDNF and NT-4/5 attenuate the down-regulation of NFM

Since BDNF and NT-4/5 suppressed the down-regulation of the neurotransmitter enzyme AChE in axotomized FMNs (chapter 3), I investigated their effect on expression of another class of molecules associated with the mature neuronal phenotype, neurofilaments. ISH was performed against mRNA for the medium neurofilament subunit (NFM). Untreated axotomized facial motoneurons expressed only 15 ± 1% of the contralateral non-injured NFM ISH signal after 7d (Fig. 12). This was about 54 ± 19% of vehicle treatment, indicating an effect of pump implantation plus vehicle treatment. The reasons for this vehicle effect are unclear. Compared to vehicle treatment, BDNF (194 ± 72% SD, median 165%, 25th-75th percentiles = 144%-242%) and NT-4/5 (158 ± 20% SD, median 164%, 25th-75th percentiles = 145%-170%) had statistically significant effects on NFM hybridization signal (p=0.05). The effects of NGF (98 ± 40% SD, median 110%, 25th-75th percentiles = 74%-123%) and NT-3 (122 ± 52% SD, median 106%, 25th-75th percentiles = 85%-159%) on NFM expression were not significantly different from vehicle (ie. 100%). The effects of BDNF were not statistically different from those of NGF (p<0.20).

4.3.2 Neurotrophin administration to axotomized motoneurons enhances, rather than suppresses, the up-regulation of GAP-43 and Tα1 α-tubulin expression

To assess whether neurotrophin treatment suppressed any changes in regeneration-associated gene expression, I performed ISH against GAP-43 and Tα1
α-tubulin mRNAs. ISH signal for GAP-43 was hardly detectable in normal FMNs, but increased by 11 ± 2 fold following axotomy. Vehicle treatment itself had no apparent effect (102 ± 6%). Treatment of FMNs with any of the neurotrophins, including BDNF (Fig. 13), also failed to prevent the rise in GAP-43 expression. Similarly, the low baseline expression of Tau α-tubulin (Fig. 14A) was increased by 5 ± 0.5 fold following injury, and this increase was not suppressed by neurotrophin treatments, including BDNF (Fig. 14c). A similar situation was found when in situ hybridization was performed for c-jun (not shown).

Quantification of the preceding ISH signals was performed in a blinded manner by using number coded slides and a computerized image analysis system. Contrary to our initial hypothesis, quantifications revealed that BDNF and NT-4/5-treated FMNs consistently expressed greater levels of GAP-43 and Tau α-tubulin ISH signals than the contralateral vehicle-treated FMNs (Fig. 15). In the case of GAP-43, there was no significant difference in ISH signal between contralateral vehicle treatment and motoneurons that were untreated (102 ± 11% SD, median 107%, 25th-75th percentiles=94%-109%), NGF-treated (79 ± 21% SD, median 85%, 25th-75th percentiles=65%-94%) or NT-3-treated (97 ± 22% SD, median 98%, 25th-75th percentiles=82%-112%). However, motoneurons treated with BDNF (155 ± 40% SD, median 148%, 25th-75th percentiles=129%-191%) or NT-4/5 (211 ± 76% SD, median 198%, 25th-75th percentiles=155%-267%) had significantly more GAP-43 ISH signal. The significance levels for NT-4/5 were p<0.02 versus NGF, and p<0.20 versus NT-3 and no pump. The significance levels for BDNF were p<0.10 versus NGF, and p<0.20 versus NT-3 and no pump. There was no significant difference between BDNF and NT-
4/5 treatments, and no significant difference between no pump, NGF, and NT-3 treatments.

In the case of Tα1 α–tubulin expression, axotomized motoneurons ("no pump") had less ISH signal than those receiving vehicle (64 ± 1%; p<0.01). Compared to vehicle, NGF (129 ± 56% SD, median 144%, 25th-75th percentiles=97%-162%) and NT-3 (127 ± 23% SD, median 120%, 25th-75th percentiles=112%-143%) treatments had no significant effect. On the other hand, BDNF (173 ± 14% SD, median 177%, 25th-75th percentiles=164%-184%) and NT-4/5 (168 ± 18% SD, median 168%, 25th-75th percentiles=153%-183%) both enhanced the Tα1 α–tubulin increase. Compared to no pump treatment, the effects of BDNF (p<0.01) and NT-4/5 (p<0.05) were highly significant, and the effects of NT-3 (p<0.20) were only very weakly significant.

ISH was also performed for a third gene associated with axonal regeneration, c-jun. Compared to vehicle, there were no significant differences detected for no pump (78 ± 14% SD, median 76%, 25th-75th percentiles=69%-87%), NGF (95 ± 22% SD, median 100%, 25th-75th percentiles=78%-110%), BDNF (92 ± 20% SD, median 85%, 25th-75th percentiles=78%-112%), or NT-3 (146 ± 79% SD, median 116%, 25th-75th percentiles=94%-205%). In the case of NT-3, the data is skewed due to one outlying datum. No data is shown for NT-4/5 as there were insufficient tissue sections left and no remaining NT-4/5 trophic factor available.

Analysis of the cross-sectional areas of the axotomized FMNs demonstrated that BDNF and NT-4/5-treated FMNs were also hypertrophied compared to vehicle-treated FMNs (Table 2). Seven days after axotomy, motoneurons had increased in volume by approximately 16% compared to contralateral intact motoneurons. As shown in table 2,
this was not changed by treating the axotomized motoneurons with vehicle, or with either 1.2 or 12 μg/d treatments of NGF or NT-3. However, axotomized motoneurons treated with these concentrations of BDNF and NT-4/5 were further increased in volume by 12-13% (BDNF) and 17% (NT-4/5) (1.2 and 12 μg/d treatments not significantly different).

Increased ISH signals for RAGs in response to BDNF or NT-4/5 were difficult to identify by visual inspection alone, but were readily revealed by the more sensitive computer-assisted quantifications of ISH signals. These increases were difficult to identify on a purely visual basis for two reasons. Firstly, increases in grain density were partially offset by the BDNF and NT-4/5-induced increases in the size of the FMN cell bodies. Secondly, the RAG expression by untreated axotomized FMNs was extremely robust, so that further trophic factor-induced increases were not readily apparent. Such a robust up-regulation results in overlapping silver grains, and as I refrained from the use of correction factors for overlapping silver grains, it is possible that the quantification criteria (area covered by grains) might actually underestimate the amount of increase. This is not a consideration when quantifying less highly expressed signals (ie. NFM, Fig. 12). One possibility for addressing these issues might be the use of a shorter ISH exposure time than is typically used. Unfortunately, there were insufficient tissue sections left from the treated animals to test this possibility. More animals were not operated to either test this possibility or perform Northern Blots due to the limited amount of NTFs and the high cost of both osmotic pumps and NTFs.
To circumvent these issues, and to further investigate whether endogenous release of NTFs at an axon injury site might be a biological correlate of our exogenous NTF application, I used a chronic axotomy model. The advantage of this model is that the initially high expression of RAGs in acutely axotomized motoneurons undergoes a gradual decline with time. Thus, use of chronically axotomized neurons may allow for a more direct visual proof of the neurotrophic factor-induced increases in RAG expression.
Figure 12. Effects of neurotrophins on NFM expression by axotomized motoneurons. In situ hybridization signals for NFM are abundant over normal motoneurons (A), but strongly down-regulated by 7d following axotomy (B). NT-3 infusion onto the proximal nerve stump had no significant effect (C), but infusion of the trkB ligand BDNF (D) attenuated the down-regulation. The effects of the neurotrophin family members are summarized in (E), and show that only the trkB ligands, BDNF and NT-4/5, had significant effects on NFM expression. Empty circle are the mean ISH signals/neuron for individual animals, filled circles are the mean +/- SEM for each treatment group. Scale bar = 30 μm.
Figure 13. BDNF fails to suppress GAP-43 expression by axotomized FMNs. Normal (uninjured) motoneurons do not express detectable levels of GAP-43 hybridization signal (A), but signals are highly increased over axotomized motoneurons (B). Treatment of the axotomized facial nerve with BDNF fails to prevent this up-regulation of GAP-43 expression (C). Scale bar = 50 μm.
Figure 14. NT-4/5 fails to suppress Tα1 α-tubulin expression by axotomized FMNs. Brightfield micrographs of cresyl violet-stained sections, showing that normal (uninjured) motoneurons express a low baseline level of Tα1 hybridization signal (A), which is increased by 7d after axotomy (B). In motoneurons treated with NT-4/5 infusion onto the proximal nerve stump over the 7d following injury, this up-regulation is not suppressed, but rather, appears increased (C). Scale bar = 30 μm.
Figure 15. Quantification of regeneration-associated gene expression by neurotrophin-treated axotomized FMNs. Computer-aided quantification of the GAP-43 (A), Tα1 α-tubulin (B), and c-jun (C) hybridization signals over neurotrophin-treated FMNs. BDNF and NT-4/5-treated motoneurons are found to express higher levels of both GAP-43 (A) and Tα1 α-tubulin (B), compared to contralateral vehicle treatment (set at 100% in each graph). In all cases, NGF and NT-3 are found to have no significant effect compared to contralateral vehicle treatment (set at 100%). Empty circles show the mean ISH signal/neuron for individual animals, filled circles show the mean ISH signal/neuron ± standard deviation for each treatment group.
GAP-43

Tα1 α-tubulin

c-jun

FIGURE 15
Table 2. Changes in cell sizes of axotomized facial motoneurons following neurotrophin treatment for 7 days.

<table>
<thead>
<tr>
<th>TREATMENT GROUPS (experimental/contralateral control)</th>
<th>Δ volume (contralateral = 100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>axotomy/ intact</td>
<td>116% ± 5% (n = 14)</td>
</tr>
<tr>
<td>axotomy/ axotomy + vehicle</td>
<td>96% ± 3% (n = 5)</td>
</tr>
<tr>
<td>axotomy + NGF (12 μg/d)/ axotomy + vehicle</td>
<td>104% ± 5% (n = 4)</td>
</tr>
<tr>
<td>axotomy + NGF (1.2 μg/d)/ axotomy + vehicle</td>
<td>96% ± 3% (n = 4)</td>
</tr>
<tr>
<td>axotomy + BDNF (12 μg/d)/ axotomy + vehicle</td>
<td>113% ± 2% (n = 9)</td>
</tr>
<tr>
<td>axotomy + BDNF (1.2 μg/d)/ axotomy + vehicle</td>
<td>112% ± 4% (n = 5)</td>
</tr>
<tr>
<td>axotomy + NT-3 (12 μg/d)/ axotomy + vehicle</td>
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</tr>
<tr>
<td>axotomy + NT-3 (1.2 μg/d)/ axotomy + vehicle</td>
<td>103% ± 6% (n = 2)</td>
</tr>
<tr>
<td>axotomy + NT-4/5 (12 μg/d)/ axotomy + vehicle</td>
<td>117% ± 2% (n = 4)</td>
</tr>
</tbody>
</table>
4.3.4 Axonal injury reverses the atrophy and RAG expression by chronically axotomized motoneurons

To assess the possibility that the effects of the exogenous neurotrophins on motoneuron RAG expression were also elicited by endogenous NTFs from an axonal injury site, I used a chronic axotomy model. By permanently separating the cut axon from its target musculature, the effects of a second axonal injury can be attributed to signals arising at an injury site rather than to negative signals from loss of target contact. Furthermore, the gradual reduction in RAG expression that occurs with time may make an injury-induced increase more apparent than following an acute injury. The buccal branch of adult mice were resected (ie. axotomized and a 4 mm segment of the nerve removed. After 10 weeks, the mice were re-aneasthetized, and the resected facial nerves examined. In each case, the proximal nerve ends had developed a neuroma, ie. an enlarged club-like ending of the proximal nerve stump. In four of the six animals, the nerve was axotomized proximal to the neuroma, and the neuroma removed. In the remaining two control animals, the neuroma was left intact. One week later, all mice were sacrificed. In the following analyses, the two control mice and two of the four re-axotomized mice (randomly selected) are examined in detail.

Cresyl violet stained sections through the facial nucleus of each mouse were examined under brightfield illumination (Fig. 16). Shown are samples of sections through the two control animals, which had been chronically axotomized for 11 weeks ("chronic AX", A–D), sections through two representative mice which had received a second axonal injury after 10 weeks ("chronic AX + 2nd AX", E–H), and sections through an uninjured contralateral nucleus ("contralateral", I–J). In each row, the boxed
area indicates the location of the resected facial motoneurons that projected through the buccal branch of the facial nerve. The boxed area is enlarged in the right panel of each row. Note that in each of the two chronically axotomized animals (A-D), there are relatively few large facial motoneurons remaining when compared to a non-injured nucleus (I-J). In contrast, the facial nuclei of chronically axotomized mice that received a second axonal injury (E-H) contained a greater number of large motoneurons than those which were not re-axotomized. Furthermore, these facial motoneurons appeared hypertrophied when compared to contralateral motoneurons.

Quantification of the cell sizes of 80-170 motoneurons, measured from 3 sections through each nucleus (Fig. 17), confirmed the above observations, showing that the chronically axotomized motoneurons in control mice were $57 \pm 33\%$ and $53 \pm 28\%$ (standard deviations) the size of their respective contralateral motoneurons, while those that received a second axonal injury were $120 \pm 54\%$ and $129 \pm 55\%$ of contralateral. Binning of the cell size data (Fig. 18) further revealed a marked shift towards smaller cell body sizes in the chronically axotomized mice (A and B), which was reversed by a second axonal injury (C and D).

To estimate the amount of neuronal loss that occurred after these chronic injuries, the number of neuronal profiles were counted by a blinded observer in every second $20\mu m$ section through the facial nuclei of four 11 week chronically injured mice, two of which received a second axonal injury one week prior to sacrifice. In the control chronically injured mice, there were $42\%$ and $45\%$ the number of neuronal profiles as compared to contralateral. However, the two chronically injured mice receiving a second axonal injury had $75\%$ and $81\%$ of the neuronal profiles compared to
contralateral. Thus, the second axonal injury reversed the apparent loss of motoneurons, indicating that the majority of the “lost” neurons were not dead, but highly atrophied, and recovered in response to a second injury.

To determine the status of the regeneration-associated gene expression by these chronically injured motoneurons, sections through each nucleus were processed for GAP-43 and α-tubulin in situ hybridizations. Expression of α-tubulin was detectable at baseline levels in chronically axotomized mice (Fig. 19A-D), but strongly up-regulated following a second axonal injury (F, H). Quantification of α–tubulin ISH signals using a computerized image analysis system (Fig. 20) revealed that lower α–tubulin hybridization signals were detectable in chronically axotomized motoneurons than in their contralateral non-injured motoneurons (A, C). Following a second axonal injury, the majority of chronically injured motoneurons showed from 3 to 10 times more α–tubulin signal than contralateral (E, G). GAP-43 hybridization signals were negligible in the contralateral and chronically axotomized facial nuclei of both control mice (Fig. 21A-D). In contrast, a robust up-regulation of GAP-43 hybridization signal was observed in chronically axotomized motoneurons which had been re-axotomized (F, H). Quantification of the in situ hybridization signals revealed that chronically axotomized motoneurons displayed approximately equal levels of GAP-43 hybridization signal as their contralateral non-injured counterparts (Fig. 22B, D), but this was up-regulated by between 10 and 100 fold by a second axonal injury (F, H).

Note that the intensity of the hybridization signals between acute and chronic experiments cannot be directly compared to each other due to the species difference and the protocols being performed in separate experimental series.
Figure 16. Cresyl violet-stained sections of facial nuclei of chronically axotomized mouse motoneurons: effect of a second axonal injury. Five facial nuclei are shown in the left column (A,C,E,G,I), and the boxed area enlarged in the adjacent right column (B,D,F,H,J, respectively). Two animals which have been chronically axotomized for 11 weeks are shown in A/B and C/D. Note the high degree of neuronal atrophy/loss in the boxed area of the facial nucleus, which projects via the chronically axotomized buccal branch of the facial nerve. Facial motoneurons projecting via the non-axotomized branches of the facial nerve are located to the left of the boxed areas, and in all animals appear healthy. Two animals which were also chronically axotomized for 11 weeks, but had received a second axonal injury after 10 weeks, are shown in E/F and G/H. Note that the re-injured facial motoneurons appear larger and more numerous than in A/B and C/D. The re-injured motoneurons appear comparable in number and even larger in size than those of the contralateral non-injured facial nucleus (I/J). Scale bar = 60 μm.
Figure 16.
Figure 17. Average cell body sizes of chronically axotomized FMNs: effect of a second axonal injury. Quantification of the sizes of chronically axotomized facial motoneurons confirmed that a second axonal injury reversed the atrophy of chronically injured motoneurons. In two animals which had been chronically injured for 11 weeks ("chronic ax"), the average facial motoneuron size was 55-60% of the contralateral non-injured motoneurons. In mice which received a second injury after 10 weeks ("chronic ax + 2nd ax"), the facial motoneurons were hypertrophied to 120-130% of the contralateral motoneurons. Error bars show the standard deviation in cell sizes.
Figure 17
Figure 18. Cross-sectional areas of chronically axotomized FMNs binned according to size. Analysis of the cell size distributions in chronically injured facial motoneurons (A and B), shows that the axotomized motoneuron cell bodies (filled bars) are shifted towards smaller sizes compared to the contralateral non-injured motoneurons (empty bars). However, in facial motoneurons receiving a second injury (C and D), the shift towards smaller sizes is prevented.
Figure 19. Effect of a second axonal injury on ISH signal for α-tubulin in chronically axotomized FMNs. In each case, the right column shows the axotomized facial nucleus, and the left column shows the contralateral non-injured facial nucleus. A/B and C/D are chronically axotomized animals, E/F and G/H are chronically axotomized animals that received a second axonal injury after 10 weeks. Low baseline levels of in situ hybridization signals for α-tubulin are found in the facial nuclei contralateral to axotomy (A,C,E,G). Low levels are also found in chronically axotomized facial nuclei (B, D). However, in chronically axotomized facial nuclei which have received a second axonal injury (F, H), there is a marked up-regulation of ISH signal. Note that there is variability in the degree of increase between the animals shown in F and H, but that it is consistently significantly higher than in B and D. Scale bar = 60 μm.
α-tubulins

Figure 19
Figure 20. Quantification of α-tubulin ISH signals in chronically axotomized FMNs: effect of a second axonal injury. Quantifications are shown from two chronically axotomized animals (A and B) and two chronically axotomized animals receiving a second axonal injury (C and D). Computer-aided quantification of the α-tubulin ISH signals reveal that the ISH signal/neuron in chronically injured motoneurons (filled circles in A and B), are decreased compared to contralateral (empty circles). However, in animals receiving a second axonal injury (C and D), the ISH signal for α-tubulin is highly increased. Note that the Y-axis scale is logarithmic, with the zero identifying the average ISH signal/neuron of the contralateral non-injured motoneurons.
FIGURE 20

ISH Signal X Contralateral (log10)

D

ISH Signal X Contralateral (log10)

C

ISH Signal X Contralateral (log10)

B

ISH Signal X Contralateral (log10)

A

α-tubulin mRNA

X-sectional area (microns²)
Figure 21. Effect of a second axonal injury on ISH signal for GAP-43 in chronically axotomized FMNs. In each case, the right column shows the axotomized facial nucleus, and the left column shows the contralateral non-injured facial nucleus. A/B and C/D are chronically axotomized animals, E/F and G/H are chronically axotomized animals that received a second axonal injury after 10 weeks. Baseline levels of GAP-43 hybridization signal very low in the facial nuclei contralateral to axotomy (A,C,E,G) and in the chronically axotomized facial nuclei (B, D). However, in chronically axotomized facial nuclei which have received a second axonal injury (F, H), there is a significant up-regulation of ISH signal. Note that, as in the case of α-tubulin, there is variability in the degree of increase between the animals shown in F and H, but that it is consistently significantly higher than in B and D. Scale bar = 60 μm.
Figure 21
Figure 22. Quantification of GAP-43 ISH signals in chronically axotomized FMNs: effect of a second axonal injury. Quantifications are shown from two chronically axotomized animals (A and B) and two chronically axotomized animals receiving a second axonal injury (C and D). Computer-aided quantification of the GAP-43 ISH signals reveal that the ISH signal/neuron in chronically injured motoneurons (filled circles in A and B), are similar to contralateral (empty circles). However, in animals receiving a second axonal injury (C and D), the ISH signal for GAP-43 is highly increased. Note that the Y-axis scale is logarithmic, with the zero identifying the average ISH signal/neuron of the contralateral non-injured motoneurons.
4.4 DISCUSSION

4.4.1 Neurotrophic regulation of gene expression in axotomized motoneurons

The primary objective of these experiments was to test whether regeneration-associated changes in gene expression by axotomized motoneurons are due directly to the interruption of target-derived neurotrophic support. Since function-blocking antibodies against the trkB neurotrophin receptor were not available, and the trkB, BDNF, and NT-3 knockout mice do not survive into adulthood, the paradigm I used was to infuse exogenous neurotrophins onto the cut facial nerve (ie. to mimic the trophic support normally received by the non-injured motoneurons). It is difficult to directly compare the concentrations of recombinant neurotrophins used in this study with those that the uninjured nerve would normally be exposed to at synaptic contacts with its target muscles (ie. given factors such as injury-induced changes in neuronal responsiveness and spatial distribution of receptors on axons versus nerve terminals). I made the assumption that the neurotrophin concentration that normalized the decline in acetylcholinesterase expression in the previous chapter would approximate the biological actions of the target-derived stores of trophic factors. Similar concentrations have been used to simulate neurotrophins from muscle targets in previous studies (Chiu et al., 1994, Friedman et al., 1995, Wang et al., 1997, Yan et al., 1994), and sequestration of endogenous muscle-derived neurotrophins has been shown to modify the electrophysiological properties of motoneurons (Munson et al., 1997), confirming that they regulate motoneuron functions. Nevertheless, in discussing these results it
should be kept in mind that effects elicited by addition of exogenous neurotrophins may not be a completely accurate reflection of the effects of the endogenous factors.

Neurotrophin administration onto the proximal nerve stump was found to modify the gene expression of the axotomized motoneurons. The partial suppression of the neurofilament down-regulation, together with the complete suppression of the acetylcholinesterase down-regulation in the previous chapter, support a model in which markers of the mature neuronal phenotype are positively regulated by target-derived neurotrophic support. The neurotrophin effects on the neurofilament expression of motoneurons are reminiscent of findings with sensory neurons (Verge et al., 1990). In contrast, changes in expression of markers of the regenerative phenotype, such as GAP-43, α-tubulins, and c-jun, do not appear to be suppressed by target-derived neurotrophic support (at least not by itself). Rather, the up-regulation of GAP-43 and α-tubulins was enhanced by exogenous neurotrophins. These data indicate that while target-derived neurotrophins may enhance the mature neuronal phenotype, they are probably not a target-derived signal for growing motoneurons to switch from the growth to mature phenotype. Furthermore, the genetic “programs” for growth and neurotransmission are not mutually exclusive, but can in fact be expressed simultaneously. Such a situation may occur during collateral sprouting, which can be triggered by activity-dependent release of target-derived neurotrophins (Funakoshi et al., 1995) and can be associated with increased expression of growth-associated tubulin isotypes (Mathew and Miller, 1990, Paden et al., 1995, Represa et al., 1993) and GAP-43 (Mearow et al., 1994; but see Bisby et al., 1996).
Interestingly, while GAP-43 and Tα1 α-tubulin expression were elevated by neurotrophins, the expression of c-jun was not significantly changed. Although the functions of GAP-43 and α-tubulins during axonal outgrowth are at least partially understood, the role of c-jun has remained ambiguous (discussed in detail in section 1.3.4), and its classification as a RAG may be questionable. The present results suggest that c-jun expression, at least in axotomized motoneurons, is relatively insensitive to changes in levels of exogenous neurotrophins.

The apparent stimulation of the RAGs GAP-43 and Tα1 α-tubulin by exogenous neurotrophins suggested that endogenous neurotrophic factors released in the vicinity of an axon injury site, such as neurotrophins, CNTF/LIF/IL-6 family members, IGFs, and GDNF, may contribute to the regenerative cell body responses of motoneurons. The next experiment in this chapter tested this prediction.

4.4.2 Chronically injured motoneurons are “rescued” by a second axonal injury

We found that axotomized motoneurons that were chronically deprived of target contact underwent substantial cell body atrophy and no longer expressed elevated levels of RAGs after 11 weeks. Our neuron counts suggested approximately 40-45% neuronal survival, which is consistent with the reports by others (Serpe et al., 1999). However, my data show that the majority of neuronal “loss” was due to massive atrophy and not neuronal death, as re-injuring the axons of chronically axotomized motoneurons increased the number of apparent remaining neurons to 75-80% of contralateral. Furthermore, the second injury reversed the cell body atrophy and stimulated an increase in both GAP-43 and α-tubulin mRNA levels. One possible
interpretation for this finding is that the neuroma acted as a substitute target, producing target-like molecules that down-regulated RAG expression. Accordingly, the second axonal injury could have simply interrupted the neuron—"target" interactions, up-regulating RAG expression. However, this seems unlikely given the massive atrophy of the motoneuron cell bodies, which does not occur when their axons successfully reach muscle targets. It is more likely that these regenerative cell body responses were triggered by axotomy-induced release of endogenous neurotrophic factors at the site of axonal injury. This interpretation is based on three lines of evidence. Firstly, it was found that exogenous neurotrophins applied to the nerve stump induced a similar increase in the cell body size and RAG expression by acutely axotomized motoneurons. Secondly, numerous endogenous neurotrophic factors are known to be released at a site of axonal injury, and are critical for the survival of axotomized motoneurons. For instance, axotomized motoneurons up-regulate the trkB neurotrophin receptor (Ernfors et al., 1993, Kobayashi et al., 1996a, Piehl et al., 1994, Tonra et al., 1998), whose ligands BDNF and NT-4/5 are produced by reactive Schwann cells (Funakoshi et al., 1993), and mice carrying a targeted deletion of the trkB gene have 50% more motoneuron loss than wild-type mice following neonatal facial nerve axotomy (Alcantara et al., 1997). Axotomized motoneurons also up-regulate gp130, the common signal transducing component for the receptor complexes of the CNTF/LIF/IL-6/cardiotoaphin family of cytokines (Heinrich et al., 1998, Murphy et al., 1997), and elevated levels of both CNTF and LIF are found at an axonal injury site (Curtis et al., 1994, Sendtner et al., 1992). In 4 week old mice carrying targeted deletions of both CNTF and LIF, axotomy induces a 35% greater loss of motoneurons
than wild-type mice by 2 weeks after injury (Sendtner et al., 1996a). Other neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF) and IGFs, have also been found to be produced at a site of axon injury (Hammarberg et al., 1996, 1998). Thus, endogenous neurotrophic factors at the lesion site are critical determinants of neuronal survival. Thirdly, recent studies have shown that weak regenerative cell body responses of CNS neurons can be strengthened by elevating the levels of neurotrophic factors in the immediate vicinity of the neuronal cell bodies. This has been experimentally accomplished by either directly infusing BDNF into the red nucleus (Kobayashi et al., 1997), or by placing a peripheral nerve segment into the vitreous of the eye, thereby exposing retinal ganglion cell bodies to the numerous neurotrophic factors produced by reactive Schwann cells (Berry et al., 1996).

4.4.3 Conclusions

Expression levels of regeneration-associated genes are an indicator of the regenerative potential of axotomized neurons, which varies widely between neurons in the mature mammalian nervous system (section 1.3.1). The present results indicate that the intrinsic regenerative potential of axotomized neurons can be critically regulated by levels of endogenous neurotrophic factors within the axonal environment. In comparison to axotomized peripheral neurons, which have access to a rich source of lesion site neurotrophic factors in the form of reactive Schwann cells and macrophages, the axons of axotomized CNS neurons may not be exposed to abundant amounts or varieties of neurotrophic factors. In the following experiments, I therefore investigated whether the typically weak cell body responses of axotomized CNS neurons can be strengthened by raising neurotrophic factor levels at a CNS lesion site.
EFFECTS OF SPINAL CORD INJURY AND LESION SITE
BDNF ADMINISTRATION ON THE REGENERATIVE CELL
BODY RESPONSES OF RUBROSPINAL NEURONS IN THE
ADULT MOUSE

5.1 SUMMARY

Results from the previous chapter suggest that endogenous neurotrophic factors (NTFs), known to be released by Schwann cells at a peripheral axonal injury site, can retrogradely enhance the cell body responses of axotomized motoneurons. Such lesion site-derived NTFs may be missing for axotomized CNS neurons, as oligodendrocytes have not been reported to be significant sources of NTFs. I therefore investigated whether supplementing a CNS lesion site with exogenous neurotrophic factors can improve the cell body responses of axotomized CNS rubrospinal neurons. To investigate this question, I first analyzed the cell body responses of adult mouse rubrospinal neurons to cervical and thoracic spinal cord injury, and then investigated the effects of lesion site treatment with Brain-Derived Neurotrophic Factor (BDNF). Transection of the cervical rubrospinal tract in adult mice induced strong mRNA in situ
hybridization signals for the regeneration-associated genes (RAGs) GAP-43 and α-tubulin within axotomized rubrospinal cell bodies. Hybridization signal intensity decreased between 7 and 21 days after transection, concomitant with atrophy of the cell bodies. In comparison, transection of the thoracic rubrospinal tract induced neither RAG expression nor cell body atrophy. When Gelfoam soaked in BDNF was inserted into the cervical lesion site at the time of injury, rubrospinal cell body responses were greatly improved. Examination of BDNF-treated rubrospinal neurons after 14 days revealed reduced cell body atrophy. BDNF-treated neurons also displayed higher levels of GAP-43 and α-tubulin, and an increased capacity for retrograde labelling with dilute concentrations of the fluorescent tracer, Fast Blue. These results demonstrate that mouse rubrospinal neurons have weak and transient cell body responses to cervical axotomy that can be strengthened by lesion site treatment with BDNF.
5.2 INTRODUCTION

The genes expressed by injured neurons are a critical determinant of their intrinsic capacity for axonal growth (detailed in chapter 1). Axotomized CNS neurons typically express only low levels of RAGs. For example, direct comparison of the injury responses of axotomized motoneurons with axotomized rubrospinal neurons demonstrated that rubrospinal RAG expression is limited in both magnitude and duration (Fernandes et al., 1999, Tetzlaff et al., 1991).

RAG expression and axonal regeneration by axotomized CNS neurons can be stimulated by treating the neuronal cell body with NTFs. Insertion of a peripheral nerve segment (a rich source of multiple NTFs) into the vitreous of the eye stimulated GAP-43 expression by axotomized retinal ganglion cells, and enabled these retinal ganglion cell axons to regenerate into a peripheral nerve transplant or even within the growth-inhibitory optic nerve itself (Berry et al., 1996, Ng et al., 1995). Similarly, direct infusion of Brain-Derived Neurotrophic Factor (BDNF) into the midbrain stimulated GAP-43 and tubulin gene expression by axotomized rat rubrospinal neurons, prevented their atrophy, and increased the number of rubrospinal axons found regenerating into a peripheral nerve transplant placed into the cervical spinal cord (Kobayashi et al., 1997). These studies support the principle that treatment of axotomized neuronal cell bodies strengthens the cell body responses to injury and increases the ability of axotomized neurons to regenerate their axons.

Results from the previous chapter suggested that endogenous NTFs, known to be released by Schwann cells at a PNS injury site (reviewed in section 1.3), can
retrogradely affect the cell body responses of axotomized motoneurons. In comparison, the corresponding CNS cell type, the oligodendrocyte, is not known to be a significant source of neurotrophic factors (NTFs). However, recent studies have shown that exogenous sources of NTFs applied to a CNS lesion site can elicit axonal growth (Grill et al., 1997a, Grill et al., 1997b, Liu et al., 1999). The mechanism by which lesion-site treatment of axotomized CNS neurons affects axonal growth has not yet been investigated. NTFs may elicit axonal growth at the injury site through a number of mechanisms, such as local effects on the axons (Berninger and Poo, 1996, Cai et al., 1999), reduction of the size of the lesion cavity (Novikova et al., 1996), prevention of axonal die-back (Guest et al., 1997, Lin et al., 1997, Weibel et al., 1994, Weibel et al., 1995), or via effects on non-neuronal cells. However, it is also possible that lesion site treatment with NTFs may strengthen the cell body responses of axotomized CNS neurons, as shown in the previous chapter for motoneurons.

The overall objective of this chapter is to assess the ability of lesion-site NTF treatment to modulate the cell body responses of axotomized CNS neurons. To accomplish this, I first characterized the injury responses of axotomized rubrospinal neurons in the adult mouse. Rubrospinal neurons are located in the red nucleus of the midbrain tegmentum. Rubrospinal axons extending from the magnocellular portion of the red nucleus decussate within the midbrain and project via the rubrospinal tract to ultimately regulate the activity of ventral spinal cord motoneurons, which function in the control of fine motor behaviour. Previous studies in the rat and opposum have shown that transection of the cervical rubrospinal tract causes axotomized rubrospinal neurons to undergo severe cell body atrophy, which results in the apparent loss of
identifiable RS neuron cell bodies. In the rat, at least, this atrophy occurs concomitant with a generalized reduction in expression of RAGs (Tetzlaff et al., 1991; Fernandes et al., 1999). While previous studies on rubrospinal neurons have primarily been carried out using rat and opposum models, here I have established the mouse model to take advantage of transgenic mouse technologies in future studies.
5.3 RESULTS

5.3.1 Organization of cervical- and lumbar-projecting populations of rubrospinal neurons in the adult mouse

Neurons within the red nucleus project to cervical and lumbar levels of the rat spinal cord, an arrangement that is likely to be similar in the mouse. To establish the projection patterns of mouse rubrospinal neurons to the spinal cord, retrograde labelling was performed from both the cervical and lumbar levels of the spinal cord within individual mice. This double retrograde labelling paradigm consisted of FluoroRuby application to a T10 hemi-section injury site combined with Fast Blue injection into the mid-cervical spinal cord. Examination of sections through the red nucleus revealed a consistent dorso-ventral segregation of cervical and lumbar projecting rubrospinal neurons, respectively (Fig. 23). This segregation was evident throughout the rostro-caudal extent of the red nucleus, and in both the magnocellular and parvocellular divisions. Since pronounced size differences between the larger magnocellular and smaller parvocellular rubrospinal neurons were noted, all subsequent analyses were limited to the caudal-most 300 µm of the red nucleus, ensuring that equivalent levels of the magnocellular red nucleus were always compared. Furthermore, within this region, the dorsomedial (cervical projecting) and ventrolateral (lumbar-projecting) rubrospinal neurons were sufficiently separated to be distinguished on the basis of cresyl violet staining alone. In the following experiments, the cell body responses of rubrospinal neurons to cervical spinal cord injury were examined. Since not all cervical-projecting rubrospinal neurons are injured by a
cervical spinal cord injury (i.e. some terminate more rostral to the lesion), we focused on the responses of the lumbar-projecting sub-population.

5.3.2 RAG expression by mouse rubrospinal neurons following cervical and thoracic spinal cord injuries

To analyze Regeneration-Associated Gene (RAG) expression in lumbar-projecting rubrospinal neurons, in situ hybridization (ISH) was performed using radioactive oligonucleotides complementary to GAP-43 and α-tubulin mRNAs. The rubrospinal tract was severed by a lateral hemi-section of the spinal cord at the C3 level. Gelfoam soaked in 2.5% Fast Blue was inserted into the lesion site at the time of injury. Mice were sacrificed after 7, 14, or 21 days. Seven days following axotomy, strong increases in hybridization signals were detected both for GAP-43 (Fig. 24) and for α–tubulins (Fig. 26). RAG expression was primarily localized to lumbar-projecting magnocellular RS neurons. Although many neurons retained elevated RAG expression after 14 days (not shown), GAP-43 signals were virtually undetectable by 21 days after axotomy (Fig. 24C), with only rare neurons maintaining expression. ISH signal for α–tubulins was similarly decreased by 21 days, with a few RS neurons continuing to express elevated levels, but the majority containing even lower ISH signals than the contralateral non-injured RS neurons (Fig. 26C).

Similar to our previous findings using rats (Fernandes et al., 1999), rubrospinal neurons that were axotomized at the T10 level of the spinal cord showed no change in RAG expression and no significant atrophy (not shown).
Figure 23. Double retrograde labelling to identify cervical and lumbar-projecting mouse rubrospinal neurons. Gelfoam containing 16% Fluoro-Ruby was placed into a T10 lateral hemi-section injury site, and 2.5% Fast Blue was injected into the cervical spinal cord. 20 μm cryostat sections through the red nucleus were visualized under fluorescence illumination using a broad spectrum filter that revealed both Fluoro-Ruby and Fast Blue fluorescence. Sections shown are at 100 μm intervals in a caudal to rostral direction (A-F) through the red nucleus. Lumbar-projecting rubrospinal neurons are located ventrally throughout the extent of the red nucleus. All sections are displayed with a dorsomedial (DM)/ventrolateral (VL) orientation as shown in A. Scale bar = 60 μm.
Figure 24. Autoradiographic signals in the mouse red nucleus following \textit{in situ} hybridization for GAP-43 mRNA. Hybridization signals are shown from the axotomized ("ax") and contralateral uninjured ("con") red nuclei, either 7 (A, B) or 21 (C, D) days after axotomy. Increased GAP-43 hybridization signals were detected in axotomized rubrospinal neurons after 7 days (A) but not after 21 days (C). Scale bar = 50 μm.
Figure 25. Quantification of GAP-43 ISH signals in axotomized mouse rubrospinal neurons. Hybridization signals for GAP-43 were quantified from the red nucleus of representative animals 7 (A) or 21 (B) days after hemi-section of the mouse spinal cord at the C3 level. Note that the majority of cervically axotomized rubrospinal neurons display highly elevated levels of GAP-43 hybridization signals after 7 days (A), but relatively few maintain this expression after 21 days (B). Filled circles represent neurons in the ventrolateral (lumbar-projecting) sub-group of the axotomized red nucleus; open circles represent the corresponding neurons in the contralateral uninjured red nucleus.
GAP-43

FIGURE 25
Figure 26. Autoradiographic signals in the mouse red nucleus following 
*in situ* hybridization for α-tubulin mRNA. Hybridization signals are shown 
from the axotomized ("ax") and contralateral uninjured ("con") red nuclei, either 
7 (A, B) or 21 (C, D) days after axotomy. Increased α-tubulin hybridization signals 
were detected in axotomized rubrospinal neurons after 7 days (A), but not after 21 
days (C). Scale bar = 50 μm.
5.3.3 Atrophy of axotomized mouse rubrospinal neurons

The axotomized red nucleus was examined for morphological changes up to 21 days after C3 level axotomy. Sections were stained in cresyl violet, examined with brightfield microscope settings, and cell sizes quantified using a computerized image analysis program. After 7 days, RS neuron cell bodies appeared slightly atrophic (Fig. 27B) compared to control mice (Fig. 27A). At both 14 (not shown) and 21 days (Fig. 25C) post-injury, the axotomized red nucleus was markedly shrunken in size, and a substantial proportion of the RS cell bodies appeared to have disappeared from the ventrolateral red nucleus. Measurement of the average cross-sectional area of rubrospinal neurons revealed little change in the cervical-projecting sub-group. In contrast, the average cross-sectional area of lumbar-projecting neurons declined from 345 $\mu$m$^2$ to 311 $\mu$m$^2$ by 7 days after axotomy, and was 165 $\mu$m$^2$ and 185 $\mu$m$^2$ by 14 and 21 days post-injury, respectively (Fig. 32). These latter measurements are likely to be an under-estimate of the actual rubrospinal atrophy, as only neurons that had not already shrunken to less than 4 microns in diameter were measured; retrogradely labelled neurons smaller than 4 microns were difficult to reliably discriminate from peri-neuronal glial cells that might have phagocytosed fluorescent RS neurons.
Figure 27. Axotomy-induced atrophy of cervical axotomized mouse rubrospinal neurons. Cresyl violet stained sections through the lumbar-projecting (i.e. ventrolateral) regions of the red nuclei in control mice (A) and C3 hemi-sected mice after 7 days (B) or 21 days (C). Note the reduction in size and apparent number of neurons in the ventrolateral region (arrows). VL=ventrolateral, DM=dorsomedial. Scale bar = 50 μm.
5.3.4 Retrograde labeling of rubrospinal neurons with Fast Blue

Analysis of corresponding cresyl violet (Fig. 28A) and Fast Blue (Fig. 28B) images from the same sections demonstrated that the entire cervical-projecting (dorsomedial) population of RS neurons was strongly labeled with Fast Blue. Upon closer examination, however, it was found that the lumbar-projecting (ventrolateral) population was heterogeneously labelled, including both Fast Blue negative (arrows) and positive cell bodies. To confirm that the unlabelled rubrospinal neurons were indeed axotomized by the lesion, Gelfoam soaked in both 2.5% Fast Blue and a 16% Fluoro-Ruby was inserted into cervical lesion sites. By virtue of its large size (MW=10000), the dextran amine Fluoro-Ruby is known to be taken up only by cut axons. Sections through the red nucleus of these animals were analyzed as above, and it was found that unlike Fast Blue, Fluoro-Ruby labeled virtually the entire lumbar-projecting ventral sub-group. Comparison of the Fluoro-Ruby fluorescence (Fig. 28C) with the Fast Blue fluorescence of the same section (Fig. 28D) clearly revealed that many of the axotomized lumbar-projecting neurons (Fluoro-Ruby positive) failed to be retrogradely labeled with Fast Blue (arrowheads). A number of the cervical projecting rubrospinal RS neurons appeared normal, and these were Fluoro-Ruby negative (Fig. 28C, arrows), indicating that they terminated rostral to the injury site and were therefore non-axotomized; however, they were still labelled with Fast Blue (arrows, Fig. 28D). Only very rarely was a Fast Blue or Fluoro-Ruby labelled neuron found in the contralateral red nucleus.

In light of the unexpected retrograde labelling pattern obtained with Fast Blue, a 400 nl bolus of Fast Blue was injected into the cervical spinal cord of several non-
injured mice, to help clarify the conditions under which Fast Blue retrogradely labels rubrospinal neurons. In one group of mice, the Fast Blue was dissolved in sterile water, and in a second group it was dissolved in 0.2% dimethylsulfoxide, which is sometimes used to help tracer penetration (Giehl and Tetzlaff, 1996). Fluorescence images revealed that, in both cases, Fast Blue labelled the cervical-projecting rubrospinal population exclusively (Fig. 29B), suggesting that in the absence of injury Fast Blue is taken up only by those neurons with synaptic terminals in the region of the injection.

Since axonal transport is known to mediate a number of injury signals from the injury site and target (section 1.4), the distribution of Fast Blue retrograde labelling was compared with the expression of RAGs in the same sections (Fig. 30). As described above, the lumbar-projecting area of the red nucleus contained RS neurons that were both Fast Blue positive (arrows) and negative (arrowheads) (Fig. 30A). Following in situ hybridization for α-tubulins, the same section displayed elevated ISH signals over both Fast Blue positive and negative rubrospinal neurons (Fig. 30C). The contralateral red nucleus did not contain fluorescent rubrospinal neurons (Fig. 30B) and displayed only low baseline levels of α-tubulin ISH signal (Fig. 30D). Similar findings were obtained following ISH using the GAP-43 probe (not shown). Thus, neither the presence nor the lack of Fast Blue retrograde labelling was correlated with the ability to undergo changes in expression of these RAGs.
Figure 28. Retrograde labelling characteristics of rubrospinal neurons. Two weeks following C3 axotomy, many neurons within the lumbar-projecting sub-group of the axotomized red nucleus can be identified by cresyl violet staining (A, arrows) but do not exhibit any Fast Blue fluorescence (B, arrows; same section as A). Double labelling with both Fluoro-ruby (C) and Fast Blue (D) from the C3 injury site confirms that the neurons not labelled by Fast Blue (arrowheads, D) do project to the spinal cord and have been axotomized, as they are successfully labelled by Fluoro-ruby (arrowheads, C; same section as D). Scale bar = 50 μm.
Figure 29. Retrograde labelling in the red nucleus following injection of Fast Blue into the uninjured cervical spinal cord of the mouse. Comparison of cresyl violet staining (A) and fluorescence (B; same tissue section as A) 7d following injection of Fast Blue into the cervical spinal cord reveals that, in the absence of injury, Fast Blue only retrogradely labels the dorsomedial rubrospinal neurons, which have terminations in the vicinity of the Fast Blue injection site. Scale bar = 50 μm.
Figure 30. Comparison of Fast Blue retrograde labelling with α-tubulin gene expression 7d after C3 hemi-section. Fluorescence images of the axotomized (A) and contralateral (B) red nuclei, and darkfield images of the same section following in situ hybridization for α-tubulins (C,D). Rubrospinal neurons that are labelled (arrows) and not labelled (arrowheads) with Fast Blue in (A) exhibit elevated levels of α-tubulin in situ hybridization signal in (C). The contralateral nucleus is not labelled (B) and has only background levels of α-tubulin gene expression (D). Scale bar = 50 μm.
5.3.5 Effects of BDNF on rubrospinal neuron atrophy

To determine whether exogenous BDNF at a lesion site could alter the cell body responses of lumbar projecting rubrospinal neurons, BDNF was administered to cervical spinal cord injury sites. In previous studies, BDNF has been shown to modify rubrospinal cell body responses when administered at a concentration of 500 ng/μl directly to the cell bodies of rubrospinal neurons (Kobayashi et al., 1998). In pilot experiments, it was found that infusion of 500 ng/μl of BDNF into the cervical spinal cord injury site of rats had no effects on the atrophy of the axotomized rubrospinal neurons. Recent in vitro studies have shown that neurons are about one order of magnitude less responsive to neurotrophins at their distal axons than at their cell bodies (Toma et al., 1997). In the case of BDNF, this is compounded by the presence of truncated trkB receptors within the spinal cord (Fryer et al., 1997), which are thought to sequester BDNF. In the present study, we therefore used a 20x higher concentration of BDNF (i.e. 10 μg/μl) than what was found effective for the stereotaxic injections directly into the red nucleus (500 ng/μl).

BDNF was applied to the cervical spinal cord injury site using Gelfoam soaked in 2.5 μl of 2.5% Fast Blue ± 10 μg/μl BDNF. Mice were perfusion fixed after 14 days. Cresyl violet staining of sections through the red nucleus revealed that more rubrospinal neuron cell bodies of larger size were present in the lumbar-projecting ventrolateral regions of the red nuclei of BDNF-treated mice (Fig. 31A,B). Quantifications of the cell sizes are shown in Table 3 below, and summarized in Fig. 32.
Table 3. Quantification of rubrospinal neuron cell body sizes following C3 axotomy and lesion site treatment with BDNF. Results shown are from a total of 60 to 80 cells for each treatment group, measured from 3 animals per group.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean size ± SD (μ²)</th>
<th>Median size (μ²)</th>
<th>25th-75th percentiles (μ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>346 ± 116</td>
<td>330</td>
<td>265-417</td>
</tr>
<tr>
<td>7d axotomized</td>
<td>312 ± 121</td>
<td>297</td>
<td>222-376</td>
</tr>
<tr>
<td>14d axotomized</td>
<td>166 ± 74</td>
<td>155</td>
<td>104-207</td>
</tr>
<tr>
<td>21d axotomized</td>
<td>185 ± 89</td>
<td>156</td>
<td>124-221</td>
</tr>
<tr>
<td>14d axotomized + BDNF</td>
<td>277 ± 138</td>
<td>244</td>
<td>170-341</td>
</tr>
</tbody>
</table>
Comparison of the cell size distributions of BDNF-treated rubrospinal neurons after 14 days (Fig. 32F) with those of 14 day vehicle-treated (Fig. 32D) and control rubrospinal neurons (Fig. 32B) illustrated that the lesion-site BDNF treatment partially prevented the axotomy-induced cell body atrophy. The BDNF and vehicle-treated samples were significantly different in size from each other after 14 days (p<0.0001).

5.3.6 Influence of BDNF on GAP-43 and α-tubulin expression

To assess the intrinsic growth potential of the BDNF-treated rubrospinal neurons, ISH was performed for the regeneration-associated genes α-tubulin and GAP-43. Since the size of rubrospinal neurons varies at different levels of the red nucleus, care was taken to ensure that comparisons were made using tissue sections at equivalent levels of BDNF and vehicle treated animals; specifically, within the caudal 200 μm of the magnocellular red nucleus. Examination of the red nuclei revealed more numerous and intense ISH signals for both GAP-43 and α–tubulins in BDNF- treated mice than in vehicle-treated mice (Fig. 33).

5.3.7 Influence of BDNF on Fast Blue retrograde labelling

Unexpectedly, examination of 20μm cryostat sections through the red nucleus under fluorescence illumination (Fig. 34A,B) revealed a strongly enhanced Fast Blue retrograde labelling intensity in animals that had received BDNF treatment. The strong labelling intensity was evident in both the dorsomedial and ventrolateral regions of the magnocellular red nucleus. In sections that were lightly stained in cresyl violet, combined brightfield/fluorescence imaging (Fig. 34C,D) illustrates that BDNF-treated
rubrospinal neurons did not exhibit the deficits of Fast Blue retrograde labelling that were present in vehicle-treated mice.
Figure 31. Lesion site BDNF application attenuates the atrophy of 14d cervical axotomized rubrospinal neurons. Examination of the ventrolateral (VL) red nucleus at equal distances from the caudal pole of the red nucleus reveals that BDNF (B) but not vehicle (A) treatment at the lesion site reduced the atrophy of the rubrospinal cell bodies. Scale bar = 50 μm.
Figure 32. Quantification of cell body sizes of 14d cervical axotomized rubrospinal neurons: effects of lesion site BDNF application. (A) Summary of the mean cross-sectional areas for lumbar-projecting rubrospinal neurons of control mice ("con"), vehicle-treated mice 7, 14, or 21 days after C3 hemi-section, and BDNF-treated mice after 14 days. Error bars show standard deviations. Sizes are measured from 3 animals in each category. In B-F, the rubrospinal neuron cross-sectional cell sizes are binned in 100 μm² categories. Note the shift towards smaller rubrospinal cell body sizes 14 (D) and 21 (E) days after injury. Comparison of cell sizes in control mice (B) with those from C3 injured mice treated with vehicle (D) or BDNF (F) demonstrates that lesion site BDNF treatment partially prevented the cell body atrophy observed 14 days after injury.
A summary of avg RS cell body size

B control

C vehicle+7d

D vehicle+14d

E vehicle+21d

F BDNF+14d

FIGURE 32
Figure 33. Effect of lesion site BDNF application on ISH signals for $\alpha$-tubulins and GAP-43 in 14d cervical axotomized mouse rubrospinal neurons. In situ hybridization for $\alpha$-tubulin and GAP-43 mRNAs reveal that the BDNF-treated rubrospinal neurons have higher expression levels of these regeneration-associated genes than the vehicle-treated rubrospinal neurons. Scale bar = 50 $\mu$m.
Figure 34. Effect of lesion site BDNF application on Fast Blue retrograde labelling of 14d cervical axotomized mouse rubrospinal neurons. The labelling intensity of the rubrospinal neurons was strongly enhanced by co-application of the BDNF with the Fast Blue at the lesion site (A, B). Combined fluorescence and brightfield cresyl violet staining revealed that BDNF-treated animals had virtually complete labelling of the ventrolateral red nucleus (C, D). Scale bar in B is 75 μm (A and B), scale in D is 50 μm (C and D).
5.4 DISCUSSION

5.4.1 Effects of cervical axotomy and BDNF on rubrospinal neuron size and gene expression

Using a double retrograde labelling paradigm that consisted of Fluoro-Ruby application to a thoracic hemi-section injury site combined with injection of Fast Blue into the cervical spinal cord, we found that the cervical and lumbar-projecting rubrospinal neurons are somatotopically segregated within the mouse red nucleus. A similar somatotopic arrangement is observed in the red nucleus of the rat, tree shrew (Murray et al., 1976), lesser bushbaby (Murray and Haines, 1975), and rhesus monkey (Castiglioni et al., 1978, Kneisley et al., 1978, Miller and Strominger, 1973); in contrast, retrograde labelling studies in the red nucleus of the North American opossum, *Didelphis virginiana*, have shown considerable overlap in the locations of the cervical and lumbar-projecting rubrospinal cell bodies (Martin et al., 1981).

Since not all cervical-projecting neurons are axotomized by a hemi-section at the C3 level of the spinal cord (ie. some may terminate higher), we focused on the injury responses of the lumbar-projecting sub-group of the red nucleus. Examination of the red nucleus 7, 14, and 21 days after C3 axotomy revealed that the mRNAs for GAP-43 and α-tubulins were up-regulated in most of the lumbar-projecting neurons at 7 days, but in relatively few neurons after 14 and 21 days. The decline in RAG expression was accompanied by a progressive injury-induced shrinkage of the rubrospinal neuron cell bodies. The atrophy was more severe for the lumbar-projecting neurons, and it also occurred mainly between days 7 and 21. The time-course of these changes in size and
gene expression is similar to previous findings for cervically axotomized rubrospinal neurons of rats (Tetzlaff et al., 1991).

Axotomy-induced atrophy of CNS neurons is generally believed to result from deprivation of trophic support from neuronal target fields. Axotomized PNS neurons rely on endogenous trkB signalling (Alcantara et al., 1997) and on CNTF/LIF/IL-6 related cytokines (Sendtner et al., 1997) (Murphy et al., 1999, Sendtner et al., 1996a) for survival. Although less intensely studied within the CNS, endogenous BDNF (a trkB ligand) also contributes to the survival of sub-cortically axotomized corticospinal neurons (Giehl et al., 1998). Exogenous BDNF applied in the vicinity of trkB-positive rubrospinal or corticospinal neuron cell bodies prevents their axotomy-induced atrophy/death, and increases their expression of regeneration-associated genes, suggesting that though they are responsive to BDNF, they are not exposed to sufficient endogenous quantities (Giehl and Tetzlaff, 1996, Kobayashi et al., 1997).

Recent studies have shown that growth factor-secreting cells transplanted into the injured rat spinal cord promote growth of spinal cord projecting neurons (Grill et al., 1997a, Grill et al., 1997b), including rubrospinal neurons (Liu et al., 1999). It is also worth noting that axotomized neurons provided with growth-permissive peripheral nerve transplants, whose Schwann cells release a variety of neurotrophic factors (Funakoshi et al., 1993, Heumann et al., 1987, Meyer et al., 1992, Zhong and Heumann, 1995), exhibit increased GAP-43 expression (Chong et al., 1996, Wouters et al., 1998). While application of growth factors to the spinal cord lesion site has been shown to reduce neuronal atrophy (Bregman et al., 1998), it is unclear whether these lesion site treatments stimulated axonal growth via local effects on the axons.
(Berninger and Poo, 1996) or via retrograde effects on the cell bodies of the axotomized neurons. In the present study, BDNF application to the cervical spinal cord injury site ameliorated the cell body atrophy and decline in RAG expression by axotomized mouse rubrospinal neurons, indicating that BDNF-treated rubrospinal neurons have a higher intrinsic growth potential. Similar cell body effects, and axonal growth, have been observed in rat rubrospinal neurons following BDNF infusion into the red nucleus (Kobayashi et al., 1997), and in rat retinal ganglion cells exposed to a degenerating peripheral nerve at their cell bodies (Berry et al., 1996). Thus, axonal growth observed following treatment of spinal cord injury sites with sources of growth factors (e.g. Liu et al., 1999) is probably at least partly due to a strengthening of intrinsic neuronal cell body responses to injury.

5.4.2 Retrograde labelling of rubrospinal neurons with Fast Blue

An unanticipated finding of the present experiments was the failure of many of the lumbar-projecting rubrospinal neurons to be retrogradely labelled with Fast Blue from a cervical injury site. Before discussing the possible significance of this finding, it is useful to summarize the conclusions that can be drawn from the present experiments regarding the characteristics of Fast Blue retrograde labelling (i) normally, (ii) after axotomy, and (iii) after axotomy + BDNF treatment.

Firstly, in non-injured subjects, 2.5% Fast Blue dissolved in sterile water or dilute DMSO (0.2%) can be taken up and retrogradely transported only by the synaptic terminals of rubrospinal axons, but is not taken up by axons passing through the
region. This is demonstrated by the finding that injection of Fast Blue in sterile water into the cervical spinal cord labelled only the cervical-projecting population.

Secondly, if Fast Blue is placed into a C3 injury site, it still labels the entire cervical population of rubrospinal neurons, but in addition, now labels the majority of lumbar-projecting rubrospinal neurons as well. Thus, in addition to axonal terminals, Fast Blue is taken up by injured axons. However, a significant sub-population of the axotomized lumbar-projecting rubrospinal neurons failed to be retrogradely labelled by Fast Blue. The fact that these unlabelled neurons were indeed axotomized was confirmed by examination of the extent of cervical spinal cord lesion sites; by their up-regulation of the regeneration-associated genes GAP-43 and α1-tubulin; and by their labelling with the 10,000 MW dextran amine, Fluoro-Ruby. The significance of the failure of these neurons to be retrogradely labelled with Fast Blue is unclear (discussed below).

Thirdly, retrograde labelling of the lumbar-projecting rubrospinal neurons was substantially enhanced when Fast Blue was co-applied with BDNF at the lesion site. Animals which received BDNF and Fast Blue together into the cervical lesion site had almost complete labelling of the lumbar-projecting rubrospinal sub-population. Furthermore, these neurons were more intensely labelled in the Fast Blue/BDNF treated than in the Fast Blue only-treated mice. It is unclear whether the BDNF-induced improvement in retrograde labelling was due to enhanced tracer uptake, retrograde transport, or both.

The significance of retrograde labelling failure, and its improvement in response to BDNF, is uncertain. Retrograde tracing with the Fast Blue has been reliably used in
numerous studies (ie. Giehl and Tetzlaff, 1996). However, declines in efficiency of retrograde labelling have been correlated with various pathological changes. For instance, reduced retrograde labelling (i.e. tracer uptake and/or retrograde transport) has been reported in basal forebrain cholinergic neurons of the aged rat (Cooper et al., 1994, De Lacalle et al., 1996). Axonal transport deficiencies have also been measured in mutant and transgenic mouse models of motoneuron degeneration, such as the wobbler (Bird et al., 1971, Mitsumoto et al., 1994) and progressive motor neuropathy (pmn) (Sagot et al., 1998) mice, and mice over-expressing neurofilament-H (Collard et al., 1995) or superoxide dismutase (Tu et al., 1996). Sagot and colleagues recently demonstrated that pmn mice exhibit impaired retrograde transport of Fast Blue and FluoroGold to their motoneuron cell bodies, and that this impaired transport precedes the observed onset of motoneuron degeneration; thus, reduced retrograde labelling appeared to be an early indicator of motoneuron degeneration. Furthermore, retrograde labelling, motoneuron survival, and the life span of these pmn mice were all increased by the co-application of specific neurotrophic factors with the retrograde tracers (Sagot et al., 1998). Since we also found enhanced retrograde labelling by co-applying BDNF and Fast Blue at the lesion site, we speculate that the uptake/retrograde transport mechanisms may be similarly compromised in axotomized rubrospinal neurons as in the above models of degeneration. Consistent with this notion, we note an apparent relation between severity of injury and efficiency of retrograde labelling with Fast Blue. Following C3 axotomy, the lumbar-projecting neurons are more severely injured than the cervical projecting neurons (in terms of amount of axon lost), and many of the lumbar-projecting sub-group fail to be
retrogradely labelled. Furthermore, using a more severe double-lesion paradigm in rats, Tseng and colleagues have reported that the entire lumbar sub-group fails to be retrogradely labelled when crystals of Fast Blue are placed into a C2 injury site if those neurons have received a prior C5 or T10 axotomy (Tseng et al., 1996).

How might BDNF improve the retrograde labelling of axotomized rubrospinal neurons? One possibility is that die-back of rubrospinal axons from the injury site is reduced (Guest et al., 1997, Lin et al., 1997, Weibel et al., 1994, Weibel et al., 1995). Alternatively, BDNF-induced growth of the rubrospinal axons (Liu et al., 1999) may increase the amount of axon surface exposed to tracer, allowing them to take up a greater quantity. These possibilities cannot be distinguished in the present experiments.

5.4.3 Conclusions

In this chapter, we have used a mouse model to characterize the injury responses of cervical axotomized rubrospinal neurons. These injury responses include an initial up-regulation of regeneration-associated genes, which declines concomitant with severe atrophy of the rubrospinal cell bodies, and a partial failure to be retrogradely labelled with Fast Blue. Application of BDNF to the spinal cord injury site enhances the regeneration-associated gene expression, ameliorates the cell body atrophy, and improves the Fast Blue retrograde labelling. Taken together, these results reveal that a variety of rubrospinal neuron cell body responses to injury can be improved by trophic factor application to the cervical spinal cord injury site. The
underlying mechanisms of these injury and BDNF-induced changes will be further investigated using transgenic mouse models.
GENERAL DISCUSSION

In the following sections, I summarize my principal findings, place them into a larger context, and attempt to address some of the questions they raise.

6.1 ROLES OF TARGET-DERIVED NTFS FOR MATURE NEURONS

In chapters 3 and 4, I investigated whether axotomy-induced loss of target-derived neurotrophic factors may be the signal triggering specific changes in neuronal gene expression. Two genes whose expression decrease upon axotomy were studied, the cholinergic enzyme acetylcholinesterase (AChE) and medium molecular weight neurofilament (NFM), and three genes whose expression increase after axotomy were studied, Growth Associated Protein-43 (GAP-43), α-tubulin, and c-jun. Supplementing the cut facial nerve with exogenous neurotrophins revealed that trkB ligands (i.e. BDNF and NT-4/5) are capable of completely preventing the axotomy-induced down-regulation of AChE, and partially prevent the down-regulation of NF-M. In contrast, neurotrophin administration did not prevent the up-regulation of GAP-43, α-tubulin, and c-jun. Rather, quantification of the hybridization signals suggested that trkB ligands actually caused an increase in GAP-43 and α-tubulin mRNA levels. Thus, while
target-derived neurotrophins may enhance the mature neuronal phenotype, they aren't by themselves a molecular switch from the growth phenotype to the mature neuronal phenotype.

It is important to note that these experiments have not specifically identified neurotrophins as the target-derived factors responsible for the phenotypic maturation of motoneurons. Rather, these findings indicate that the signalling pathways activated by neurotrophins, most likely via the trkB receptor tyrosine kinase, promote the maturation of the motoneuron phenotype; it remains possible that other target-derived NTFs using common signalling pathways also contribute.

While neurotrophins do not appear to be the target-derived repressors of regeneration-associated gene (RAG) expression for motoneurons, it is still considered likely that the target is a source of repression for RAGs. For example, studies using both sensory and motoneurons have shown that GAP-43 is up-regulated to a similar level regardless of how far axotomy occurs from the neuronal cell body - thus, the source of GAP-43 repression does not appear to be from the ensheathing cells. In contrast, I found that mouse rubrospinal neurons up-regulated RAGs after cervical but not thoracic axotomy (chapter 5; see also Fernandes et al., 1999). However, this finding may still be explained by invoking a target-derived repressor model: rubrospinal axons projecting to the thoracic level have collaterals to targets at more proximal levels of the spinal cord as well, which may continue to supply repressors to thoracically axotomized rubrospinal neurons. Interestingly, dependence of RAG expression on the axotomy-to-cell body distance has been described in other CNS models as well, which cannot simply be accounted for by axonal collaterals; for example, retinal ganglion
cells, which do not have collaterals, only up-regulate GAP-43 if axotomized within 3 mm of their cell bodies (Doster et al., 1991). A possible model to reconcile these findings is presented in section 6.4.

6.2 LESION SITE-DERIVED NTFs ELICIT REGENERATIVE CELL BODY RESPONSES FROM CHRONICALLY AXOTOMIZED MOTONEURONS

Exogenous trkB ligands, BDNF and NT-4/5, administered to the proximal stump of the facial nerve were found to accentuate the axotomy-induced up-regulation of GAP-43 and α-tubulin mRNAs. Recent studies have shown that synthesis of these neurotrophins is up-regulated by Schwann cells in the vicinity of an axonal injury (Funakoshi et al., 1993). In addition, numerous additional neurotrophic factors are also synthesized by reactive Schwann cells and macrophages at a peripheral injury site (including CNTF, LIF, GDNF, IGFs, and interleukins; reviewed in section 1.3), and are likely to use many overlapping signalling pathways. I therefore hypothesized that endogenous NTFs at an axonal injury site, such as BDNF and NT-4/5 (Funakoshi et al., 1993), CNTF (Sendtner et al., 1992), LIF (Curtis et al., 1994), GDNF and IGFs (Hammarberg et al., 1996, 1998), enhance the regenerative responses of the axotomized neuronal cell body. To test this hypothesis, I re-axotomized motoneurons which had been chronically separated from their target musculature, and found that this axonal injury was sufficient to rescue and induce regenerative changes in neuronal cell body gene expression and size. I suggest that these regenerative changes are mainly stimulated by the endogenous neurotrophic factors released at the injury site, as the
effects on cell body size and gene expression were reminiscent of those I obtained by infusion of exogenous neurotrophins. However, it cannot be excluded that other processes participated as well, such as electrophysiological changes induced by the axonal injury. Nevertheless, these findings have two important implications.

Firstly, regeneration-associated gene expression appears to require at least two types of molecular “signals”. Interruption of target-derived repression is only one of the signals, as motoneurons whose axons were resected in order to prevent regeneration (“chronically axotomized”) were found to have only low levels of RAG expression remaining after 11 weeks. A second axonal injury to the proximal nerve stump of these chronically axotomized motoneurons supplied a second required signal, as it re-induced an up-regulation of RAG expression. A possible model to explain these findings is presented in section 6.4 below.

Secondly, the cell bodies of axotomized neurons can remain in a highly atrophic, unrecognizable state for long periods of time, and this atrophy may be reversed by NTFs. We found a remarkable reversal of atrophy of the chronically axotomized motoneurons by a second axonal injury after 10 weeks. The atrophy reversal was dramatic enough to actually increase the number of countable motoneurons from about 40-45% to 75-80% of controls. Since axotomized CNS neurons, including rubrospinal neurons, undergo massive cell body degeneration, it is possible that the apparent “loss” of neuronal cell bodies may often not be neuronal death but severe neuronal atrophy. In support of this theory, recent work in our laboratory has found that infusion of BDNF into the red nucleus of the rat 52 weeks following cervical spinal cord hemi-section restores a majority of the rubrospinal cell bodies that had apparently
disappeared (N. Kobayashi and W. Tetzlaff, unpublished). These findings have important implications for the potential of treating chronic spinal cord injuries.

6.3 ADMINISTRATION OF NTFs TO THE LESION SITE OF ACUTELY AXOTOMIZED CNS NEURONS REDUCES THEIR ATROPHY AND MAINTAINS THEIR RAG EXPRESSION

Previous studies have demonstrated that sources of neurotrophic factors within the vicinity of axotomized CNS neuronal cell bodies are capable of enhancing their regenerative cell body responses (Berry et al., 1996, Kobayashi et al., 1997). Since I had found that supplying neurotrophic factors to the site of axonal injury enhanced the cell body responses of acutely axotomized motoneurons, and that endogenous neurotrophic factors released by axonal injury appear to have a similar effect, I hypothesized that administration of appropriate NTFs to the lesion site of axotomized CNS neurons would enhance their regenerative cell body responses as well. Administration of BDNF to the cervical lesion site of axotomized rubrospinal neurons was found to have several biological effects on the rubrospinal cell bodies, including reduction of soma atrophy, longer expression of regeneration-associated genes, and enhanced retrograde labelling with dilute amounts of Fast Blue tracer from the lesion site. Thus, lesion site treatment with NTFs may be an effective means of stimulating regenerative cell body responses by axotomized CNS neurons.

Whether lesion site treatments with NTFs can replace direct treatments of the neuronal cell bodies is not yet established. For example, since the reasons for RAG
expression after cervical but not thoracic axotomy are not known (Fernandes et al., 1999), it is conceivable that lesion site treatment with BDNF will be effective after cervical but not thoracic axotomy. Also, it is possible that some injured axons might “die-back” from the injury site, particularly in the chronic situation.

In addition to retrograde effects on the axotomized neuron’s cell body responses, lesion site NTF application can have several local beneficial effects at the injury site itself. These effects may include (i) a reduction of axonal die-back from the injury site (Guest et al., 1997, Lin et al., 1997, Weibel et al., 1994, Weibel et al., 1995); (ii) a reduction in the size of the lesion-induced cavity (Novikova et al., 1996); and (iii) an increased axonal resistance to growth cone collapse in response to myelin-associated growth inhibitors, mediated by a putative cAMP-PKA-Rho pathway (Cai et al., 1999, Lang et al., 1996).

6.4 HYPOTHETICAL MODEL FOR REGULATION OF RAG EXPRESSION

There is currently no simple explanation for the stronger up-regulation of regeneration-associated genes following axotomy proximal than distal from the cell body of CNS neurons. Transgenic mouse studies using the lacZ reporter gene attached downstream of either the GAP-43 (Vanselow et al., 1994) or Tα1 α–tubulin (Gloster et al., 1994) promoter regions indicate that these genes are normally transcriptionally repressed by molecules from neuronal target tissues. One possible explanation for the distance-dependent expression in the CNS is continued transcriptional repression supplied from axonal collaterals located more proximal to an injury site. However, this simple model
does not fit for the optic system, where there are no collaterals. It has also been proposed that CNS myelin along the proximal axon is an additional source of transcriptional GAP-43 repression (Kapfhammer and Schwab, 1994), so that shorter proximal axons exert less inhibition of GAP-43 expression than longer axons. However, myelin fractions do not repress GAP-43 expression in developing cortical neurons (Karimi-Abdolrezaee and Schreyer, 1998), and treatment of Purkinje cell axons with antibodies to the NI-35/250 components of myelin fail to induce expression of GAP-43 (Zagrebelsky et al., 1998).

An alternative explanation, proposed here, is that prolonged injury-induced expression of GAP-43 involves two processes: (i) axotomy-induced release from target-derived transcriptional repressors, and (ii) additional positive signals to stabilize the newly transcribed mRNAs. Such positive signals may occur in the form of neurotrophic factors (Kobayashi et al., 1997) or inflammatory factors (Lu and Richardson, 1991, Lu and Richardson, 1995, Richardson and Lu, 1994), both of which have been shown to increase GAP-43 mRNA levels. Interestingly, recent in vitro studies of sensory and sympathetic neurons appear consistent with a two signal hypothesis, as primary DRG cultures show increased galanin expression in response to NGF withdrawal (negative signal) or LIF application (positive signal) (Corness et al., 1998). Similarly, explants of sympathetic ganglia explants show an increase in STAT signalling after NGF withdrawal or treatment with LIF (Rajan et al., 1998).

Post-transcriptional processes do indeed appear to regulate mRNA stability of both GAP-43 (Neve et al., 1999) and tubulins (Bhattacharya et al., 1991, Moskowitz and Oblinger, 1995b). In the case of tubulin, for example, the dramatic reduction in β-II tubulin mRNA expression between postnatal day 5 and adult rats has been shown to be
accompanied by only a minor reduction in the rate of transcription, suggesting that
decreased mRNA stability is the predominant regulatory process involved (Bhattacharya
et al., 1991, Moskowitz and Oblinger, 1995b). Some evidence also exists for suggesting
that neurotrophic and inflammatory factors modulate mRNA stability, as NGF has been
shown to increase GAP-43 expression \textit{in vitro} via post-transcriptional mechanisms
(Nishizawa, 1994, Perrone-Bizzozero et al., 1993, Tsai et al., 1997).

Using this model, we would interpret the preceding observations in the following
fashion: (i) Axotomy of PNS neurons de-represses GAP-43 transcription, and the variety
of neurotrophic factors and cytokines to which PNS neurons are exposed (section 1.2)
enhance stabilization of the GAP-43 mRNAs; (ii) Cervically axotomized rubrospinal
neurons show an initial up-regulation of growth-associated genes due to loss of target-
derived repression, but this is attenuated and transient due to limited quantities of local
trophic/inflammatory factors to stabilize the growth-associated mRNAs - thus, they
atrophy and undergo a decline in RAG expression after 7 days; (iii) Thoracically
axotomized rubrospinal neurons have more proximal sustaining collaterals that continue
to supply both target-derived GAP-43 repressors \textit{and} adequate trophic support - thus,
they undergo very limited atrophy, and fail to increase GAP-43 transcription; (iv) Retinal
ganglion cells are predicted to increase GAP-43 transcription after axotomy, but are
found to only show increased GAP-43 mRNA levels after lesions that are within 3 mm
from the retina (Doster et al., 1991) because injury-related cytokines and trophic factors
reach the neuronal cell bodies from the intra-orbital lesion sites but not from the more
distant intracranial lesion sites; (v) Furthermore, positive signals arising from peripheral
nerve transplants may explain why only those neurons regenerating into them maintain high expression of axotomy-induced genes such as GAP-43, tubulins, and c-jun.

Conceptually, this model provides an attractive mechanism (i.e. enhancement/stabilization of the pre-existing transcriptional state of the neuron) with which to explain the diverse effects of neurotrophic factors over the course of neurodevelopment (proliferation, growth, terminal differentiation). However, at present this model is speculative.

6.5 CONCLUSIONS

The work presented in this thesis provides insights into the signals regulating axotomy-induced changes in neuronal gene expression. Injured PNS motoneurons decrease their gene expression for neurotransmitter enzymes and neurofilaments, markers of the mature neuronal phenotype, and this appears attributable to loss of target-derived NTFs. However, following axotomy, non-target-derived NTFs present at the lesion site can act as "positive" signals to stimulate regenerative cell body responses. Their effects are potent enough to even reverse the atrophy and decline in regeneration-associated gene expression by chronically axotomized motoneurons. By supplementing a CNS injury site with exogenous BDNF, it was possible to strengthen the weak and transient regenerative cell body responses of axotomized CNS rubrospinal neurons. Since recent studies have shown that improving neuronal cell body responses to injury increases the success of axon regeneration by CNS neurons, future work can investigate whether lesion site treatment with BDNF by itself enables injured CNS neurons to sprout and/or regenerate within the growth-inhibitory CNS environment. However, it remains
unclear whether large-scale axonal regeneration can be achieved within the CNS solely by enhancing neuronal growth capabilities. For example, even if neurons having a high intrinsic growth capacity can overcome myelin-associated growth inhibitors, the physical and chemical barrier presented by the lesion site scar and cavitation may still require additional treatments (Davies et al., 1997, Davies et al., 1999, Davies and Silver, 1998). Furthermore, proper functioning of restored circuits will necessitate remyelination of the regenerated axons and appropriate sensori-motor retraining (Muir and Steeves, 1997). Thus, it appears that lesion site treatment with neurotrophic factors could be part of a combinatorial therapeutic strategy that addresses both intrinsic neuronal and extrinsic glial aspects of spinal cord injury.


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