THE ROLE OF BRAIN-DERIVED NEUROTROPHIC FACTOR IN THE INJURED/REGENERATING SENSORY NEURON

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Anatomy and Cell Biology

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ABSTRACT

Peripheral nerve injury induces a robust regenerative state in sensory neurons that includes elevated expression of injury/regeneration-associated genes. The molecular signal(s) underlying the transition to the regenerating state are largely unknown. Brain-derived neurotrophic factor (BDNF) is the sole identified neurotrophin that is upregulated in sensory neurons following peripheral nerve injury. As members of the neurotrophin family exert a profound influence on the intact phenotype of sensory neurons, I hypothesize that injury-associated alterations in BDNF expression play a similar role in the injured/regenerating response. Antagonizing endogenous BDNF with a function-blocking antibody prevented increases in injury/regeneration-associated gene expression and decreased the growth capabilities of the injured sensory neurons. However, BDNF was not important for maintaining this cell body response in injured neurons. The elevation of BDNF expression in injured sensory neurons either through intrathecal infusion or electrical stimulation was associated with increased injury/regeneration-associated gene expression in a dose dependent manner and the latter corresponded to increased sensory axonal regeneration. Though BDNF was able to induce and enhance the intrinsic cell body response of injured sensory neurons, exogenous BDNF was not sufficient to induce an injury phenotype in intact sensory neurons. Thus, additional signals are likely induced by the injury response. In conclusion, BDNF plays a critical role in inducing the regenerative state in sensory neurons following injury and strategies aimed at elevating levels of BDNF available to the injured sensory neuron during the inductive phase improve the cell body response.

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LIST OF ABBREVIATIONS

AC	alternating current
ATF3	activating-transcription factor 3
BDNF	brain-derived neurotrophic factor
cAMP	3’5’ cyclic adenosine monophosphate
CGRP	calcitonin gene-related protein
CNS	central nervous system
CREB	3’5’ cyclic adenosine monophosphate response element binding protein
DC	direct current
DRG	dorsal root ganglia
ERK	extracellular-signal-related kinase
FG	fluorogold
FR	fluororuby
GAP-43	growth-associated protein 43
GDNF	glial cell line-derived neurotrophic factor
HSP-27	heat shock protein 27
IL-6	interleukin-6
LIF	leukemia inhibitory factor
MAG	myelin-associated glycoprotein
NGF	nerve growth factor
NT-3	neurotrophin-3
NPY	neuropeptide Y
PNS	peripheral nervous system
SP	substance P
STAT3	signal transducer and activator of transcription 3
TNFR	tumor necrosis family receptor
trk	tropomyosin-related kinase
TTX	tetrodotoxin

1.0 INTRODUCTION

1.1 Primary Sensory Neurons and Neurotrophins.

1.1.1 Function of Sensory Neurons.

The peripheral nervous system (PNS) detects and responds to specific variables in the internal and external environment. In order to interact with the environment; peripheral nerves run throughout the body and are less protected leaving them vulnerable to damage. Neurons of the PNS have the ability to robustly regenerate after peripheral nerve injury unlike those of the central nervous system (CNS). The propensity for peripheral neurons to regenerate is likely a consequence of their function.

The peripheral nervous system includes sensory, motor neurons, sympathetic and parasympathetic neurons. The sensory neurons are important detectors of change in the environment. Sensory neurons are pseudounipolar, with a single axon arising from the cell body bifurcating to form a peripheral branch and a central branch (Tandrup, 1995). The peripheral branch innervates peripheral tissue (i.e. skin, muscle or joint) while the central branch enters the spinal cord. The cell bodies of the sensory neurons are located outside of the spinal cord in clusters referred to as the dorsal root ganglia (DRG). Sensory neurons acquire information about the environment from sensory receptors and relay it to the central nervous system (CNS). Response to the environment occurs when the information is processed by our CNS and in some instances transmitted to the motor neurons.

Sensory neurons can detect and respond to a variety of different stimuli. The modality of the peripheral stimulus that the sensory neuron can detect is an important characteristic of that neuron. For instance, nociceptive neurons are important in the perception of pain. This includes the detection of pain producing stimuli that are thermal, chemical or mechanical in nature. Nociception is important because it protects the body from injury and tissue damage. Other neurons can detect innocuous mechanical input, i.e. mechanoreceptive sensory neurons detect input such as touch,

muscle stretching, and pressure. Finally, some sensory neurons are proprioceptive in function. Proprioception refers to kinesthetic awareness and this includes the capacity to locate limbs in space. This involves the sensation of joint motion and acceleration important for motor control and posture.

1.1.2 Organization of Sensory Neurons.

Distinct subpopulations of sensory neurons are associated with each specific modality of peripheral stimulus that the cell detects. The modality that the sensory neuron subserves can to a large degree be correlated with cell body size/diameter of axon, conduction velocity, biochemical characteristics and trophic responsiveness (Lewin, 1996; reviewed in Lindsay, 1996). As a result, sensory neurons of the DRG comprise a heterogeneous population of cells. For simplicity, general assumptions are used to categorize sensory neurons. Although sensory neurons present in each category can be further subdivided, such distinctions are not required for this thesis.

1.1.2.1 Axon Diameter and Conduction Velocity.

Sensory neurons can be classified according to axon diameter and conduction velocity. Axon diameter is also related to soma size, although there is not always a precise correlation. Sensory neurons that transmit nociceptive information consist of Aδ fibers and C-fibers. Aδ fibers are medium size and are covered with thin myelin sheaths. Aδ fibers transmit are associated with the sensation of sharp, pricking pain. C-fibers have small diameters and are not myelinated; as a result their conduction velocity is slow. C-fibers are associated with the sensation of dull pain. C-fibers are also polymodal and can detect more than one modality, such as heat and chemical stimuli (reviewed in Millan, 1999). An innocuous stimulus is transmitted by Aβ-fibers and a subset of Aδ-fibers. Aβ-fibers are medium-large in diameter and since they are myelinated they have rapid conduction velocities. Aβ-fibers are responsive to touch, vibration, pressure and proprioception (reviewed in Millan, 1999). A portion of Aδ-

fibers also respond to low threshold mechanosensation (reviewed in Lewin and Mendell, 1993).

1.1.2.2 Biochemical Markers.

Sensory neurons that are responsive to a select modality also express distinguishing enzymes, proteins and genes. Though the function of most of these molecules are not known, their expression can be used as biochemical markers to identify the distinct subpopulation of sensory neurons (Carr and Nagy, 1993). The expression of these biochemical markers is not mutually exclusive and may overlap in some subpopulations. The pattern of expression of these biochemical markers under homeostatic conditions is referred to as an intact phenotype. The expression of many neuronal markers neuron is altered in response to pathologic conditions such as inflammation and injury. Understanding the modulators of expression of these molecules is important in understanding the regulation of the inflammation and/or regenerative response.

1.1.3 Neurotrophins.

The neurotrophin family, whose function in sensory neurons have been well studied, include nerve growth factor (NGF; Levi Montalcini, 1951; Levi-Montalcini et al., 1954; Cohen, 1960), neurotrophin-3 (NT-3; Ernfors et al., 1990a; Hohn et al., 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), and brain-derived neurotrophic factor (BDNF; Barde et al., 1982, Leibrock et al., 1989). The members of the neurotrophin family share a high degree of structural homology and are biologically active as noncovalent homodimers. This closely related family of trophic factors regulates neuronal survival, differentiation and phenotype (reviewed in Lewin, 1996; reviewed in Lindsay, 1996). Neurotrophins are predominately synthesized and released by target tissues and retrogradely transported by receptor mediated processes. The neuronal fate of sensory neurons likely arises during development in response to neurotrophins (reviewed in Lewin, 1996). Gene deletion studies reveal that distinct

functional subpopulations of DRG neurons are critically dependent on select neurotrophins for their survival and development. In the adult DRG these subpopulations of sensory neurons are largely associated with distinct sensory modalities and are maintained by select neurotrophins (i.e. NGF for nociceptive and NT-3 for proprioceptive neurons). However, the action of the neurotrophins themselves can extend beyond the subpopulation and regulate other modalities (see below).

1.1.3.1 Nerve growth factor.

In development, NGF is an important survival factor for the nociceptive subpopulation of DRG neurons. Genetic deletion of NGF or administration of NGF antibodies *in utero* results in a loss of the small diameter neurons (Ruit et al., 1992; Crowley et al., 1994). NGF deficient mice display ~70% fewer neurons which reflects the predominance of small diameter neurons in wild type DRGs (Crowley et al., 1994). In axotomized DRG neurons, NGF rescues the small nociceptive neurons but does not rescue the large proprioceptive neurons (Miyata et al., 1986).

In adults, NGF is no longer essential for neuronal survival. Treatment of sensory neurons in the early postnatal period with NGF does not influence cell death (reviewed in Lewin, 1992). Instead, NGF maintains the phenotype of the small-medium diameter nociceptive sensory neurons (reviewed in Lewin and Mendell, 1993; Verge et al., 1989a; 1990a;b; 1995). Virtually all neurons that are NGF-responsive also express calcitonin gene-related peptide (CGRP; Verge et al., 1989a; Averill et al., 1995). Infusion of exogenous NGF in intact DRG neurons increases the level of CGRP and substance P (SP) in intact DRG neurons (Inaishi et al., 1992; Verge et al., 1995), whereas infusion of an NGF antibody decreases these neuropeptides, such as SP (Schwartz et al. 1982).

Though ~ 70% of sensory neurons require NGF for survival in development, only 40-45% of sensory neurons in adult DRG are responsive to NGF (Verge et al., 1989b; Verge et al., 1992; Karchewski et al., 1999). Postnatally there is a change in some of the small diameter neurons and these neurons are no longer responsive to any member of the neurotrophin family. Instead they express the glial cell-line derived

neurotrophic factor (GDNF) receptor, which can be recognized by its ability to bind isolectin B4 (Bennett et al., 1998). It is this population of DRG neurons that no longer expresses detectable levels of neurotrophin receptors and is presumably no longer responsive to the neurotrophin family (McMahon et al., 1994).

1.1.3.2 Neurotrophin-3.

NT-3 is important for the survival of the medium-large diameter (Aβ-fibers) proprioceptive and mechanoceptive sensory neurons in development. Mice lacking NT-3 lack the proprioceptive muscle spindle afferent neurons and display abnormal posture and movement (Ernfors, 1994b; Farinas et al., 1994; Klein et al., 1994). NT-3 also supports survival of developing sensory neurons that project to muscle *in vitro* (Hory-Lee et al., 1993). However, NT-3 does not influence survival of all sensory neurons that project to the muscle. For example, nociceptive neurons that innervate the muscle are not influenced by NT-3 (Farinas et al., 1994). NT-3 also supports the survival of mechanoceptive neurons that innervate the skin. A subset of cutaneous afferents required for mechanoception are absent in heterozygous NT-3 null mutant mice (Airaksinen et al., 1996).

In adults, NT-3 maintains the phenotype of the medium-large diameter proprioceptive neurons that constitute ~43% of the DRG neurons (reviewed in Verge et al., 1996; Karchewski et al., 1999). However, NT-3 can also influence the function and phenotype of other subpopulations, most notably nociceptive neurons, presumably due to its capability to bind to the high affinity receptor for NGF (Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003; Gandhi et al., 2004; Wilson-Gerwing et al., 2005).

1.1.3.3 Brain-derived neurotrophic factor.

In development, mice lacking brain-derived neurotrophic factor (BDNF) have smaller sized DRGs than wildtype animals (Ernfors et al., 1994a), but the loss of neurons is less profound than observed in mice lacking NGF or NT-3. The function or

type of neurons that respond to BDNF in development remains enigmatic. A possibility is that the BDNF-responsive subpopulation of sensory neurons is also responsive to the other neurotrophins (McMahon et al., 1994; Farinas et al., 1998; Karchewski et al., 1999).

In mature DRG neurons BDNF maintains a subset of medium-large size sensory neurons (Mu et al., 1993; Carroll et al., 1998) which represents ~33% of the DRG neurons (Karchewski et al., 1999). These neurons are transducers of slow-adapting mechanoreception (touch). BDNF is important for the functional maturation of the slow-adapting mechanoreceptive neurons, but not for their survival in development (Carroll et al., 1998).

Adult DRG neurons can survive in the absence of exogenous neurotrophins *in vitro*, including cells cultured at low density or as single neurons in microwells (Lindsay, 1988). The capability of adult DRG neurons to survive independently of their target-derived neurotrophic factors protects the neurons from neuronal cell death in response to target damage. Peripheral tissues, specifically skin, are exposed to the external environment and their risk of damage is high; thus to decrease neuronal death in response to target damage requires another source of trophic support that is not target derived (reviewed in Lindsay, 1996). BDNF is unlike the other neurotrophins in that it is both target-derived and synthesized by the sensory neurons themselves. BDNF mRNA is expressed in the small-medium nociceptive DRG neurons in the intact state (Ernfors et al., 1990b; Wetmore and Olson, 1995; reviewed in Verge et al., 1996). In this state, there is little overlap between the neurons that synthesize BDNF and the neurons that are responsive to BDNF (Kashiba et al., 1997). Disruption of BDNF synthesis by treatment with antisense oligonucleotides induces cell death in single cell cultures. The antisense induced death can be rescued by addition of exogenous BDNF. This suggests that BDNF promotes survival of adult neurons by an autocrine loop or a paracrine loop in the presence of other neurons (Acheson et al., 1995).

BDNF also plays a neuromodulatory role in pain transduction. BDNF is expressed by nociceptive neurons and is upregulated in response to inflammation or infusion of NGF (Apfel et al., 1996; reviewed in Verge et al., 1996; Cho et al., 1997; Michael et al., 1997). In these neurons, BDNF is synthesized and stored in dense-core

vesicles and transported anterogradely to the central terminals of the sensory neurons where it can act on cells in the dorsal horn expressing its receptor (Zhou and Rush, 1996; Michael et al., 1997; Tonra et al., 1998). BDNF is found in vesicles that also contain the neuropeptides CGRP and SP, whose synthesis can also be regulated by NGF. The activation of trkB receptors at the dorsal horn by endogenous BDNF contributes to hyperalgesic states and can cause sensitization of dorsal horn neurons to activation through N-methyl-D-aspartic acid glutamate receptors (Kerr et al., 1999; Mannion et al., 1999; Heppenstall and Lewin, 2001)

1.1.3.4 The tropomyosin-related kinase receptors.

The specificity of neurotrophin actions is mediated through binding and activation of their high affinity receptors, the tropomyosin-related kinase (trk) receptors (reviewed in Barbacid, 1994). NGF binds to trkA (Kaplan et al., 1991; Klein et al., 1991; Meakin et al., 1992); BDNF binds to trkB (Klein et al., 1989; Middelmas et al., 1991), and NT-3 preferentially binds to trkC (Lamballe et al., 1991). NT-3 can also bind to trkA and trkB, but with lower affinity (Soppet et al., 1991; Squinto et al., 1991; Ip et al., 1993; Farinas et al., 1998). Binding of neurotrophin ligands to their receptors induces autophosphorylation and activates several intracellular signaling pathways which promote cell survival and phenotypic responses.

The expression of individual trk receptors is restricted to select subpopulations of DRG neurons and confers the selectivity of the individual neurotrophin actions on specific subpopulation of sensory neurons. For example, NGF influences the nociceptive neurons via trkA (Verge et al., 1992; Mu et al., 1993; McMahon et al., 1994). Although NT-3 acts on the medium-large diameter proprioceptive neurons via trkC (Mu et al., 1993; McMahon et al 1994; reviewed in Verge et al., 1996), it can also antagonize nociceptive phenotype and pain states presumably by binding to an isoform of trkA (Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003; Gandhi et al., 2004; Wilson-Gerwing et al., 2005). BDNF acts on medium-large sized neurons via trkB (Mu et al., 1993; Wright and Snider, 1995). There is a high degree of colocalization of trk receptors in DRG neurons. Neurons expressing mRNA for both

trkA and trkC represent ~20% in lumbar sensory neurons, while trkA and trkB are coexpressed in ~10%, and trkB and trkC are co-expressed in ~19% of DRG neurons. Only ~3-4 % of DRG neurons express all trk receptors (Karchewski et al., 1999).

Within the trk family members there are multiple isoforms of each trk. TrkA has two variants that differ in their extracellular domains (Barker et al., 1993). The different transcripts of trkC encode both full length protein receptors with variable small inserts in their kinase domain and also a transcript that codes for a receptor that lack the kinase domain (truncated trkC; Velenzuela et al., 1993). The trkB gene encodes several isoforms as well, including transcripts encoding a protein that lacks a catalytic kinase domain (truncated trkB; Klein et al., 1991). These truncated trkB receptors may function to inhibit signaling through full-length trkB (Biffo et al., 1995). For example they can form inactive heterodimers with full-length trkB when coexpressed with trkB (Eide et al., 1996). Truncated trkB can also sequester BDNF when expressed in nonneuronal cells such as astrocytes, oligodendrocytes and Schwann cells (Friesen et al., 1993; Rubio, 1997), acting as a molecular sponge to soak-up excess BDNF, rapidly internalizing BDNF and preventing diffusion (Biffo et al., 1995). The truncated trkB may also store BDNF in the extracellular space and present this molecule when necessary such as to growing axons (Beck et al., 1993). However, recent research suggests that truncated trkB is also involved in cell signaling. Despite the lack of a catalytic domain, cell lines transfected with truncated trkB receptors are capable of mediating BDNF signaling (Baxter et al., 1997). As well, overexpression of the truncated trkB receptors in postnatal hippocampal neurons can initiate dendritic outgrowth. This effect is reduced when the truncated trkB receptor is coexpressed with the p75 neurotrophin receptor lacking the intracellular domain or in p75 knockout of exon III which gives rise to a short p75 receptor lacking most of the extracellular domain. (Hartmann et al., 2004). Truncated trkB receptors may signal through a p75 interaction. Another possibility is trkB may signal by activating a G protein-coupled receptor. When BDNF was applied to cultures of astroglia and brain slices containing truncated trkB, calcium release was generated inside the glial cells through a G protein interaction (Rose et al., 2003). Thus, truncated trkB may have an active role in neuronal and glial signaling despite lacking a catalytic domain it may interact with other proteins

similar to that observed for p75 signaling (see below; Kryl and Barker, 2000; Rose et al., 2003; Hartmann et al., 2004).

1.1.3.5 The common neurotrophin receptor -p75.

The p75 neurotrophin receptor is capable of binding all neurotrophins with low affinity (Johnson et al., 1986; Radeke et al., 1987; Ernfors et al., 1990a; Rodriguez-Tebar et al., 1990) and is expressed in up to 80% of DRG neurons (Zhou et al., 1993; 1996; Wetmore and Olson, 1995; Karchewski et al., 1999). The p75 receptor is a member of the tumor necrosis factor receptor (TNFR) superfamily. The TNFR do not have intrinsic enzymatic activity and signaling requires association with cytoplasmic adaptor proteins. The p75 receptor is an unusual member of the family in that it dimerizes rather than trimerizes and the neurotrophins are structurally unrelated to other ligands that bind to the TNFR (Roux and Barker, 2002). The p75 receptor can elicit a variety of responses, including increased ligand specificity, induction of apoptosis, cell survival, and peripheral nerve myelination.

When p75 is expressed in the presence of the trk receptors, there is increased ligand specificity and affinity of neurotrophin binding (reviewed in Hempstead, 2002; Roux and Barker, 2002; reviewed in Chao, 2003). Increased expression of p75 in the presence of trkA or trkB increases the specificity for NGF or BDNF respectively (Hempstead et al., 1991; Beneditti et al., 1993; Ip et al., 1993; Bibel et al., 1999). NT-3 can bind to both trkA and trkB, but p75 expression may restrict signaling to its corresponding neurotrophin receptor. p75 can also increase the affinity of NGF binding to trkA and enhance activation of trkA in response to low concentrations of this neurotrophin (Hempstead et al., 1991; Rodriguez-Tebar et al., 1992), while BDNF-mediated trkB activation is not altered by p75 coexpression (Bibel et al., 1999). p75 may enhance the affinity by which NGF binds to trkA and increase trk activation through an allosteric effect. p75 may interact with the trk receptor through its extracellular domain since truncated p75 enhanced trk responsiveness when compared to the full-length p75 receptor in fibroblasts (Hantzopoulos et al., 1994). However, even though the p75 receptor is truncated it still contains a transmembrane domain and may

contain a shortened functional cytoplasmic domain. Other research suggests that the p75 and trk receptor may interact through a cytoplasmic domain as co-expression of trkA in the presence of p75 containing an altered extracellular domain did not alter high affinity binding of NGF to trkA (Esposito et al., 2001). This interaction may result in conformational changes of the trk receptor to provide additional points of contact with its ligand (Zaccaro et al., 2001) or adaptor proteins to augment trk tyrosine phosphorylation (Epa et al., 2004). Interestingly, BDNF can bind to p75 and attenuate trkA signaling. Binding of BDNF to p75 induces ceramide-mediated phosphorylation of serine residues in the trkA intracellular domain and correlates with reduced NGF-trkA activation (MacPhee and Barker, 1997)

As a member of the TNF receptor superfamily, it is no surprise that p75 can induce programmed cell death (reviewed in Baker and Reddy, 1998; reviewed in Hempstead, 2002; reviewed in Chao, 2003). Similar to other members of the tumor necrosis family, p75 contains an intracellular death domain. The death domain of p75 is structurally different from that of other members of the TNFR family and p75-mediated apoptosis may occur independently of this domain (Liepinsh et al., 1997; Salehi et al., 2000). p75-mediated cell death occurs during certain developmental stages and pathologic states (reviewed in Hempstead, 2002). Overexpression of the cytoplasmic domain of the p75 receptor in transgenic mice induced apoptosis in peripheral neurons during development (Majdan et al., 1997). As well, reducing p75 expression with antisense oligonucleotides rescued developing axotomized sensory neurons from cell death (Cheema et al., 1996). The induction of cell death can be determined by the ratio of p75 to trk receptors (Barrett and Bartlett, 1994). p75 induces cell death in embryonic sensory neurons that express little or no trk receptors (Davies et al 1993). The signaling of trk can dominate and block the p75-mediated death signal (Yoon et al., 1998). The idea that survival or death of a neuron depends on the predominance of signaling of one receptor over another is an overgeneralization. High expression of p75 does not always cause death. Cell death is absent in cultured Schwann cells that are exposed to neurotrophins, express high levels of p75, and do not express trk receptors (Yoon et al., 1998). Furthermore, trk activation can also induce cell death (Kim et al., 2003). Thus, signaling of one receptor or another cannot predict cell fate. In addition to proapoptotic

signaling, p75 can also mediate a survival response. Blocking of NGF binding to p75 reduced survival of developing trigeminal neurons (Hamanoue et al., 1999). As well, p75 can induce downstream signaling molecules involved in survival (Hamanoue et al., 1999; Foehr et al., 2000; Roux et al., 2001; Epa et al., 2004). Thus, the p75 neurotrophin receptor is an important player in the fine regulatory control between cell survival and death.

Another functional role of p75 is myelin formation. The p75 receptor regulates myelination of Schwann cells in the presence of neurotrophins. p75 is expressed at high levels in Schwann cells during development and regeneration of the peripheral nerve (Cosgaya et al., 2002). Binding of BDNF to p75 induces myelination, whereas NT-3 inhibits myelination likely via a trkC interaction (Chan et al., 2001; Cosgaya et al., 2002).

1.2. Effect of peripheral nerve injury on sensory neurons and nerve environment.

Understanding the organization of the sensory neurons of the DRG is important in understanding the response of the neurons to injury since each class of sensory neurons exhibits its own unique pattern of change. After peripheral nerve injury, DRG neurons switch from an intact phenotype to an injury/regenerating phenotype. The injury/regenerating phenotype promotes survival and regeneration of adult DRG neurons. Axotomy of the peripheral nerve creates a distal nerve stump, proximal nerve stump and changes in the corresponding DRG ganglia. The changes that occur in response to injury create a permissive environment for growth and also drive the intrinsic robust cell body response that is sufficient for regeneration.

1.2.1. Distal nerve stump response to axotomy.

A well-defined series of cellular changes termed Wallerian degeneration occurs in the distal nerve stump following nerve injury. In the PNS, the degenerative changes are complete in a few weeks, while in the CNS the changes proceed over several months (reviewed in Frostick et al., 1998). In the first few days after injury, the axons

degenerate and the neuronal-myelin segments become fragmented and begin to shrink. Schwann cells become active and initially help remove myelin and axon debris. By day 3-4, macrophages infiltrate the area and clear the myelin and axonal debris leaving behind proliferating Schwann cells inside the basal lamina tubes that surround the original nerve fiber. The clearance of myelin and axonal debris is complete by 15-30 days. The loss of contact with their axons and macrophage infiltration induces Schwann cells to proliferate. As Schwann cells cease to proliferate they begin to extend slender cytoplasmic processes that form a cell strand surrounded by basal lamina, known as the band of Büngner (reviewed in Ide, 1996). The bands of Büngner will accept regenerating axonal sprouts from the proximal stump. Regenerating axons do not elongate through a graft if Schwann cell migration is inhibited (Hall, 1986). In addition to clearing debris, macrophages secrete cytokines which induce Schwann cell proliferation and NGF synthesis (Bandtlow et al., 1987). NGF is present at low levels in the rat sciatic nerve but is rapidly increased after nerve injury. There is a biphasic increase in NGF expression; with an initial and transient increase peaking ~6 hours postaxotomy followed by a second increase which starts at 2 to 3 days and lasts several weeks (Heumann et al., 1987). The second phase coincides with macrophage infiltration and is likely a result of interleukin-1 released by macrophages (Rotshenker et al., 1992). The increase in NGF mRNA is mimicked by the addition of recombinant interleukin-1 to culture explants of sciatic nerve and blocked by interleukin-1 antibodies (Lindholm et al., 1987). There is also an increase in fibroblasts in the distal stump that secrete factors that also induce NGF synthesis.

NGF is not the only neurotrophin produced by the nerve distal to the injury. BDNF is normally found at low levels in the sciatic nerve. After injury BDNF levels increase slowly; BDNF is detected at 4 days postaxotomy reaching maximum levels at 2-4 weeks. The maximal levels of BDNF are 10 times higher than those of NGF mRNA in the lesioned nerve (Meyer et al., 1992). BDNF is likely produced by Schwann cells as the maximum macrophage invasion and activation occurs 2-6 days after injury while BDNF mRNA reaches a maximal level 2 weeks after lesion (Meyer et al., 1992). NT-3 mRNA is normally expressed in the sciatic nerve following injury and is downregulated 6-12 hours after axotomy, but returns to control levels after 2 weeks (Funakoshi et al.,

1993). There are also changes in trk receptor expression, while trkA is not detectable in the injured sciatic nerve, levels of trkB and trkC decrease to undetectable levels in the injured distal stump. In contrast, the levels of truncated trkB expression appear to increase slightly in the distal stump 1 day after sciatic nerve transection and then return to baseline (Funaksohi et al., 1993). Expression of the common neurotrophin receptor p75 mRNA is undetectable in the intact sciatic nerve but increases following injury. The p75 receptor may act to present neurotrophins synthesized in the Schwann cells to the growing axons (Taniuchi et al., 1986).

Thus, the distal stump creates a favorable environment for regenerating axons. The Schwann cells express neurotrophic factors that will attract the regenerating axons, synthesize surface cell adhesion molecules, and basement membranes express extracellular matrix protein such as laminin (Ide, 1983; reviewed in Fu and Gordon, 1997; reviewed in Frostick et al., 1998). If the denervated distal stump is removed, axonal sprouting still occurs but forms a tangled mass or neuroma in the proximal stump (Zochodne and Cheng, 2000).

1.2.2 Proximal nerve stump response to axotomy.

The proximal stump responds to injury with varied degradation that can range from the injury site, to the next node of Ranvier, or to the cell body depending on the severity of the injury (reviewed in Burnett and Zager, 2004). Degradation occurs in the form of Wallerian degeneration. Regenerating axons begin to emerge from the proximal stump a few hours after axotomy, though some axons may not begin to regenerate until several weeks after injury. The sprouts in myelinated axons emerge from the terminal nodes of Ranvier (Fawcett and Keyes, 1990). An axon may have several sprouts which will grow down the endoneurial tube, contact the Schwann cell basal lamina on one side and the Schwann cell membrane on the other (Fawett and Keyes, 1990). Axonal elongation is slow at the beginning and then increases. Under ideal conditions the regrowing axons will grow through the distal stump within their originally occupied endoneurial tube and reinnervate their distal targets. There is an average of 5 axon sprouts that emerge from one injured axon and after target

reinnervation only one axon will remain while the other axons recede (McKinnon et al., 1991). Remyelination occurs when the axon contacts the Schwann cells in the distal stump.

NGF expression is detected in the proximal stump after injury, although NGF levels are not as high as detected in the distal stump (Heumann et al., 1987). Expression of NGF is restricted to the very end of the proximal stump but reaches only 40% of the levels of NGF expressed in the intact sciatic nerve (Heumann et al., 1987). The amount of NGF protein present within the proximal stump can be detected as early as 2 hours post-injury (Abrahamson et al., 1987). The proximal nerve stump also expresses BDNF and to a lesser extent NGF in surrounding mast cells however this level is similar in intact nerves and does not change in response to injury. Alteration of NT-3 expression was not observed (Zochodne and Cheng, 2000). Schwann cells also proliferate in the proximal stump and there is a slight increase in mRNA expression of the neurotrophin receptors p75, trkB and trkC mRNA (Funakoshi et al., 1993). The Schwann cells from the proximal and distal stump will migrate to form a continuous cable across the gap to guide axons to the bands of Büngner (Torigoe et al., 1996).

1.2.3 Cell body responses to axotomy.

Following axotomy, neuronal cell bodies undergo a variety of changes that include morphological alterations and modifications in gene expression. The injured cells undergo chromatolysis, a cell reaction which includes the dispersal of the larger Nissl bodies (stacked endoplasmic reticulum with attached ribosomes) due to the disintegration of larger granular condensations of rough endoplasmic reticulum. It is characterized under a light microscope by clear areas of cytoplasm lacking Nissl bodies (reviewed in Cragg, 1970). Another characteristic change is the movement of the nucleus to an eccentric position within the cell (reviewed in Cragg, 1970). It is unknown what the purpose is of the morphological changes of the cell body following injury. Chromatolysis is not necessarily a marker of regeneration, since DRG neurons undergo very little chromatolysis (Hall, 1982; Perry et al., 1983)

The onset of injury is also associated with changes in expression of genes and proteins. The expression of these genes is believed to limit damage to the injured neurons and promote survival and recovery of these neurons. Though peripheral nerve injury in neonates results in the death of a majority of sensory neurons (Himes and Tessler, 1989), axotomy of adult DRG neurons are more resistant to cell death. In the adult, neuronal death begins within 1 day postaxotomy and approximately ~20-30% of sensory neurons die within 2-3 weeks, while a small amount of additional cell death occurs by 6 months (Arvidsson et al., 1986; Himes and Tessler, 1989; Verge et al., 1989b; Hart et al., 2002).

Alterations in neuronal gene expression following peripheral nerve injury are referred to as the injury/regeneration response. These changes can occur as early as 12 hours after nerve transection (Miller et al., 1989). The switch of the DRG neurons from an intact to an injury/regenerating phenotype is likely influenced by changes in target and nerve derived trophic and cytokine factor availability as they play an important role in maintaining the intact phenotype.

1.2.3.1 Injury/regeneration -associated genes.

Peripheral nerve injury induces alterations in injury/regeneration-associated gene expression. These genes are associated with the regenerative response and their expression is correlated with axon regeneration (Skene and Willard, 1981; Bisby, 1985; Hoffman, 1989). While distinct patterns of gene expression are attributed to subpopulations of injured sensory neurons, there are also global phenotypic markers of the injured state that are expressed by all injured sensory neurons (Verge et al., 2002). The expression of these genes is used as markers of the injury/regenerative state and can monitor the impact of therapeutic strategies or experimental interventions. There are several factors that could regulate the expression of these genes, including the loss of target-derived neurotrophins and molecules generated at the site of injury or at the cell body. The following injury/regeneration molecules discussed below are not a comprehensive list, but includes the molecules used in this thesis to define the injury/regeneration state and/or play a key role in this modulation of this state.

1.2.3.1.1 Growth-associated protein 43.

Growth-associated protein 43 (GAP-43) can play a role in neural development, synaptic modulation and axonal regeneration (reviewed in Benowitz and Routtenberg, 1987). GAP-43 accumulates in the growth cone and is an important regulator of growth cone motility (Meiri et al., 1986; Skene et al., 1986; Goslin et al., 1990). The growth cone is the motile organelle of the regenerating axon and its movement determines the rate of regeneration. GAP-43 is considered an intrinsic determinant of the growth state of the neuron and is a reliable marker of nerve regeneration (Skene and Willard, 1981; Benowitz and Lewis, 1983; Redshaw and Bixby, 1984; reviewed in Skene, 1989; Schreyer and Skene, 1991; Schreyer and Skene, 1993). GAP-43 expression can enhance the initiation of axon elongation and reduce the frequency of branching, a hallmark of effective regeneration (Smith and Skene, 1997). However, neurite outgrowth does not absolutely require GAP-43 expression (Strittmatter et al., 1995; Andersen et al., 2000) nor does expression of GAP-43 absolutely indicate regenerative growth (Jacobson et al., 1986; Buffo et al., 1997).

GAP-43 is expressed at high levels during development (Karns et al., 1987), generally declines with maturity, and increases following peripheral nerve injury (Basi et al., 1987; Hoffman 1989; Tetzlaff et al., 1989; Chong et al., 1992). GAP-43 levels begin to decline as axons innervate their target (Bisby, 1988; Reynolds et al., 1991; Schreyer and Skene, 1991; Chong et al., 1992). GAP-43 is expressed constitutively at low levels in intact DRG neurons in the small-medium sized neurons (Verge et al., 1990b; Schreyer and Skene, 1993). It is this group of small-medium diameter DRG neurons that respond rapidly to peripheral nerve injury and have a capacity for collateral sprouting (Jackson and Diamond, 1984). Following peripheral nerve injury, within 24 hours GAP-43 labeling of neurons begins to increase (Bisby, 1988) and is upregulated in all axotomized DRG neurons (Verge et al., 1990b; Schreyer and Skene, 1993).

1.2.3.1.2 Tα1 tubulin.

Tubulin is a cytoskeletal protein and consists as a dimer composed of an alpha and a beta subunit. The tubulins are the major constituent protein of the microtubules (Hoffman, 1989). Microtubules are a major structural element of the axon. Thus, Tα1 tubulin is expressed at high levels during development in the PNS and in the CNS at a time when neurons are extending processes (Miller et al., 1987). Tα1 tubulin expression is also increased after peripheral nerve injury to rebuild the regenerating axon and is decreased following reinnervation (Hall, 1982; Miller et al., 1989; Wu et al., 1997).

Tα1 tubulin is expressed at low levels in all neurons and is found at slightly higher levels in the small-medium intact DRG neurons. These neurons also constitutively express GAP-43 and have a high propensity for growth. After peripheral nerve injury the expression of Tα1 tubulin is upregulated in all neurons of the DRG (Mohiuddin and Tomlinson, 1997).

1.2.3.1.3 Heat shock protein 27.

Heat shock proteins act as molecular chaperones and are involved in protein translocation or folding (Becker and Craig, 1994). In stressed or injured cells, heat shock proteins contribute to the repair process by refolding denatured proteins (Becker and Craig, 1994; Ryan and Jensen, 1995; Hartl 1996). Induction of heat shock proteins results in a protective effect of various cell types to exposure to stress (Lowenstein et al., 1991; Rodorf et al., 1991).

Peripheral nerve injury in adult DRG neurons results in a low amount of cell death (~20-30%; Lekan et al., 1997). A possible reason is the upregulation of heat shock protein 27 (HSP-27) after peripheral nerve injury. HSP-27 is constitutively present at low levels in the small-medium sized intact DRG neurons (Plumier et al., 1997) and then upregulated in virtually all injured sensory neurons within 1 day following injury (Costigan et al., 1998). HSP-27 can act as an intrinsic survival factor in DRG neurons. Overexpression of HSP-27 in neonatal DRGs protects neurons from NGF withdrawal induced apoptosis and in adult DRG neurons from ischemia and thermal stress (Lewis

et al., 1999; Wagstaff et al., 1999). Furthermore, knockdown of HSP-27 with antisense RNA in adult sensory neurons results in apoptosis (Benn et al., 2002). HSP-27 may promote survival by acting as a molecular chaperone (Becker and Craig, 1994), by indirectly stabilizing mRNA (Carper et al., 1997), and/or act as an actin capping protein to stabilize the cytoskeleton (Lavoie et al., 1995). HSP-27 can also inhibit apoptosis by interacting with cytochrome c and prevent its activation of downstream caspases and cell death (Bruey et al., 2000). HSP-27 needs to be phosphorylated in order to protect the cell from death and non-phosphorylated HSP-27 has no survival promoting activity (Benn et al., 2002).

In addition to promoting neuronal survival, HSP-27 can promote axonal regeneration. The ability of HSP-27 to act as an actin-capping protein can prevent the polymerization of actin. Phosphorylation of HSP-27 prevents its association with actin and can increase the rate and extent of actin polymerization (Landry and Huot, 1995; Lavoie et al., 1995). Inhibition of HSP-27 phosphorylation in DRG neurons was found to result in aberrant neurite outgrowth (Williams et al., 2005).

1.2.3.1.4 Neuropeptide Y.

The exact role of neuropeptide Y (NPY) is not known, but is assumed to have an analgesic effect. In NPY deficient mice there is an increase in autotonomy (self-mutilation) following peripheral nerve injury (Shi et al., 1998). Furthermore, intrathecal administration of NPY represses the spinal nociceptive flexor reflex (Hua et al., 1991).

NPY is not expressed in intact sensory neurons. Instead NPY is upregulated dramatically after peripheral nerve injury and is expressed at high levels in the medium-large DRG neurons (Wakisaka et al., 1991; Wakisaka et al., 1992). The *de novo* expression of NPY following nerve injury makes it an ideal marker of this state.

1.2.3.1.5 Activating transcription factor 3.

Activating transcription factor 3 (ATF3) is a member of the 3'-5'-cyclic adenosine monophosphate (cAMP) response element-binding protein/ATF family

transcription factors. Contrary to the implication of its name, ATF3 is a repressor of transcription when it is bound to deoxyribonucleic acid (Chen et al., 1994). However, when ATF3 dimerizes with Jun, the ATF3/Jun heterodimer can activate transcription (Chu et al 1994). ATF3 is induced in cells during stress (Liang et al., 1996). In superior cervical ganglion neurons, expression of ATF3 during a time in which these cells are normally killed, induces HSP-27 and thus promotes survival and neurite elongation (Nakagomi et al., 2003). In pheochromocytoma 12 and neuroblastoma cell lines, the combination of ATF3 and c-Jun provides a synergistic effect where ATF3 enhances c-Jun mediated neurite sprouting (Pearson et al., 2003). Expression of ATF3 in the peripheral neurons may play a similar role in survival and regeneration.

In response to peripheral nerve injury, ATF3 is expressed in almost all axotomized DRG neurons (Tsujino et al., 2000). Expression of ATF3 can be used as a marker of the injury state (Tsuzuki et al., 2001; Obata et al., 2003).

1.2.3.2 Cytokines.

Members of the cytokine family are pleiotropic glycoprotein molecules with widespread actions on a variety of cells. The neuropoeitic cytokine family generally refers to the molecules of the interleukin-6 family. The members of the neuropoeitic cytokine family bind to a common receptor component, the gp130 glycoprotein (Ip et al., 1992). Many neuropoeitic cytokines do not bind gp130 directly but bind to an associated ligand binding subunit to exert their action. The neuropoeitic cytokines can regulate survival and gene expression in the peripheral neurons and have been shown to exert trophic effects. The cytokines may compensate for the loss of retrograde supply of neurotrophins and help regulate the response of the DRG neurons to injury.

1.2.3.2.1 Leukemia inhibitory factor.

The leukemia inhibitory factor (LIF) has many actions on various cell types including lipid metabolism, myoblast proliferation, and maintenance of hematopoietic stem cells (for more examples see Hilton and Gough, 1991). LIF is also expressed in the

nervous system. Under normal conditions LIF is not detectable in the peripheral nerve or DRG. After peripheral nerve injury, LIF mRNA is increased in both proximal and distal stumps. This increase in expression is rapid, occurring within 24 hours and is maintained for ~2 weeks (Banner and Patterson, 1994; Curtis et al., 1994). LIF is retrogradely transported and accumulates in the small diameter, trkA positive and isolectin B4 positive (GDNF responsive) neurons (Thompson et al., 1997). The rapid increase of LIF in the distal stump is similar to induction of NGF after peripheral nerve transection. LIF is also induced by interleukin-1 from macrophages in cultured ganglia (Shadiak et al., 1993) and promotes a similar neuropeptides phenotype to that promoted by NGF (Verge et al., 1995, Corness et al., 1996; Sun and Zigmond, 1996). LIF has also been shown to be involved in the regenerative response of injured DRG neurons. LIF knockout mice failed to increase their intrinsic growth capacity in response to a conditioning lesion (See 3.2.1.) and the addition of exogenous LIF enhanced growth of the small-medium sensory neurons *in vitro* of LIF knockout mice (Cafferty et al., 2001)

1.2.3.2.2 Interleukin-6.

Interleukin-6 (IL-6) mediates various actions including immune response and inflammatory reactions (reviewed in Hirano et al., 1997). It also plays a role in the peripheral nervous system. IL-6 and IL-6 receptor are expressed in DRG neurons during development (Gadient and Otten, 1996). Postnatally, IL-6 is not detectable in intact DRG neurons but IL-6 expression increases in response to peripheral nerve injury (Gradient and Otten, 1996). Sciatic nerve injury induces IL-6 mRNA expression in nonneuronal cells in the sciatic nerve at the distal and proximal stump. IL-6 mRNA synthesis is also stimulated in the medium and large lumbar DRG neurons within 1 day with maximal expression after 2-4 days, and then decreases below threshold detection by 7 days following axotomy (Murphy et al., 1995; reviewed in Zhong and Heumann, 1995; Reichert et al., 1996). Macrophages and fibroblasts are major inducers of IL-6 in the injured sciatic nerve (Bolin et al., 1995; Reichert et al., 1996). IL-6 is important in sciatic nerve regeneration. In IL-6 knockout mice, regeneration following sciatic nerve injury is impaired (Zhong et al., 1999) and the conditioning lesion response fails to

occur (Cafferty et al., 2004), whereas mice that overexpress IL-6 and its receptor exhibit enhanced PNS regeneration (Hirota et al., 1996)

1.2.3.3 Neurotrophins.

Changes in availability of neurotrophin factors after injury are a major factor that underlies the phenotypic changes observed in sensory neurons after axotomy. Following axotomy of the peripheral nerve parent neuronal cell bodies experience a loss of the target-derived source of neurotrophins. There is also a change in neuronal-derived BDNF and expression of neurotrophin receptors by the neuronal cell body. Axotomy of the sciatic nerve results in a reduction of the p75 neurotrophin receptor (Verge et al., 1992; Zhou et al., 1996) and the trk receptors (Verge et al., 1990b; 1992; Karchewski et al., 1999) in the DRG neurons.

1.2.3.3.1 NGF and NT-3.

Peripheral nerve injury or crush reduces the supply of target-derived neurotrophins resulting in a dramatic decrease in NGF and NT-3 retrograde transport. The loss of target-derived neurotrophins is believed to provide a 'negative' signal which induces the cell body response. Blockade of axonal transport with microtubule-depolymerizing drugs, such as colchicines or vinblastine, produces a response similar to that found after axotomy (Kashiba et al., 1992; Cougnon-Aptel et al., 1999). After axons re-establish contact to their target organs the injury/regenerating phenotype returns to an intact phenotype (Bisby and Keen, 1986; O'Hara et al., 1994).

Despite the upregulated local production of NGF at the proximal stump, the amount of NGF remains lower than that delivered by retrograde transport. Axotomy results in almost a 10-fold decrease in NGF (Ravich et al., 1991). There is also a decrease in expression in NGF receptors and phenotypic neuropeptides (CGRP and SP) normally found in the small-medium sized DRG neurons (Verge et al., 1989b; 1992; 1995; Shen et al., 1999). Administration of NGF directly on the proximal nerve or intrathecal infusion of NGF immediately following sciatic nerve injury was found to

suppress the expression of GAP-43 and ATF3 in small-medium size DRG neurons (Mohiuddin et al., 1999; Hirata et al., 2002; Averill et al., 2004). Similarly, intrathecal infusion of NGF or administration of NGF directly on the proximal nerve also restore NGF receptor expression and phenotypic markers of the NGF responsive population, such as SP and CGRP (Verge et al., 1989b; 1992; 1995; Zhang et al., 1995). Conversely, use of an NGF antibody to suppress endogenous NGF was able to promote aspects of an injury phenotype in intact DRG neurons (Shadiack et al., 2001).

The loss of the retrograde supply of NT-3 influences the medium-large DRG neurons. The delivery of NT-3 onto the proximal stump appears to reverse the NPY upregulation in DRG neurons but does not affect the neuropeptides (i.e. SP or CGRP) normally expressed in the small-medium DRG neurons (O'Hara et al., 1995; Sterne et al., 1998).

1.2.3.3.2 BDNF.

After peripheral nerve injury there is a loss of target-derived BDNF and a phenotypic change in BDNF expression in the DRG neurons. As previously mentioned, in the intact state, BDNF is synthesized in small-medium sized DRG neurons (Ernfors et al., 1990b, Wetmore and Olson, 1995; reviewed in Verge et al., 1996). Following peripheral nerve injury, there is an increased anterograde transport of BDNF (Tonra et al., 1998). BDNF is initially expressed in ~80 % of injured DRG neurons and by 1 week postinjury BDNF remains elevated in the medium-large (trkB/C) DRG neurons while exhibiting decreased expression in small neurons (Michael et al., 1999; Zhou et al., 1999; Karchewski et al., 2002). The upregulation of BDNF in the injured DRG neurons may serve as a neuroprotective role (Acheson et al., 1995; Murphy et al., 2000) and/or trigger a positive signal in the cell body response.

1.3 Axonal regeneration of sensory neurons.

Peripheral nerve injury results in a robust regenerative response of DRG neurons and injured axons can grow vigorously over long distances. The environment is one

critical factor determining whether PNS neurons regenerate. The PNS lacks many of the inhibitory molecules expressed by the glia of the CNS. When peripheral nerve segments were used as a bridge, CNS axons were able to grow into the peripheral nerve segment even though they normally would not regenerate within the CNS environment (Richardson et al., 1980; David and Aguayo, 1981). After peripheral nerve injury, the distal stump creates a favorable environment for growth. Axon growth inhibiting myelin is quickly removed and Schwann cells proliferate to produce a trophic environment that is permissive for axonal growth. However, a permissive environment is not enough for regeneration. Dorsal column axons only grow into a peripheral nerve graft if there has also been a peripheral axon injury (Richardson and Issa 1984; Richardson and Verge, 1986)

Peripheral axotomy also induces a change in gene expression at the cell body that contributes to the robust regenerative response. Axotomy of the central axon branch of the DRG results in only mild cell body change with no detectable change in chromalytic reaction. These are slight changes in the expression of cytoskeleton genes/proteins but the effect is not as dramatic as observed following axotomy of the peripheral branch (Hall, 1982; Greenberg and Lasek, 1988). Furthermore, GAP-43 expression fails to increase in response to central axotomy (Schreyer and Skene, 1993; Chong et al., 1994). The inability of central axotomy to induce a similar cell body response as a peripheral axotomy correlates with a poor regenerative response of the cell body (Richardson and Issa, 1984; Richardson and Verge, 1986). These findings suggest that the intrinsic regenerative response induced in the cell bodies of the peripheral neurons is important for successful regeneration.

1.3.1 Reasons for poor functional outcome.

Despite the capacity of the peripheral neurons to regenerate, functional recovery is often less than optimal. For optimal recovery of function, axons need to reinnervate their original targets. However, the distance an axon needs to travel is long and as time passes the regenerating neurons are likely to deal with progressively poorer growth conditions.

A major problem is the delay of regenerating axons before they cross the repair site (reviewed in Fu and Gordon, 1997). Not all regenerating axons cross the repair site in a synchronous manner, some axons may cross the repair site only to retract or die-back into the proximal stump. Brushart et al., (2002) found that axons regenerate into the distal stump in 'staggered' fashion, as a result some axons can take weeks to cross the repair site. Axotomy creates a gap between the disrupted nerve ends that axons need to transverse to reach their appropriate target. As the axons enter the gap they may come across many obstacles such as scar tissue. If the regenerating axons do not reach a distal endoneurial tube they will form a neuroma or a tangle of nerve endings. Axotomy of peripheral nerve results in disruption of the axon, endoneurial, epineurial and perineurial tubes. When regenerating axons do reach the distal stump they do not have the original tubes to guide their way back to their target, as a result they may fail to reach their target or reinnervate an inappropriate target. A crush injury is less destructive: only the axons but not the endoneurial tubes are disrupted and the growing axons are led back to their appropriate target organ (Gordon et al., 2003). Despite microsurgical techniques to eliminate the gap, reduce the amount of scar tissue, and re-align the proximal to the distal stump, the regenerating axons do not always succeed in entering the appropriate fascicles. The regenerating axons may send sprouts down multiple endoneurial tubes which can result in misdirection of regenerating axons and inappropriate reinnervation (Gordon et al., 2003). Inappropriate pathway choice also excludes appropriate axons from the pathways they normally occupy (Brushart and Seiler, 1987). However, when regenerating motor axons were given equal access to sensory or motor targets, the regenerating motor axons preferentially reinnervate muscle (Brushart, 1988; 1996). Thus, regenerating axons may be able to appropriately reinnervate their target, but axons may fail to reach their target possibly due to delays in entering the distal stump.

The estimated rate of axonal regeneration in humans is 1-3 mm per day (Gutmann et al., 1942). Often peripheral axons need to travel long distances to reach their target organ and it could take a regenerating axon over a year to reach its target. By this time the growth permissive environment, target organs and the intrinsic regenerative response may have begun to deteriorate (reviewed in Fu and Gordon,

1997). In less than a month, the denervated muscle degenerates and fibrosis gradually replaces the muscle fibers (reviewed in Fernandez et al., 1997). Thus, the lag in regeneration and/or slow growth can result in unsuccessful regeneration. As time passes the growth support provided by the distal nerve stump and the growth capacity of the axotomized neurons deteriorates (Fu and Gordon, 1995a, b). As a result, the regenerating axons may fail to reach and reinnervate their target, or reinnervate an inappropriate target resulting in a chronic loss of sensation, loss of muscle and neuropathic pain states. To improve functional outcome the regenerative capacity of the neurons and/or the growth permissive environment must be sustained.

1.3.2 Strategies to enhance the cell body response.

To improve functional recovery, strategies and approaches include influencing the intrinsic regenerative response, as the rate of growth is determined by changes within the cell body (reviewed in Burnett and Zager, 2004). Enhancing the injury/regeneration response at the cell body will enhance the growth states of regenerating axons and decrease the delay in crossing the repair site and increase the rate of regeneration. Increasing the number of axons that enter the growth permissive environment before it deteriorates increases the chance that an axon will enter the distal stump and return to its original target.

1.3.2.1 Conditioning lesion.

A conditioning lesion encourages growth by reducing the delay before initiating regeneration and increasing the rate of regeneration. The conditioning lesion involves injuring (crush or cut) the peripheral branch of the DRG prior to a second (test) lesion. When the peripheral branch of the DRG axon is cut and this is followed by a second injury to the peripheral branch the regeneration of the axon is more rapid (McQuarrie and Grafstein, 1973; McQuarrie et al., 1977). A conditioning lesion can also improve regeneration of central processes of DRG neurons so that the central axons regenerate more quickly into peripheral nerve grafts (Richardson and Issa, 1984), into the dorsal

root entry zone (Richardson and Verge, 1987), or into the spinal cord which is normally a hostile environment for growth (Neumann and Wolf, 1999). A conditioning lesion has been associated with accelerated functional recovery (Bisby, 1985).

The conditioning lesion can enhance regeneration by altering the local environment and/or by altering the cell body response. There is evidence that a conditioning lesion can alter the local growth environment. One possibility is that Wallerian degeneration that occurs in the distal stump prior to the test lesion leaves a pathway vacated by pre-degenerated axons for axons to grow along (Sjoberg and Kanje, 1990). However, the effect of a conditioning lesion in altering the growth capabilities of the neurons can not be ignored, since in most cases a conditioning lesion is able to enhance regeneration into a hostile environment (Richardson and Verge, 1984; 1987; Neumann and Woolf, 1999). As well, a conditioning lesion can enhance neurite outgrowth *in vitro* (Smith and Skene, 1997; Lankford et al., 1998), a situation which lacks the growth environment found *in vivo*. The ability of a conditioning lesion to alter the cell body response can enhance outgrowth since nerves are in a state of regeneration at the time of the second lesion. The levels of GAP-43 expression remain elevated 14 days following the first injury (Van der Zee et al., 1989), whereas, the second lesion does not amplify the levels of GAP-43 expression nor tubulin (Redshaw and Bisby, 1987; Van der Zee et al., 1989; Tetzlaff et al., 1996). There is a reduction in neurofilament which reduces the neurofilament/tubulin ratio and allows more tubulin available for transport and presumably a higher rate of axonal elongation (Tetzlaff et al., 1996). The primed upregulation of regeneration-associated genes (i.e. GAP-43 and Tα1 tubulin) allows for a more rapid regeneration by reducing the delay before outgrowth of newly formed sprouts (McQuarrie et al., 1977). Regeneration is enhanced when the conditioning lesion occurs weeks, days or even 14 hours before the test injury as compared to when the two lesions occur at the same time (Forman et al., 1980; Jenq et al., 1988; Arotz et al., 1989; Smith and Skene, 1997). In the case of central axon regeneration the reduction of a delay in regeneration and increased regeneration rates may allow the regenerating axons to grow into the hostile environment before the onset of the glial scar formation (Oudega et al., 1994; reviewed in Filbin, 1999).

A conditioning lesion is capable of enhancing the intrinsic regenerative response beyond the normal expected degree. However, the use of a conditioning lesion to promote regeneration is clinically impossible. Instead, other strategies are required that mimic the underlying cellular and molecular mechanisms of the conditioning lesion response.

1.3.2.2 FK506.

FK506 is an immunosuppressant that is primarily used to prevent graft rejection and has also been shown to increase regeneration of sensory fibers in the rat sciatic nerve. The immunosuppressant action of FK506 arises from its ability to inhibit calcineurin. FK506 is active after binding to a member of the immunophilin family, FK506-binding protein-12 (FKBP-12). It has been proposed that this complex inhibits the activity of calcineurin, a calcium-activated phosphatase, thereby activating GAP-43 (Lyons et al., 1994; Skene, 1990). Numerous studies have shown that FK506 can increase the rate of axonal elongation of sensory neurons and accelerate functional recovery (Gold et al., 1994; Gold et al., 1995; Doolabh and McKinnon, 1999; Chunasuwankul et al., 2002; Kvist et al., 2003; Udina et al., 2003). Following peripheral nerve injury there is an increase in FKBP-12 which coincides with the increase of GAP-43 in the sensory neurons (Lyons et al., 1995). As well, FK506 increases the axotomy induced up-regulation of GAP-43 mRNA in DRG neurons (Gold et al., 1998). FK506 may enhance regeneration by increasing the activity of GAP-43 (Gold et al., 1995). However, the immunosuppressant drug cyclosporine A which also produces its immunosuppressant effect by inhibiting calcineurin does not alter nerve regeneration (Wang et al., 1997; Lee et al., 2000) and FKBP-12 increases the rate of axonal regeneration in a calcineurin-independent manner (Gold et al., 1997). Perhaps the ability of FK506 to associate with calcium channels (Lyons et al., 1995) enhances intracellular calcium which has been shown to regulate growth cone motility (Kater and Mills, 1991). Even though it is not known how FK506 enhances nerve regeneration, it is an example of how increased rate of regeneration can promote functional recovery.

1.3.2.3 cAMP.

cAMP is a second messenger that is produced by adenylyl cyclases in response to extracellular stimulation. Intracellular calcium and calmodulin can also modulate adenylyl cyclases, suggesting a role for cAMP in plasticity (reviewed in Xia and Storm, 1997). cAMP can regulate transcription including the cAMP response element binding transcriptional pathway. Recent studies implicate cAMP as a potential mechanism to overcome inhibitory signals of the CNS and promote regeneration (Qui et al., 2002; Neumann et al., 2002).

A conditioning lesion allows central axons to grow into the hostile CNS environment. Neumann et al. (2002) found they could mimic the conditioning lesion response without injuring the peripheral branch by elevating the cAMP levels of the DRG neurons. Elevated cAMP allows for growth over molecules such as myelin-associated glycoprotein (MAG) and myelin that are normally inhibitory to axon growth. The inhibitory signal of MAG on DRG outgrowth *in vitro* is switched to attraction by addition of cAMP agonist to the culture medium (Song et al., 1998; Cai et al., 1999). Studies *in vivo* have found similar responses; the injection of a cAMP derivative, dibutyryl cAMP, at the cell body increased the distance regenerated by the central branch of DRG neurons into the dorsal column (Qiu et al., 2002; Neumann et al., 2002). cAMP levels must be elevated above basal levels prior to exposure to the inhibitory molecules or binding MAG and myelin activates an inhibitory G protein that blocks axon growth (Cai et al., 1999). The DRG neurons can be primed by prior lesion, pharmacological elevation of cAMP, or exposing the neurons to NGF, GDNF, or BDNF (Cai et al., 1999).

The elevation of cAMP in the DRG following peripheral axotomy can also enhance peripheral nerve regeneration. Elevating the intracellular levels of cAMP by application of dibutyryl cAMP *in vivo* has been found to enhance peripheral nerve regeneration (Pichichero et al., 1973; Gershenbaum and Roisen, 1980). As well, increasing adenylate cyclase activity increases cAMP concentration and the use of forskolin, a robust activator of adenylate cyclase, can stimulate sensory nerve regeneration (Kilmer and Carlsen, 1984). cAMP can enhance regeneration by blocking

inhibitory molecules in the CNS. The PNS environment is permissive to growth; lacks many of the inhibitory molecules found in the CNS and though it expresses MAGs (reviewed in Trapp, 1990), they are quickly removed by activated Schwann cells and macrophages. Regenerating peripheral neurons may encounter MAGs during early Wallerian degeneration (Martini and Schachner, 1988). As well, a conditioning lesion may increase the regeneration rate of axons so that they encounter the MAGs before they are completely removed. The elevation of cAMP following peripheral axotomy may overcome this inhibitory molecule. Motor neuron regeneration is enhanced in response to antibodies to MAG in C56BL/WldS mice, a mouse strain where Wallerian degeneration is delayed (Mears et al., 2003).

Besides altering extrinsic signals, cAMP may also influence the intrinsic regenerative capacity of the DRG neurons by activating downstream pathways through protein kinase A (Cai et al., 1999). DRGs treated with dibutyryl cAMP *in vivo* exhibited increased axon growth *in vitro* (Neumann et al., 2000). However, Han et al. (2004) found that injection of dibutyryl cAMP in DRG neurons increased the expression of tubulins but did not increase the regeneration rate of the growing axons. Whereas, cAMP is able to induce neurite outgrowth of DRG neurons *in vitro*, high levels of cAMP can repress GAP-43 expression (Andersen et al., 2000). Thus, cAMP may not be sufficient to alter the intrinsic regenerative capacity of the DRG neurons.

1.3.2.4 Electrical stimulation (alternating current).

Continuous (alternating) electrical stimulation has been shown to induce positive results in the regenerating motor neurons of the peripheral nerve. Electrical stimulation of motor neurons after a conditioning lesion accelerated muscle reinnervation (Sebille and Bondoux-Jahan, 1980) and electrical stimulation enhanced reinnervation of motor neurons to their target after a crush injury (Nix and Hopf, 1983; Pockett and Gavin, 1985). In a recent experiment, Al-Majed et al. (2000) applied brief electrical stimulation to the repaired femoral nerve following axotomy. It was found that electrical stimulation, as brief as 1 hour, could accelerate motor neuron regeneration such that regenerated axons appropriately reinnervated their targets within

3 weeks rather than 8 weeks as observed with surgical nerve repair alone (Al-Majed et al., 2000b). Electrical stimulation recruited motor axons to cross the repair site earlier by compressing the asynchronous (staggered) regeneration but had no effect on regeneration speed (Brushart et al., 2002). Brief electrical stimulation was also found to improve specificity of the regenerating sensory axons (Brushart et al., 2005). However it is unknown if electrical stimulation has a similar effect in increasing the intrinsic regenerative response of the sensory neurons. Sensory and motor neurons respond differently to axotomy. Injured sensory neurons downregulate trkB and p75 receptors, while the injured motor neurons upregulate trkB, trkC and p75 neurotrophin receptor (Wood et al., 1990; Saika et al., 1991; reviewed in Lindsay, 1994; Roberson et al., 1994; Wu, 1996).

Electrical stimulation using an alternating current (AC) should not be confused with direct current (DC). An AC flow is reversed or changed on a regular basis and DC flows in the same direction at all times, either towards an anode or a cathode. A weak DC creates a constant electrical field and can therefore affect neurite initiation and orientation. The neurites grow preferentially and rapidly towards the cathode in an applied electrical field and reabsorb neurites facing the anode (Jaffee and Poo, 1979; Roman et al., 1987). The effect of a DC is similar to a tropic effect and likely involves calcium and cAMP to coordinate the directional field response of growth cones (Palmer et al., 2000). Application of DC has been reported to either enhance peripheral nerve regeneration (Politis et al., 1988; Kerns et al., 1991) or have no apparent effect (McDevitt et al., 1987; McGinnis and Murphy, 1992).

Electrical stimulation (AC) may enhance motor neuron regeneration by exerting its effect through axon guidance. Brief periods of electrical stimulation of cultured Xenopus spinal neurons results in a turning response of the growth cone (Ming et al., 2001). As well, electrical stimulation applied to either chromaffin cells or pheochromocytoma cell line PC12 *in vitro* induced filopodia sprouting (Manivannan and Terakawa, 1994). Both of these effects require the presence of extracellular calcium which mediates an elevation of intracellular calcium and cAMP in response to depolarization (Manivannan and Terakawa, 1994; Ming et al., 2001). However, the depolarization-induced intracellular calcium increase associated with electrical

stimulation may also exert its effect on the cell body. Elevated calcium in DRG neurons *in vitro* was found to upregulate immediate early genes and induce neurite outgrowth (Kocsis et al., 1994). As well, pulse electrical stimulation for 30 minutes for 3 weeks was found to increase motor neuron nerve fiber bundles an action on the cell body (Cheng and Lin, 2004). Al-Majed et al (2000b) found that electrical stimulation mediated its effect through the cell body and not the growth cone. The acceleration of motor neuron regeneration in response to electrical stimulation was associated with an upregulation of BDNF, the trkB receptor (Al-Majed et al., 2000a) and regeneration-associated genes at the cell body of the motor neurons (Al-Majed et al., 2004). The influx of calcium associated with electrical activity can regulate BDNF expression. Calcium influx can stimulate transcription of BDNF in cortical neurons from the BDNF promoter III (Tao et al., 1998). There are two elements in this promoter upstream from the start site; one is a calcium-dependent site and the second is a cAMP response element which is required for transactivation of the promoter in the adult but not embryonic neurons (Shieh et al., 1998). The cAMP response element-dependent component can be mediated by CREB and is regulated by CaM kinase IV (Finkbeiner et al., 1997; Shieh et al., 1998). Another consequence of calcium influx mediated by electrical activity is the increase of cAMP through activation of calcium-dependent adenylate cyclase (Xia et al., 1991; Meyer-Franke et al., 1995; reviewed in Xia and Storm, 1997).

It is no surprise that BDNF is upregulated in response to electrical stimulation as BDNF is regulated by neuronal activity. Activation of glutamate receptors in hippocampal neurons leads to enhanced BDNF mRNA levels and stimulates release of the BDNF protein (Zafra et al., 1990; 1991). Long term potentiation can induce BDNF expression in hippocampal CA1 pyramidal neurons or dentate granule neurons (Castren et al., 1993; Dragunow et al., 1993) and seizures can increase BDNF mRNA expression in the forebrain (Isackson et al., 1991). Visual experience can also regulate expression of BDNF mRNA in the visual cortex (Castren et al., 1992). As well, BDNF is released in the dorsal horn by short bursts of high-frequency stimulation or capsaicin stimulation of the dorsal roots (Lever et al., 2001). In many ways BDNF acts like a neurotransmitter- it is stored in vesicles with other neuropeptides in the unmyelinated

afferents of the DRG, anterogradely transported to the dorsal horn (Zhou and Rush, 1996; Michael et al., 1997) and high levels of BDNF can downregulate its receptor, trkB (Frank et al., 1996; 1997; Knusel et al., 1997; Sommerfeld et al., 2000). The neural activity regulation of BDNF contributes to the ability of this neurotrophin to regulate synaptic plasticity in the brain and in the dorsal horn of the spinal cord (Canossa et al., 1997; reviewed in Snider and McMahon, 1998; Schumann et al., 1999; reviewed in Woolf and Salter, 2000).

With regards to peripheral nerve regeneration, upregulation of BDNF and trkB gene expression in motor neurons in response to brief electrical stimulation was associated with an altered expression of regeneration-associated genes such as GAP-43 and Tα1 tubulin.(Al-Majed et al., 2004). The upregulation of BDNF and trkB mRNA preceded the alteration in the expression of regeneration-associated genes, suggesting that BDNF plays a key role in the regulation of these genes and axonal outgrowth (Al-Majed et al., 2004).

1.3.2.5 BDNF and regeneration.

The potential role of BDNF in regulating the regenerative response in motor neurons following electrical stimulation of the injured/repaired femoral nerve is only one example of BDNF expression correlating with regenerative growth. BDNF has been shown to enhance regeneration in a variety of neuronal types. For this thesis, emphasis will be placed on studies of immediate/acute treatment of adult injured neurons *in vivo* with BDNF.

Following peripheral nerve injury, BDNF and trkB are upregulated in motor neurons (Funakoshi et al., 1993; Koliastsos et al., 1993; Piehl et al., 1994; Kobayashi et al., 1996). Application of BDNF either infused onto the proximal nerve or cross-linked to the collagen matrix was found to improve the rate of recovery of sciatic function after nerve transection (Utley et al., 1996). Infusion of both BDNF and ciliary neurotrophic factor directly onto the proximal nerve further improved functional motor recovery when compared to treatment with BDNF alone (Lewin et al., 1997). However, a single exposure of BDNF after entubulation repair of a transected peroneal nerve had no

discernable influence on motor neuron regeneration as determined by gait analysis (Shirley et al., 1996). Thus, BDNF appears to be beneficial for proximal nerve repair but its effect can vary according to dose and by how and where it is delivered.

In retinal ganglion cells, BDNF is expressed in the target tissue (Friedman et al., 1991) and these cells become dependent on BDNF during target field innervation (Rodriguez-Tebar et al., 1989). After axotomy, intravitreal injection of BDNF increases the intraretinal branch length of retinal ganglion axons (Sawai et al., 1996) but does not lead to long distance regrowth (Mansour-Robaey et al., 1994). The intravitreal administration of BDNF enhanced GAP-43 levels but had no effect on tubulins in injured retinal ganglion cells (Fournier et al., 1997; Fournier and McKerracher, 1997).

BDNF has also been used to promote regeneration after spinal cord injury. The rubrospinal tract motor neurons express trkB and therefore are responsive to BDNF (Kobayashi et al., 1997). After cervical axotomy, rubrospinal neurons initially express cytoskeletal and GAP-43 mRNAs similar to that found in motor neurons of the PNS. During the second week, the overall levels of cytoskeletal mRNAs decrease, but the neurons sustain expression of Tα1 tubulin and GAP-43 (Tetzlaff et al., 1991). However, there is no change in regeneration-associated gene expression or axonal regeneration when the rubrospinal axons are severed at the low thoracic level (Tetzlaff et al., 1994; Fernandes et al., 1999) and this correlates with an inability of these distally axotomized neurons to regenerate into a peripheral nerve graft (Richardson et al., 1984). Thus, the capacity of rubrospinal neurons to regenerate correlates with elevated GAP-43 and Tα1 tubulin expression and proximity to the lesion of the cell body (Tetzlaff et al., 1994). Infusion of BDNF into the vicinity of cell bodies of the rubrospinal neurons after cervical axotomy prevented atrophy and increased GAP-43 and Tα1 tubulin and was correlated with increased axonal regeneration (Kobayashi et al., 1997). Adeno-associated viral vector-mediated gene transfer of BDNF also enhanced rubrospinal axon regeneration (Koda et al, 2004). BDNF can also be effective when applied at the site of injury. Schwann cell grafts infused with both NT-3 and BDNF were able to enhance regeneration of the brain stem neurons and promote regeneration into the grafts at the mid-thoracic level of the spinal cord (Xu et al., 1995). As well, grafts containing

fibroblasts genetically modified to express BDNF promote regeneration of supraspinal axons after a cervical spinal cord injury (Liu et al., 1999).

The influence of BDNF on the expression of regeneration-associated genes and axonal outgrowth is distinct in different neuronal systems. The variability of BDNF to promote regeneration in some systems may be the result of different dose, application or duration of BDNF treatment. Despite some inconsistencies, the ability of BDNF to enhance regeneration correlates with altered regeneration-associated gene expression.

2.0 SPECIFIC AIMS

Sensory neurons of the DRG are an important component of the peripheral nervous system. They have the capacity to sense information from the internal and external environment and relay it to the central nervous system. Sensory neurons are organized in relation to function, soma size, and neurotrophin responsiveness. Understanding the general organization of the DRG in the homeostatic state can help understand the modulators of the regeneration response that occur following injury. Following peripheral nerve injury the DRG neurons undergo an intrinsic regenerative response that includes changes in the expression of injury/regeneration genes, cytokines and neurotrophins. This response results in robust regeneration of the injured sensory axons. Despite this capacity, functional recovery often fails to occur. A primary reason is that there is a delay before axons cross the repair site. As time passes the permissive environment created in the degenerating nerve and target organs begins to deteriorate. As a result, the regenerating sensory axons fail to reach their target resulting in loss of sensation and neuropathic pain states. Strategies to improve axonal regeneration include enhancing the intrinsic cell body response so that more axons cross the repair site in a shorter amount of time. A potential approach includes the use of electrical stimulation (AC) to improve regeneration. Brief electrical stimulation is capable of enhancing appropriate motor neuron regeneration of the peripheral nerve and accelerates the time frame required for motor axons to select the correct motor nerve branch by 3 fold. The beneficial effect of electrical stimulation on motor neurons was associated with an upregulation of BDNF mRNA expression and correlated with increased expression of injury/regeneration-associated genes. Administration of exogenous BDNF is also beneficial in promoting regeneration in many systems. However, most research on peripheral nerve regeneration has focused on the effect of BDNF on motor neuron regeneration. It is unknown if electrical stimulation is beneficial in sensory neurons or if BDNF subserves a similar role in sensory neurons.

There is some suggestion that BDNF is important in sensory neuron regeneration. The treatment of DRG neurons *in vitro* with BDNF resulted in enhanced axonal outgrowth (Lindsay, 1988). However blocking endogenous BDNF with systemic

administration of a BDNF antibody impaired sciatic nerve regeneration and myelination (Zhang et al., 2000). As well, BDNF is the sole neurotrophin that is upregulated after injury and as neurotrophins are responsible for the phenotypic maintenance of the sensory neurons, BDNF may regulate the injury/regeneration phenotype.

I hypothesize that the manipulations that enhance electrical activity of injured sensory neurons lead to improved regeneration in peripheral nerves through a mechanism which requires elevated BDNF expression. I have addressed this hypothesis through the following aims:

1) I have assessed the effects of electrical stimulation on sensory neuron regeneration. The rat femoral model of transection and surgical repair was used to determine the effect of various durations of 20 Hz electrical stimulation applied immediately following repair on axonal regeneration of sensory neurons. Retrograde labeling and cell counts were performed to determine the number of regenerating sensory axons that crossed the repair site and regenerated into muscle or cutaneous branches of the femoral nerve 21 days following injury. *In situ* hybridization and immunohistochemistry were also performed on DRG tissue removed 48 hours after repair and immediate electrical stimulation to detect changes in injury/regeneration-associated genes, including BDNF expression.

2) I have determined whether endogenous BDNF regulates induction and/or maintenance of the cell body response in injured sensory neurons and the intrinsic ability of these cells to extend neurites. A specific function-blocking BDNF antibody was infused intrathecally via a mini-osmotic pump for 3 days either immediately after an L4, L5 spinal nerve transection or 1 week post-injury. Control IgG infusion or injury alone served as controls. *In situ* hybridization was performed to detect changes in injury/regeneration-associated gene expression associated with the experimental treatments and a neurite outgrowth assay was performed to determine if endogenous BDNF can regulate the growth capacity of sensory neurons

3) I have examined whether a supply of exogenous BDNF to an injured sensory neuron can enhance the cell body response of sensory neurons to injury as assessed by examining its influence on injury/regeneration-associated gene expression. Exogenous BDNF of a high or low dose was infused intrathecally following an L4, L5 spinal nerve

transection for 3 days. Infusion of vehicle or injury alone served as controls. *In situ* hybridization was performed to detect changes in injury/regeneration-associated gene expression, including trkB expression.

4) I have determined whether an increased supply of exogenous BDNF administered to intact sensory neurons is sufficient to induce a switch in neuronal phenotype that mimics the regenerating state. Exogenous BDNF was infused intrathecally for 7 days in naïve animals. *In situ* hybridization was performed to detect changes in injury/regeneration associated gene expression to determine whether alterations in neuronal phenotype mimicked the state found after peripheral nerve injury.

3.0 BRIEF ELECTRICAL STIMULATION PROMOTES SENSORY NEURON REGENERATION AND INTRINSIC GROWTH-ASSOCIATED GENE EXPRESSION.

3.1 Abstract.

Brief electrical stimulation enhances the regenerative ability of axotomized motor (Nix and Hopf, 1983; Al-Majed et al., 2000b) and sensory (Brushart et al., 2005) neurons. Here we examined the parameter of duration of stimulation on regenerative capacity, including the intrinsic growth programs, of sensory neurons. The effect of 20 Hz continuous electrical stimulation on the number of DRG sensory neurons that regenerate their axons was evaluated following transection and surgical repair of the femoral nerve trunk. Stimulation was applied proximal to the repair site for 1 hour, 3 hours, 1 day, 7 days, or 14 days at the time of nerve repair. Following a 21-day regeneration period, DRG neurons that regenerated axons into the muscle and cutaneous sensory nerve branches were retrogradely identified. Stimulation for 1 hour led to a significant increase in DRG neurons regenerating into cutaneous and muscle branches when compared to 0 hour (sham) stimulation or longer periods of stimulation. Stimulation for 1 hour also significantly increased the numbers of neurons that regenerated axons beyond the repair site 4 days after lesion and was correlated with a significant increase in expression of GAP-43 mRNA, in the regenerating neurons at 2 days post-repair. Additional indicators of an enhanced intrinsic growth capacity following 1 hour stimulation included elevated expression of Tαl tubulin, BDNF and the BDNF receptor, trkB. The effect of brief stimulation on enhancing sensory and motor neuron regeneration holds promise for inducing improved peripheral nerve repair in the clinical setting.

3.2 Introduction.

Peripheral nerve regeneration, although more successful than central nervous system regeneration, is fraught with challenges when reinnervation is not exact or when

debilitating neuropathic pain develops. Despite surgical repair to guide regenerating axons to their appropriate target, axonal regeneration often fails to achieve significant functional recovery (reviewed in Sunderland, 1978; reviewed in Kline and Hudson, 1995). One reason is that regenerating axons frequently grow long distances to reach denervated target organs and the rate of regeneration is slow. Rapidly regenerating axons grow at 1-3 mm/day with a latent period of 3-7 days for axons to cross the surgical site (reviewed in Sunderland, 1978; reviewed in Kline and Hudson, 1995). By the time regenerating axons enter the distal nerve stumps, the growth potential of axotomized neurons and the permissive growth environment of the distal nerve stumps has begun to deteriorate (Fu and Gordon, 1995a, b; Whitworth et al., 1996). As a result regenerating axons may fail to reach their target end-organs.

Strategies to improve peripheral nerve regeneration are aimed at inducing and maintaining strong growth states in the maximum number of neurons. The signals which set up these programs in sensory neurons involve disconnection from peripheral target tissue (Richardson and Issa, 1984; Richardson and Verge, 1987) and induction of regeneration-associated gene expression (Richardson and Issa, 1984; Richardson and Verge, 1987; Bevan and Winter, 1995; Doster et al., 1991; Andersen and Schreyer, 1999; Fernandes et al., 1999; Al-Majed et al., 2004). Acceleration of these programs can be achieved in sensory neurons by a conditioning lesion (Richardson and Verge, 1987), which is effective, but not clinically desirable. Studies by Al-Majed et al., (2000b) demonstrate that 1 hour of 20 Hz electrical stimulation (alternating current) of the nerve proximal to the site of surgical repair enhances motor axonal regeneration across the surgical gap (Brushart et al., 2002) and shortens the time frame required for motor axons to select the correct motor nerve branch by 3-fold. This effect is correlated with increased expression of injury/regeneration-associated genes (GAP-43 and Tα1 tubulin), the neurotrophin BDNF and its receptor, trk B (Al-Majed et al., 2000a, 2004). Thus, brief electrical stimulation holds tremendous clinical promise for axonal regeneration of motor neurons after surgical repair. In addition, recent evidence demonstrates that 1 hour stimulation improves the specificity of sensory neuron regeneration 21 days post repair (Brushart et al., 2005). However, whether this duration

of electrical stimulation is optimal and whether it alters the intrinsic growth programs of sensory neurons is unknown.

We hypothesize that electrical stimulation increases the intrinsic regenerative capacity of sensory neurons. The present study evaluates the effect of duration of electrical stimulation on the regeneration of sensory neurons contributing to the femoral nerve following transection and repair. Our findings demonstrate that the beneficial effects of electrical stimulation observed for motor neurons extend to sensory neurons as evidenced by an increased number of sensory neurons that regenerate their axons in response to the stimulation and pronounced effect on expression of injury/regeneration-associated genes. Portions of this work have been published in abstract form (Geremia et al., 2002).

3.3 Materials and Methods

3.3.1 Experimental Design.

Experiments were performed on the adult rat femoral nerve in which regenerating motor axons preferentially reinnervate muscle pathways (Brushart and Seiler, 1988). The femoral nerve contains cutaneous sensory fibers that branch to innervate the skin via the saphenous nerve. These fibers are intermingled in the femoral nerve with sensory and motor fibers that innervate the quadriceps muscle via the quadriceps muscle nerve branch (Fig. 3-1a). One third of the axons derive from the α-motor neurons that innervate the skeletal muscle fibers (Brushart and Seiler, 1987). Experiments were approved by a local ethical committee (Health Science Laboratory Animal Services) under the Canadian guidelines for animal experimentation.

3.3.2 Nerve repair.

Experiments were performed under aseptic conditions on the left femoral nerves of young adult (220- 240 gm) female Sprague Dawley rats that were anesthetized with somnitol (30 mg/kg, i.p.). The proximal femoral nerve was sharply

cut 20 mm proximal to the bifurcation of the cutaneous and muscle nerves. The proximal and distal stumps were then carefully aligned and surgically joined within a 4 mm long silastic nerve cuff (0.03 mm inner diameter; Dow Corning) by placing a single suture of 9-0 silk (Ethicon) through the epinerium of the proximal and distal stumps under 40X magnification (Fig. 3-1b).

3.3.3 Electrical stimulation of cut and repaired femoral nerve.

Two insulated stainless steel wires (Cooner A 5632) were bared of insulation for 2-3 mm, twisted to form a small loop to secure either side of the femoral nerve stump proximal to the suture site and positioned proximal to the suture repair site for electrical stimulation. The cathode was sutured alongside the femoral nerve just below its exit from the peritoneal cavity, whereas the anode was sutured to muscle more distally, close to the nerve and just proximal to the suture repair site. The wires were connected to a custom-made biocompatible implantable stimulator that was encased in epoxy resin and covered with biocompatible silastic and contained a light-sensitive diode, which turned the stimulator on and off by an external light flash. Stimulation commenced immediately after nerve repair with supramaximal pulses (100 μsec; 3V) delivered in a continuous 20 Hz train by the implantable stimulator. The non-stimulated control group underwent nerve transection and repair alone (n=7). In the sham group of rats, the nerve was transected and repaired and the electrodes were implanted, but the stimulator was not activated (n=13). The stimulated axotomized sensory neurons and their regenerating axons were subject to short-term (1 hour) or long-term (3 hour, 1 day, 7 day or 14 day) periods of continuous low-frequency electrical stimulation (n=48).

3.3.4 Tetrodotoxin (TTX) application.

We used TTX at a dose of 60 μg/ml to block the propagation of action potentials back to the cell body as previously described (Al-Majed et al., 2000b). A sterile piece of absorbent cotton soaked with a 60 μg/ml dose of TTX was applied to the femoral nerve proximal to the position of the stimulating electrodes prior to the femoral nerve

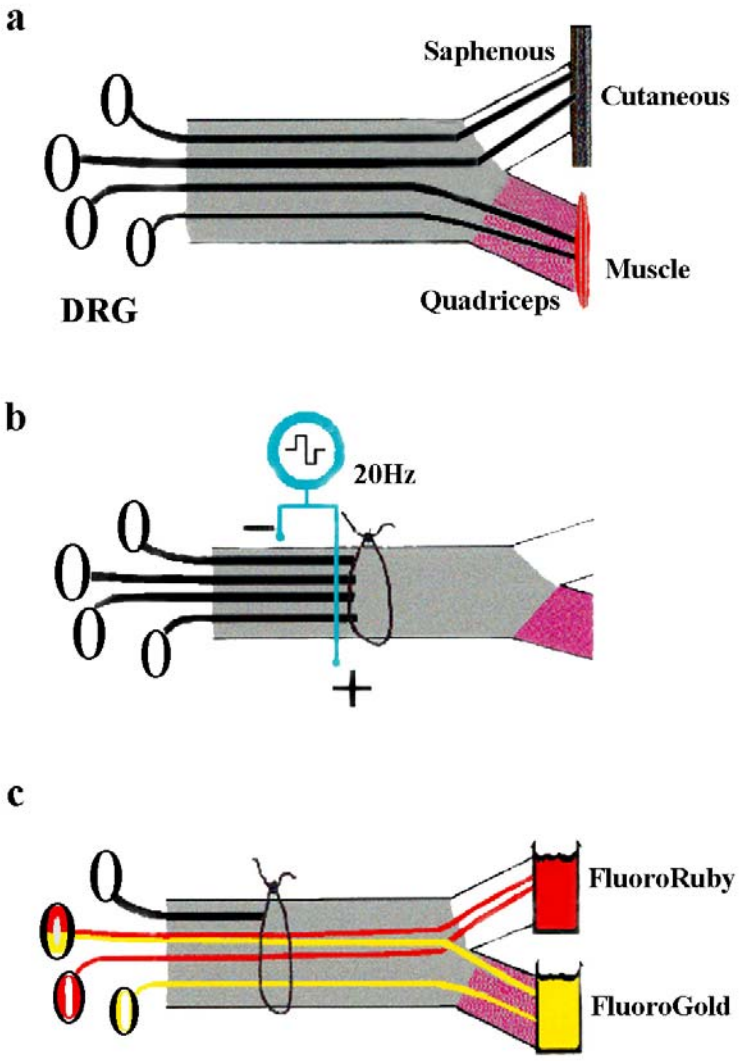

Figure 3-1. Diagrammatic representation of the regeneration model employed (as adapted from Al-Majed et al., 2000b). A. The femoral nerve has two branches: one to the quadricep muscle via the quadricep muscle nerve (motor and sensory axons) and one to the skin (cutaneous branch) via the saphenous nerve (sensory axons). B. Bipolar electrodes were placed proximal to the site of nerve transection and surgical repair. Stimulation was a continuous train of 20 Hz for various times. C. Retrograde tracers

were applied 21 -days after nerve repair to assess the number of sensory neurons that had regenerated axons into the muscle and cutaneous branches of the femoral nerve. transection, repair, and the 1 hour stimulation period (Fig. 3-5a). The electrodes and the TTX were removed before closing the wound site (n=6).

3.3.5 Retrograde labeling of sensory neurons.

At the end of the 21-day regeneration period, the muscle and cutaneous branches of the left femoral nerve were isolated, cut and backlabeled with neurotracers to identify the sensory neurons innervating each branch. Fluorogold (FG; Fluorochrome Inc., Denver, CO) and Fluororuby (FR; dextran tetramethylrhodamine, D-1817; Molecular Probes, Eugene, OR) were employed because they are effectively endocytosed and retrogradely transported (Schmued and Fallon, 1986). The muscle and cutaneous branches were cut 5 mm distal to the femoral bifurcation (25 mm from the repair site). In each rat, one branch was labeled with FG and the other with FR (the dye application was alternated between animals to control for possible differences in retrograde uptake and transport of the dyes). Backlabeling with FG was done by exposing the tip of the severed branch to 4% FG in 0.1 M cacodylic acid for 1 hour in a Vaseline well, after which it was extensively irrigated and reflected to a distant portion of the wound. Backlabeling with FR was done by placing the tip of the severed branch above a small weighing paper with FR crystals for 2 hours and then irrigating the nerve and placing it in the opposite corner of the wound to prevent cross-contamination by diffusion of tracers. Animals were kept for 72 hours after tracer application to allow the retrograde tracers to travel back to the neuronal cell bodies.

3.3.6 Backlabeling of femoral nerve axons just distal to the repair site.

Experiments were performed on the femoral nerves of young adult (250g) female Sprague Dawley rats (n=11). The animals were anesthetized by intramuscular injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Both femoral nerves of each rat were transected and repaired, but only one repair was stimulated. The femoral nerve

was sharply transected 1 mm distal to the iliacus branch, carefully aligned and sutured with 11-0 nylon under 20-40X magnification. Stimulation was delivered intraoperatively for 1 hour with animals under the same anesthetic. A Grass (Quincy, MA) SD-9 stimulator delivered continuous 20 Hz stimulation (100 μsec, 3-5 V) to fine silver wires placed at (anode) and just proximal to (cathode) the repair.

As described previously for motor neurons (Brushart et al., 2002), backlabeling of axons just distal to the repair site was used to identify the sensory neurons that regenerated their axons across the surgical repair site. After a 4 day regeneration period, the femoral nerve was exposed and crushed 1.5 mm distal to the suture line with a narrow microforceps. A micropipette was introduced through the epineurium and perineurium distal to the crush and advanced intraneurally to the crush zone. Approximately 0.5μl of 5 % FR was injected with a Picospritzer (Parker Hannefin, Fairfield, NJ) to restore the flattened crush zone to its normal rounded contour (Fig. 3-4a). Animals were kept for 48 hours after tracer application, the time required for the tracer to reach the neuronal cell body.

3.3.7 Tissue fixation by cardiac perfusion.

All rats were deeply anesthetized (somnitol, 0.12 ml/100 g of body weight) and perfused through the left ventricle prior to further histological analysis as described below. A warm saline flush (100 ml) was followed by 500 ml of ice cold 4% paraformaldehyde. L3 DRGs were dissected ipsilaterally, cryoprotected in 20-30% sucrose overnight, embedded in OCT compound (Tissue Tek, Miles Inc, Elkhart, IN) in a cryomold (Tissue Tek), frozen in isopentane cooled to -70°C and stored at –80°C until further processing.

3.3.8 Quantification of the number of regenerated sensory neurons.

The L3 DRG were serially sectioned at 10 μm using a Microm HM500 cryostat (Zeiss, Canada) and mounted on glass slides. Each section was visualized at 20-40X under ultraviolet fluorescence at barrier filters of 580 nm for FR and 430 nm for FG.

Sensory neurons containing both FR and FG were visualized using both filters. The observer who was unaware of experimental condition and which branch had received FG or FR counted the backlabeled sensory neurons. Only DRG neurons with a visible nucleus were counted. The number of backlabeled sensory neurons was determined by counting every other section to minimize the possibility of counting large neurons more than once.

3.3.9 *In situ* hybridization.

Following a 2 day regeneration period, L3 DRG were harvested from cardiac perfused rats subjected to 0 hour (sham - n=3), 1 hour (n=3) or 3 hour (n=3) stimulation at the time of nerve repair. The L3 DRGs were cryoprotected and embedded as above and stored at –80°C until further processing. Serial DRG sections were cut at 6 μm using a Microm HM500 cryostat (Zeiss, Canada), thaw mounted onto Probe-On+ slides (Fisher Scientific, Canada) and stored with dessicant at -20°C until hybridization.

In situ hybridization was carried out on tissue using 48 base pair oligonucleotide probes (University Core DNA Services, Calgary, AB, Canada) complementary to and selective for the following mRNAs: GAP-43 [complementary to bases 70-117 (Karns et al., 1987)], Tα1 tubulin [complementary to bases 1548-1594 (Lemischka et al., 1981)] and trkB (full length) [complementary to bases 1361-1408 (Middlemas et al., 1992)]. All probes were checked against the GenBank database (NIH, Bethesda, MD) to ensure that no greater than 75% homology was found to sequences other than the cognate. The probes were labeled at the 3'-end with α- [^{35}S]dATP (New England Nuclear, Boston, MA) using terminal deoxyucleotidyl-transferase (Amersham Biosciences, Piscataway, NJ) in a buffer containing 10 mM $CoCl_2$, 1mM dithiothreitol (DTT), 300 mM Tris base, and 1.4 M potassium cacodylate, pH 7.2, and purified through Bio-Spin Disposable Chromatoraph Columns (Bio-Rad Laboratories, Hercules, CA) containing 200 mg of NENSORB PREP Nucleic Acid Purification Resin (DuPont NEN, Boston MA). Dithiothreitol was added to a final concentration of 10 nM. The specific activities ranged from 2.0 to 5.0 X 10^6 cpm/ng for each oligonucleotide.

In situ hybridization was carried out according to published procedures (Dagerlind et al., 1992). Briefly, the sections were hybridized at 43°C for 14-18 hours in a buffer containing 50% formamide (Sigma Aldrich, Oakville, ON, Canada), 4 X SSC (1 X SSS = 0.15 M NaCl, 0.015 M sodium citrate), 1 X Denhardt's solution (0.02% bovine serum albumin and 0.02% Ficoll), 1% sarcosyl (N-laurylsarcosine), 0.02 M phosphate buffer, pH 7.0, 10% dextran sulfate, 500 μg/ml heat-denatured sheared salmon sperm DNA, 200 mM dithiothreitol, and 10^7 cpm/ml of probe. After hybridization, the slides were washed 4 X 15 min in 1 X SSC at 55°C, dehydrated in ascending alcohols, processed for radioautography as per Karchewski et al. (2002), and exposed for 1-42 days before developing in D-19 (Kodak, Rochester, NY).

The specificity of hybridization signal for each probe was ascertained by hybridization of labeled probe, labeled probe with a 1000-fold excess of cold probe (signal abolished), or labeled probe with a 1000-fold excess of a dissimilar cold probe of the same length and similar G-C content (signal unchanged).

3.3.10 Immunohistochemistry.

The L3 DRG sections were cut at 6 μm on a cryostat, thaw mounted onto Probe-ON$^+$ slides (Fisher Scientific), and processed for immunohistochemistry. To detect activating transcription factor 3 (ATF3) protein expression, sections were washed 3 X 10 min in TBS and blocked in 10% goat serum in TBS for 1 hour at room temperature. Primary antibody rabbit anti-ATF3 (1:300; Santa Cruz Biotech Inc, Santa Cruz, CA) in TBS was left on tissue overnight at 4°C. The next day, sections were washed at 3 X 10 min in TBS and then incubated in secondary antibody, biotinylated goat anti-rabbit IgG (1:300; Vector, Burlington, ON, Canada) in TBS. The ATF3 immunoreactivity was visualized using standard ABC procedure (Vector, Burlington, ON, Canada). To detect BDNF protein expression, immunohistochemistry was performed as previously described by Karchewski et al., 2002. The primary antibody, rabbit anti-BDNF (1:500; gift from Cindy Wetmore, Mayo Clinic, Rochester, MN, USA; Wetmore and Olsen, 1995) was left on tissue overnight at 4° C. The next day, sections were incubated in secondary antibody Cy3-conjugated donkey anti-rabbit IgG (1:300; Jackson,

Westgrove, PA) for 1 hour in the dark at room temperature. Slides were coverslipped with glycerol/PBS. Control sections were processed in the same manner, but with blocking solution or undiluted normal rabbit serum replacing the primary antibody.

3.3.11 *In situ* hybridization quantification and analysis.

Not all L3 DRG neurons contribute to the femoral nerve at the level of the nerve injury. Neurons whose axons were transected were identified by processing sections for immunohistochemistry to detect ATF3, which is expressed *de novo* in axotomized sensory neurons (Tsujino et al., 2000). The adjacent section was processed for *in situ* hybridization to detect GAP-43 mRNA, and all neurons with a detectable nucleus were identified in the serial sections and labeled as axotomized or intact based on whether or not nuclear ATF3 expression was detected. This allowed alterations in GAP-43 mRNA hybridization signal to be quantified in the axotomized (those with the potential to regenerate) versus the non-axotomized intact neurons. DRG sections from each experimental group were mounted on the same slide to ensure that slide to slide variability in hybridization signal did not impact the relative difference in hybridization signal for each experimental group. Quantitative analysis was performed on DRG sections (n=2) for each probe to detect the density of GAP-43 mRNA hybridization signal in ATF3 positive and negative neurons after 0 hour (sham), 1 hour, and 3 hours of electrical stimulation. The quantitative results were confirmed qualitatively in the remaining sections for each animal that was processed but not analysed quantitatively (n=1). Neurons were considered labeled if they had more than 5 Xs background level of silver grains, as determined by averaging grain densities over defined areas of neuropil devoid of positively labeled cell bodies. This cut-off corresponds to the level of hybridization signal that must be present in order to confidently declare the neuron labeled when observed under 63X oil immersion.

To assess whether electrical stimulation altered the number of neurons expressing relatively high levels of trkB mRNA hybridization signal, the percentage of neurons expressing abundant trkB hybridization signal was determined under 40X

objective in 6 DRG sections from animals exposed to 0 hour (sham), 1 hour or 3 hour stimulation, by an observer blind to experimental condition.

3.3.12 Statistical analysis.

A Kruskal-Wallis nonparametric ANOVA (Prism v.2.01 GraphPad Software Inc., San Diego, CA) was used to compare the number of sensory neurons projecting axons to cutaneous and muscle branches within each group. A Mann Whitney U test (Prism v.2.01) was used to compare the number of sensory neurons extending an axon across the surgical repair site 4 days after repair with or without 1 hour stimulation at the time of repair. Statistical significance was accepted at $p < 0.05$ level.

3.4 Results.

3.4.1 The effect of short versus long-term electrical stimulation on regeneration of sensory neurons *in vivo.*

In the transected and surgically repaired rat femoral nerve, a 1 hour period of continuous 20 HZ electrical stimulation was as effective as periods of 1 day to 2 weeks of electrical stimulation in significantly enhancing motor axonal regeneration (Al-Majed et al., 2000b). The identical stimulation protocol was employed to assess whether this duration of stimulation enhanced sensory neuron regeneration. We examined the number of L3 DRG neurons that regenerated their axons into the cutaneous and/or the muscle branch of the femoral nerve after 21 days. Regenerated neurons were identified by backlabeling with FR in one branch and FG in the other (Fig. 3-1c). The total number of DRG neurons that regenerated their axons down each branch and those that had grown down both branches (identified by double labeling) was determined.

Experimental control groups included nerve injury and repair and the nerve injury and repair plus placement of electrodes for 1 hour without turning on the stimulator (sham group). No significant differences were found between the control groups, so the data from the two groups were pooled and comprised the 0 hour group

(Fig. 3-2). Stimulation of the femoral nerve for 1 hour after nerve transection and repair resulted in a significant increase in the number of sensory neurons that regenerated

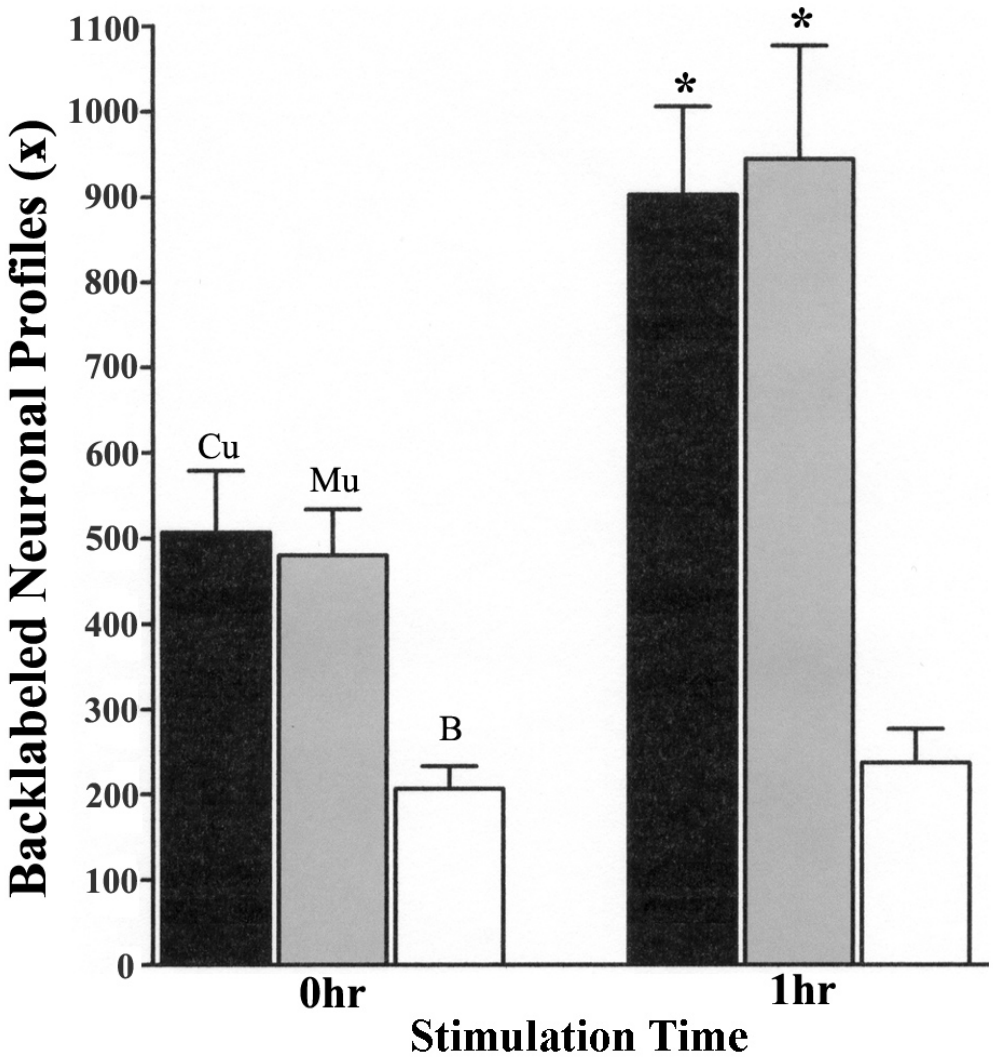

Figure 3-2. Electrical stimulation for 1 hour significantly improves sensory axon regeneration. The mean number ± SEM of backlabeled sensory neurons that regenerated their axons into the muscle *(mu - gray bar*), cutaneous branch (*cu - black bar*), or both (*B - open bar*) after femoral repair with no stimulation (0 hr) or brief stimulation (1 hr; *$p < 0.05$).

axons into either the cutaneous (507 +/- 73 SEM (0 hr) versus 904 +/-103 SEM (1hr)) or muscle branch (480 +/- 54 SEM (0hr) versus 945 +/- 133 SEM (1hr); Fig.3-2).

The increase in the number of sensory neurons that regenerated their axons into the cutaneous or muscle branch was not due to an increased incidence of double labeled

neurons – i.e. those that sent axonal collaterals to both branches, as the numbers of neurons remained relatively unchanged among treatment groups (210 +/- 45 SEM (0hr) versus 206 +/- 27 SEM (1hr)) (Fig. 3-2).

The duration of electrical stimulation at the time of nerve repair was extended beyond 1 hour to assess whether regeneration of sensory neurons might be further enhanced. Stimulation for continuous periods of 3 hours, 1 day, 7 days or 14 days was less effective than 1 hour stimulation at enhancing the number of sensory neurons that regenerated into the muscle and was not significantly different from the 0 hour control group (480 +/- 54 SEM (0hr); 945 +/- 133 SEM (1hr); 709 +/- 130 SEM (3 hr); 548 +/- 277 SEM (1d); 411 +/- 133 SEM (7d); 558 +/- 138 SEM (14d)) and cutaneous (507 +/- 73 SEM (0 hr); 904 +/- 103 SEM (1hr); 757 +/- 53 SEM (3hr); 437 +/- 331 SEM (1d); 409 +/- 118 SEM (7d); 456 +/- 131 SEM (14d)) 21 days after nerve repair (Fig. 3-3).

3.4.2 The effect of brief electrical stimulation on the ability of sensory neurons to regenerate beyond the repair site.

We next examined whether the enhanced sensory nerve regeneration observed in response to 1 hour stimulation at the time of femoral nerve repair was due to an accelerated growth of axons across the repair site into the distal nerve stump as observed for motor axons (Brushart et al., 2002). All sensory neurons that regenerated axons beyond the repair site by 4 days post repair were identified using retrograde labeling just distal to the surgical reunion of the nerve stumps in 0 hour and 1 hour stimulated rats. The number of backlabeled neuronal profiles in the 1 hour stimulation

group (758 +/- 75 SEM) was significantly greater than the 0 hour control group (461 +/- 103 SEM). These findings demonstrate that 1 hour electrical stimulation led to a 64% increase in the number of DRG neurons that rapidly regenerated axons across the repair site (Fig. 4b).

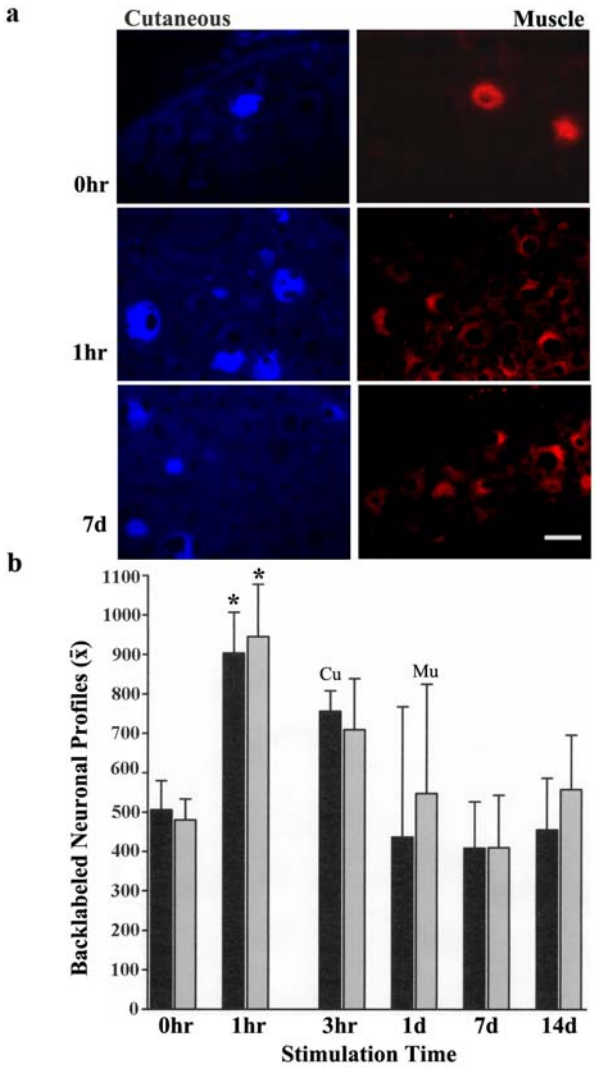

Figure 3-3. Long-term stimulation is less effective than short-term stimulation in promoting axonal regeneration of sensory neurons. A. Fluorescence photomicrographs of retrogradely labeled sensory neurons that regenerated into the muscle branch or cutaneous branch 21 days after femoral repair with 0 hr, 1 hr, or 7

days of 20 Hz continuous electrical stimulation. Scale bar = 50 μm. B. The mean number ± SEM of backlabeled sensory neurons that regenerated their axons into the cutaneous (*cu - black bar*) or muscle (*mu - gray bar*) branches after femoral repair with stimulation times as indicated (*p<0.05)

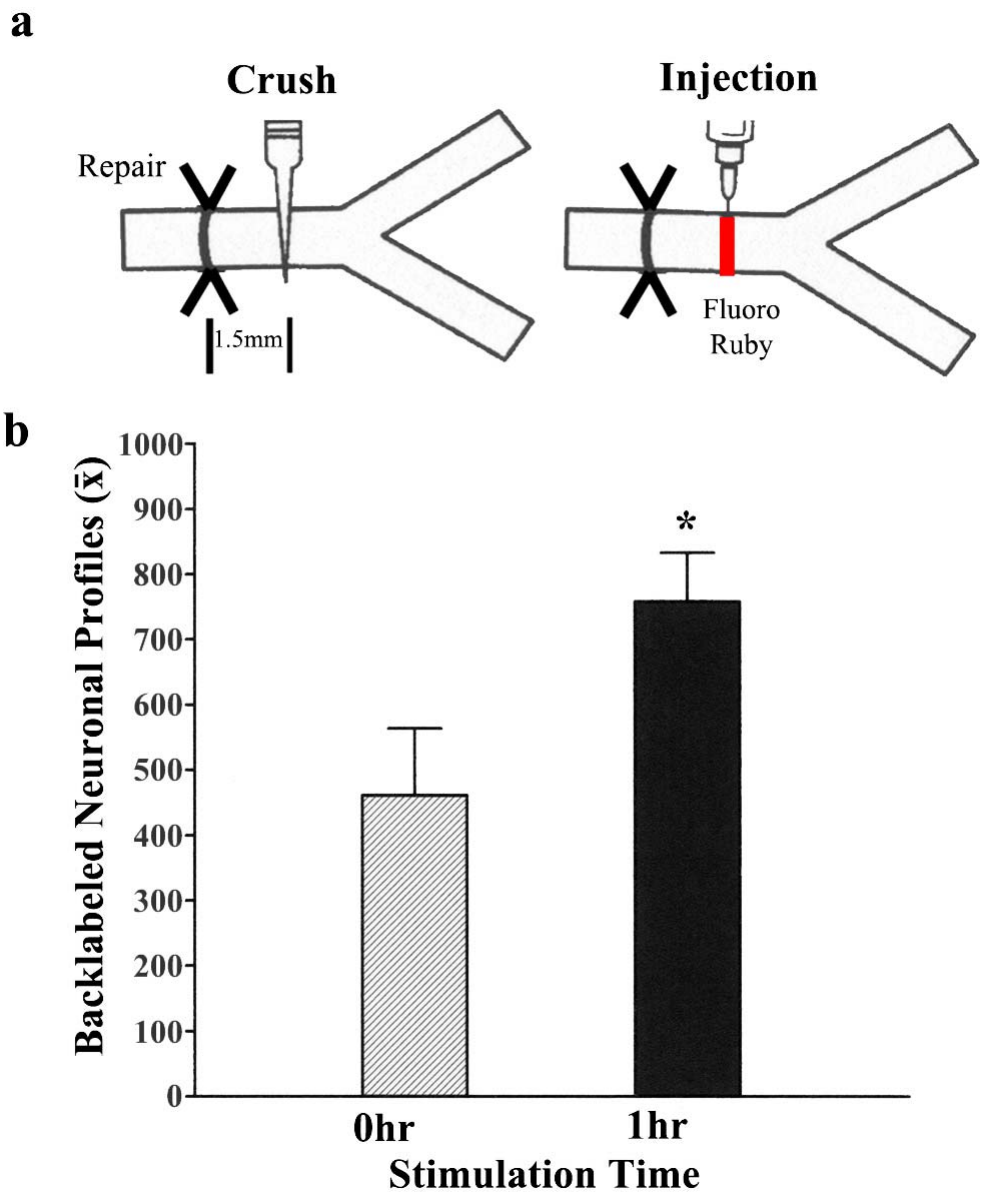

Figure 3-4. One hour of 20 Hz continuous electrical stimulation significantly increases the number of sensory neurons extending axons across the repair site 4d

following nerve transection. A. Diagrammatic representation of distal labeling (as adapted from Brushart et al., 2002). Axons were labeled just distal to the repair to identify the sensory axons that crossed the repair site. B. The mean number $\pm$ SEM of backlabeled sensory neuronal profiles that regenerated axons beyond repair site 4d after injury with no stimulation as compared to 1 hr stimulation (*$p<0.05$).

3.4.3 Influence of action potential blockade on sensory neuron regeneration following brief electrical stimulation.

To assess the effect of increased electrical activity on the sensory neurons, retrograde conduction of action potentials to the cell body was blocked using a TTX-mediated blockade of sodium channels proximal to the stimulating electrode (Fig. 3-5a). As expected from the corresponding motor neuron data (Al-Majed et al., 2000b), TTX application abolished the positive influence of 1 hour electrical stimulation on sensory axon regeneration (cutaneous-904 +/- 103 SEM ; muscle- 945 +/- 133 SEM; Fig. 3-5b). There was no significant difference on the regenerative response of sensory neurons in the stimulated + TTX-treated group (cutaneous- 437 +/- 106 SEM; muscle- 403 +/- 98 SEM) when compared to 0 hour control group (cutaneous- 507 +/- 73 SEM; muscle- 480 +/- 54 SEM).

Abolishing sodium channel activity with TTX resulted in a similar incidence of regenerating sensory neurons extending axons down both the motor and cutaneous nerve branches (double-labeled neurons; 237 +/- 40 SEM) as observed for the 0 hour group (210 +/- 45 SEM) and 1 hour electrical stimulation group (206 +/- 27 SEM; Fig. 3-5b).

3.4.4 The effect of short-term electrical stimulation on the cell body response of sensory neurons.

To assess whether 1 hour electrical stimulation at the time of femoral nerve repair results in elevated expression of the injury/regeneration-associated genes, GAP-43, Tα1 tubulin and BDNF and the BDNF receptor trkB, alterations in expression of these markers were evaluated in L3 DRG removed 2 days following femoral nerve

section and repair with 0 hour (sham), 1 hour or 3 hour electrical stimulation. The shorter 2 day regeneration time was selected for examination rather than the previous 21-day regeneration period because the electrical stimulation promotes the outgrowth of axons across the suture site within 4 days and by 21 days post-repair discernible

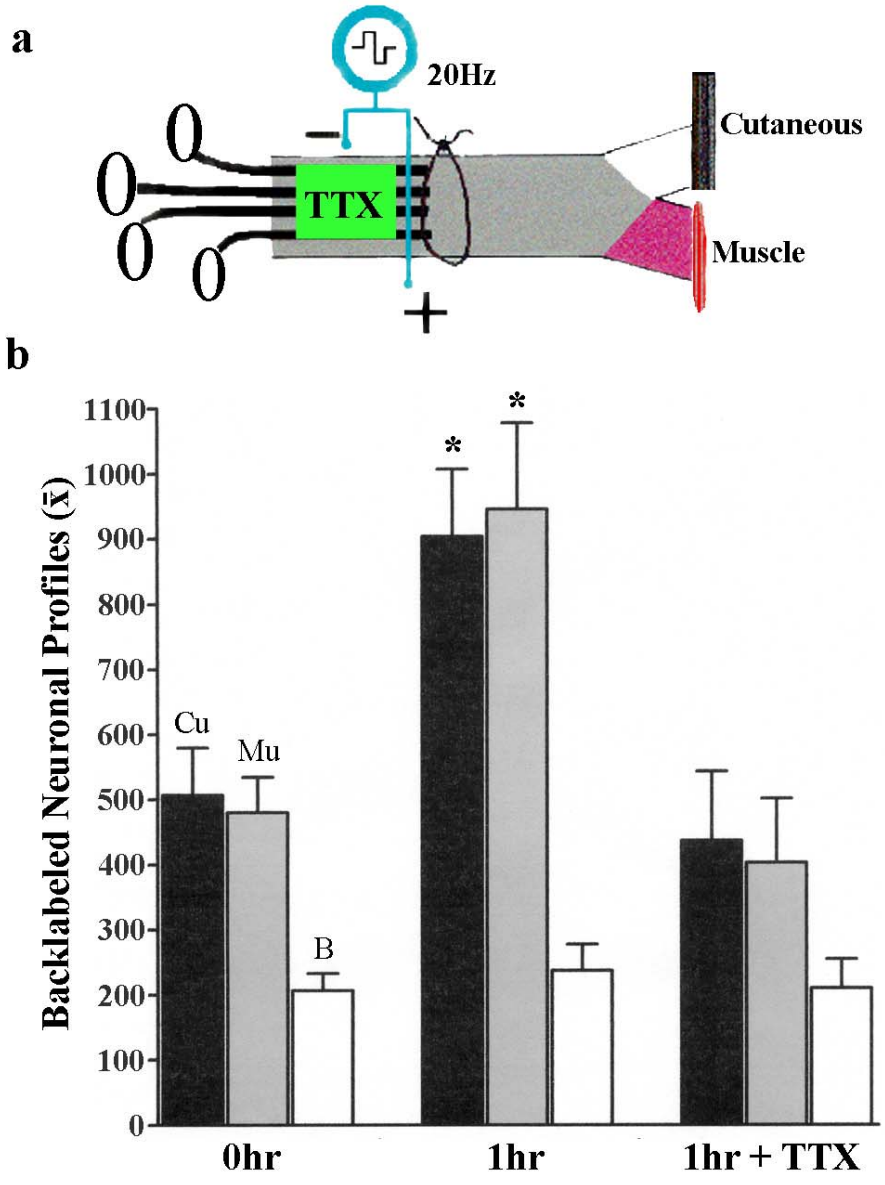

Figure 3-5. TTX blocks the effect of 1 hour of 20 Hz continuous electrical stimulation on 21 day regeneration after nerve transection and surgical repair. A. Diagrammatic representation of the application of TTX (60 μg/ml; as adapted from Al-Majed et al., 2000b). B. The mean number ± SEM of backlabeled sensory neurons that regenerated their axons into the cutaneous (*cu - black bar*), muscle (*mu - gray bar*), or both (*B - open bar*) branches after nerve transection and repair with stimulation paradigms as indicated (*$p<0.05$).

differences in expression of these markers are no longer evident (Fig. 3-4).

At 2 days post-nerve repair, 1 hour stimulation resulted in an enhanced level of GAP-43 and Tα1 tubulin mRNA expression in some but not all L3 sensory neurons when compared to the L3 sensory neurons that received 0 hour or 3 hour stimulation at the time of nerve repair (Fig. 3-6). Since only a proportion of L3 DRG neurons send their axons to the femoral nerve at the level of the nerve section and repair, we expected such a partial effect. To specifically identify axotomized neurons, we processed serial sections of the L3 DRGs for ATF3 immunoreactivity or GAP-43 mRNA in situ hybridization. ATF3 serves as a marker for sensory neurons that are axotomized (Tsujino et al. 2000) and allows identification of the neurons that have the potential to regenerate. Using serial sections, the same DRG neurons were identified in the adjacent sections and allowed quantification of GAP-43 mRNA hybridization signal over ATF3-positive (injured) versus the ATF3–negative (non-injured) neuronal subpopulations of the L3 DRG (Fig. 3-6).

Quantification of the GAP-43 mRNA hybridization signal over the individual ATF3-negative neurons revealed that moderate to high levels of signal (>10 X background) were detected almost exclusively over small-to medium sized intact DRG neurons (Fig. 3-7). This distribution of GAP-43 mRNA in the ATF3-negative (non-injured) neurons is similar to the pattern of expression of GAP-43 mRNA previously described for intact sensory neurons (Verge et al., 1990). Stimulation for 1 hour at the time of femoral nerve transection and repair had no effect on the level of GAP-43 mRNA expression measured in non-injured, ATF3- negative sensory neurons, when compared to 0 hour control group (Fig. 3-6, -7). Three hours of electrical stimulation at the time of repair however, resulted in a slightly lower level of injury/regeneration-

associated gene expression when compared to 1 hour of stimulation or the 0 hour group (Fig. 3-6). These observations are supported by slightly lower incidence in the number of neuronal profiles expressing detectable GAP-43 mRNA hybridization signal in the 3 hour stimulated group (i.e >5X background). Approximately 61.6% of neuronal profiles quantified expressed detectable GAP-43 mRNA two days following 3 hour electrical stimulation, as compared to ~68.6% or ~67.2% of neurons following 0 hour or 1 hour electrical stimulation respectively (Fig. 3-7).

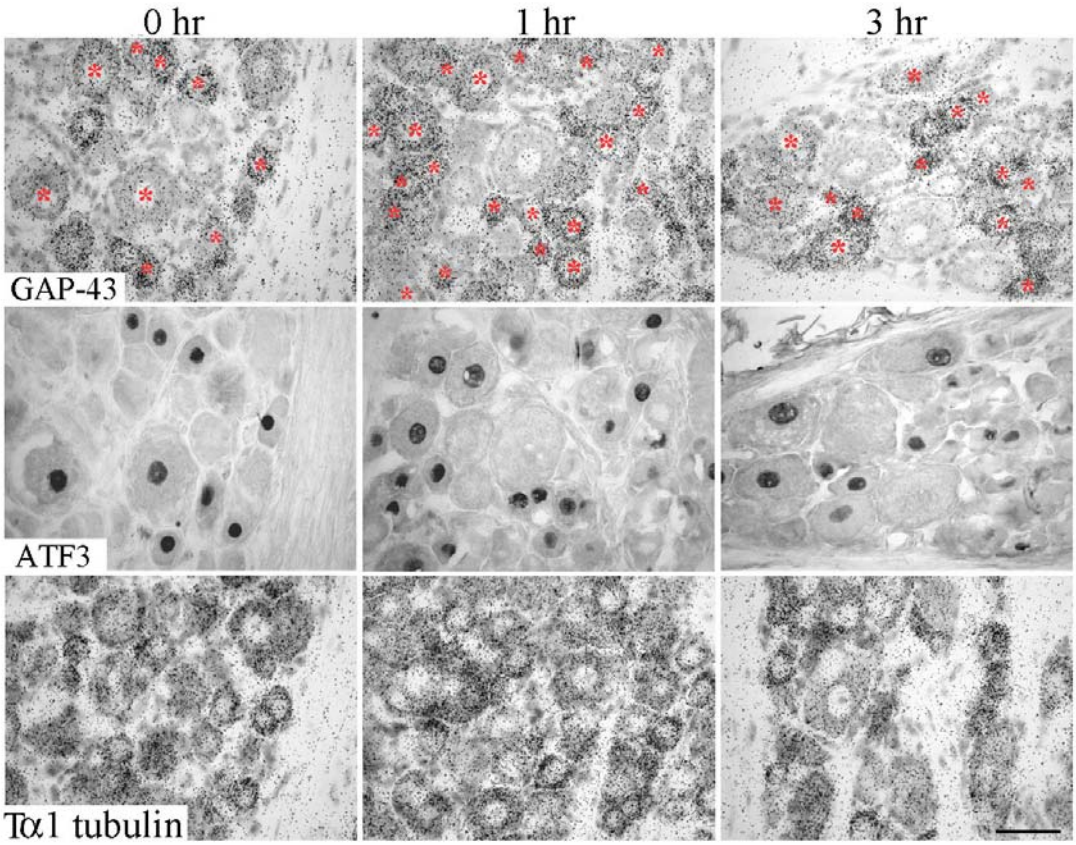

Figure 3-6. Brief electrical stimulation induces an enhanced cell body response to injury in sensory neurons. Brightfield photomicrographs depicting levels of GAP-43 and Tα1 tubulin mRNA in L3 DRG in response to 0 hr, 1hr or 3hr electrical stimulation. ATF3 is used as a marker to identify injured sensory neurons in slides adjacent to those processed by *in situ* hybridization to detect GAP-43 mRNA. The ATF3-positive neurons are indicated by red asterisks in the adjacent GAP-43 section. Scale bar = 50 μm.

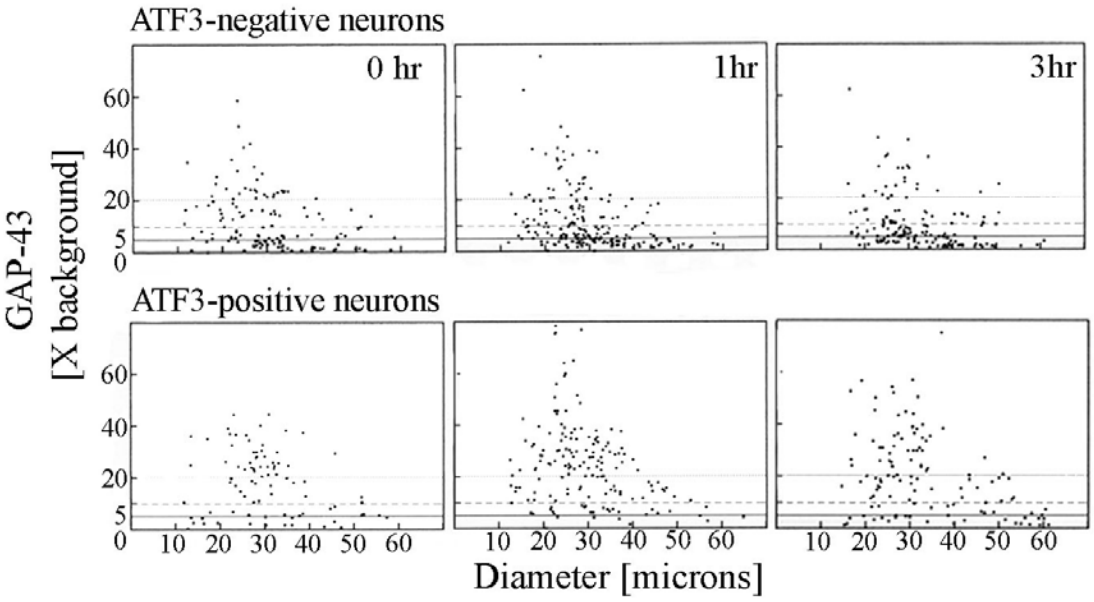

Figure 3-7. Brief electrical stimulation results in increased GAP-43 expression in injured but not in uninjured sensory neurons. Representative scatterplots depicting the level of GAP-43 mRNA hybridization signal over individual neurons of the L3 DRG that are either ATF3-positive (injured) or ATF3-negative (uninjured). Stimulation times are indicated at the top of each graph. Solid lines (5X) divide the plots into labeled and unlabeled populations; dashed lines (10X) separate lightly labeled from moderately labeled; and dotted lines (20X) separate moderately labeled from highly labeled sensory neurons.

In injured ATF3-positive neurons, GAP-43 mRNA hybridization signal was elevated when compared to expression levels in ATF3-negative neurons (Fig. 3-7). This pattern of elevated GAP-43 mRNA expression is normally observed in response to peripheral axotomy in sensory neurons (Verge et al., 1990). Quantification revealed that 1 hour stimulation at the time of repair results in significantly elevated GAP-43 hybridization signal over ATF3-positive neurons when compared to the 0 hour control group. This increased level of relative GAP-43 mRNA expression is further supported by an increased incidence of neurons expressing detectable GAP-43 expression and is most evident in those which express moderate to high levels of hybridization signal (i.e > 10X background). Approximately 81.2% of ATF-3 positive neuronal profiles expressed moderate- high levels of GAP-43 mRNA in response to 1 hour electrical stimulation, with only ~68.6% doing so in the 0 hour group (Fig. 3-6, -7). Stimulation for a longer period, 3 hours, is less effective than 1 hour stimulation in elevating the level of GAP-43 mRNA hybridization signal observed over ATF-3 positive neurons with only ~69.2% of neuronal profiles expressing moderate-high levels of GAP-43 mRNA hybridization signal (>10 X background; Fig. 3-6, -7). There was no difference in the neuronal labeling index of GAP-43 mRNA signal between the 0 hour and 3 hour stimulation in the ATF-3 positive neuronal profiles.

Qualitative analysis revealed that the relative alterations in Tα1 tubulin mRNA expression in response to 1 hour and 3 hour nerve stimulation at the time of repair mirrored those described for GAP-43 mRNA, with the highest levels of expression observed in the 1 hour electrical stimulation group (Fig. 3-6).

Brief electrical stimulation also had a marked influence on the level of BDNF protein expression detected in sensory neurons supplying the femoral nerve. Immunohistochemical analysis revealed that 2 days following 1 hour electrical stimulation at the time of femoral nerve repair, BDNF-like immunoreactivity (LI) was elevated across all size ranges of neurons, but most apparent over small-medium size neurons (Fig. 3-8). BDNF-LI was still slightly elevated in the 1 hour stimulated group 4 days following nerve repair, but did not differ from the 0 hour stimulated group by 21

days post-repair (NMG, data not shown). However, 3 hour electrical stimulation further elevated BDNF-LI when compared to 0 hour or 1 hour stimulation (Fig. 3-8).

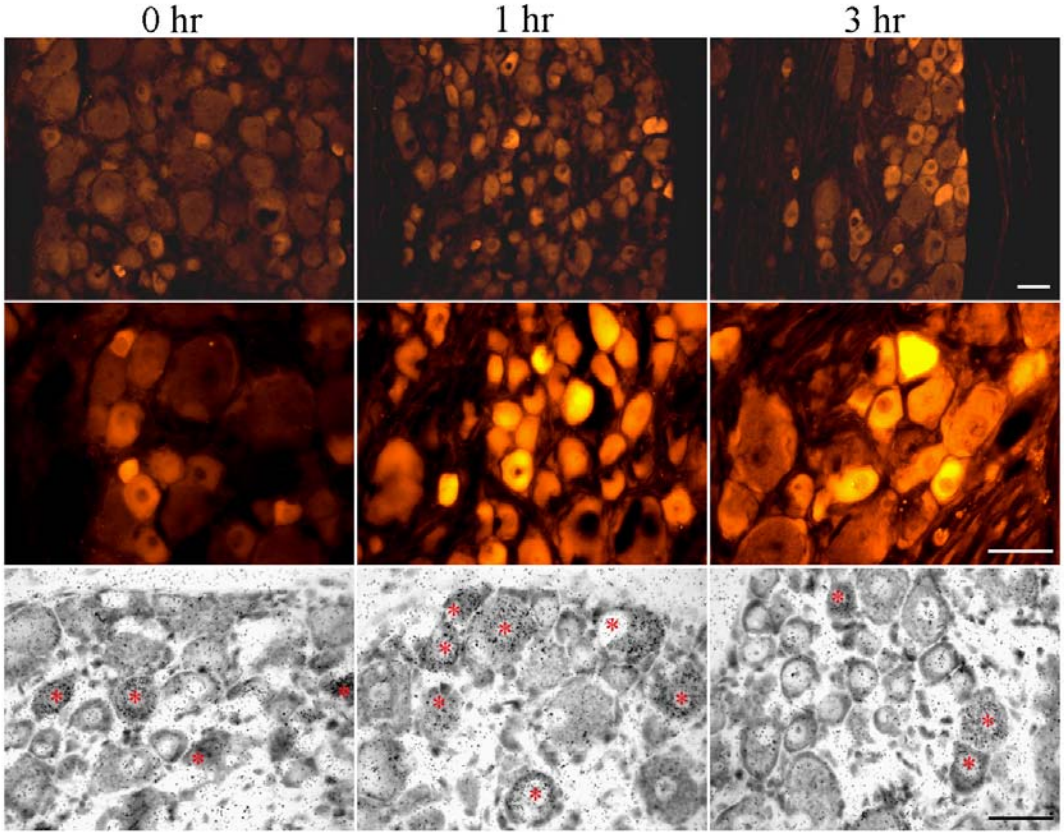

Figure 3-8. Brief electrical stimulation results in elevated BDNF and trkB expression. Fluorescence photomicrographs taken at low (top) or high power (middle) depict BDNF-LI and brightfield photomicrographs (bottom) depict the level of trkB mRNA in response to 0 hr, 1 hr or 3 hr electrical stimulation. Highly-labeled trkB expressing neurons are identified by a red asterisks. Scale bar = 50 μm.

Expression of mRNA encoding the receptor for BDNF, trkB, was also altered by electrical stimulation (Fig. 3-8). TrkB mRNA is normally expressed in medium-large sized sensory neurons and is downregulated following injury in this subpopulation of sensory neurons (Karchewski et al., 1999). After 0 hour stimulation, ~6.2% of neuronal profiles expressed relatively high levels of hybridization signal for trkB mRNA. Brief electrical stimulation resulted in an increased number of neurons expressing detectable trkB mRNA, such that ~9.7% of neuronal profiles were highly labeled for trkB. Stimulation for a longer period, 3 hours, did not augment the numbers of neurons expressing trkB mRNA, with only ~5.7% of neuronal profiles being highly labeled.

3.5 Discussion.

The present study assesses the effect of electrical stimulation on regeneration of sensory neurons following transection and repair of the rat femoral nerve under conditions that promote maximal and appropriate motor neuron regeneration. We present the novel findings that continuous electrical stimulation at 20 Hz for 1 hour significantly increases the number of regenerating sensory fibers, promotes early regeneration across the repair site and induces an enhanced regeneration-associated cell body response in the affected sensory neurons. However, extending the duration of stimulation beyond 1 hour resulted in less effective repair, unlike what has been observed for motor neurons.

3.5.1 Brief electrical stimulation promotes sensory neuron regeneration in the injured peripheral nerve.

Brief electrical stimulation applied immediately after surgical repair of the transected femoral nerve was effective in enhancing sensory axonal regeneration. Though electrical stimulation (alternating current) has been shown to enhance motor

neuron regeneration of the peripheral nerve (Nix and Kopf, 1983; Al-Majed et al, 2000b), we are the first to examine the effect of various durations of electrical stimulation on the intrinsic regenerative response of sensory neurons. Stimulation for 1 hour was optimal for sensory neuron regeneration, while longer periods of stimulation were detrimental. Brief electrical stimulation significantly increased the number of regenerating sensory axons, however there was no change in double-labeled neurons within each treatment group. The fact that the numbers of neurons extending axon collaterals to both branches remains unchanged suggests that a select subpopulation of regenerating sensory neurons is predisposed to do so regardless of treatment (Langford and Coggeshall, 1981; Taylor and Pierau, 1982; Taylor et al, 1983). To resolve this requires further characterization of the subpopulation of double-labeled neurons and is beyond the scope of this study.

The increase in sensory axonal regeneration was correlated with an enhanced cell body response. Peripheral nerve axotomy induces a regenerative state in sensory neurons (Richardson and Issa 1984; Richardson and Verge 1984; Aigner and Caroni, 1993; Aigner and Caroni, 1995; Tetzlaff et al., 1989) and is correlated with a cell body response that includes upregulation in expression of injury/regeneration-associated genes such as GAP-43, Tα1 tubulin and BDNF (Verge et al., 1990; Schreyer and Skene, 1991; Schreyer and Skene, 1993; Mohiuddin and Tomlinson 1997; Michael et al., 1999; Zhou et al., 1999; Karchewski et al., 2002). Brief electrical stimulation at the time of nerve repair induced a more robust response with respect to GAP-43, Tα1 tubulin, trkB and BDNF mRNA expression in the affected sensory neurons and this correlated with an enhanced propensity for axonal regeneration. Stimulation for longer periods (3 hours) decreased the levels of injury/regeneration-associated gene expression and this was correlated with decreased axonal regeneration.

The increase in sensory axonal regeneration is different from the findings of another study examining the effect of 1 hour electrical stimulation on the specificity of sensory neuron regeneration. Brushart et al. (2005) found that 1 hour 20 Hz electrical stimulation enhanced the specificity of target reinnervation to the muscle, but did not significantly increase the overall numbers of regenerating neurons. Brief electrical stimulation appropriately redistributed the regenerating sensory neurons to their original

targets, decreasing the number of regenerating sensory neurons to the muscle and increasing those to the cutaneous branch (Brushart et al., 2005). This is different from our current study where we show significant improvement in the overall numbers of neurons regenerating to either branch. The difference may be related to cell count strategy. To determine specificity, Brushart et al., (2005) counted all neurons contributing to the femoral nerve, including contributions from L2, L3 and some L4 DRG neurons. Our study focused on the effect of stimulation duration on sensory neuron regeneration and impact on injury/regeneration-associated gene response. As a result we only examined L3 DRG neurons. The increased number of sensory neurons regenerating to the muscle, observed in this study, may be reflective of the L3 DRG neuron population. We found that this significant increase in L3 sensory neurons regenerating in response to1 hour electrical stimulation was consistent in three separate experiments and correlated with an upregulation of injury/regeneration-associated genes. Interestingly, brief electrical stimulation may not only enhance sensory axonal regeneration but may also improve specificity.

3.5.2 Benefits of brief electrical stimulation: Reducing staggered regeneration.

Femoral nerve axons regenerating at a rate of 1-3 mm/day (Guttmann et al., 1942) would take 2- 3 weeks to regenerate from the repair site to the target tissue. This is the rate of the fastest growing axons and does not take into account the lag period of days or weeks before axons enter the distal nerve stump (Al-Majed et al., 2000b). Electrical stimulation might enhance regeneration by decreasing the delay before axons begin to elongate and therefore compress the 'staggered' regeneration period. Brushart et al. (2002) found brief stimulation significantly compressed this staggered regeneration period for motor neurons. We find the same stimulation protocol led to a significant increase in the number of sensory axons that regenerate across the repair site. It remains undetermined whether electrical stimulation forces quiescent neurons more rapidly into their regeneration program or whether that program has been modified to facilitate crossing the repair gap (see Brushart et al., 2002).

3.5.3 Potential Mechanism.

The increase in injury/regeneration-associated gene expression in response to brief electrical stimulation suggests that the beneficial effects of stimulation impact the cell body response. Blocking action potentials with TTX abolished this response in both sensory (present study) and motor neurons (Al-Majed et al., 2000b). Blocking membrane depolarization prevents the opening of voltage dependent calcium channels and calcium influx (Ming et al., 2001), thereby impairing upregulation of immediate early genes, intracellular cAMP (Ming et al., Kocsis et al., 1994), elevated BDNF and trkB expression (Shieh et al. 1998; Tao et al., 1998; Kingsbury et al., 2003) and increased neurite outgrowth (Kocsis et al., 1994). In addition, BDNF is rapidly released in response to increased neuronal activity following nerve stimulation (Lever et al., 2001) and trkB receptors are recruited to the cell membrane (Meyer-Franke et al., 1999; Goldberg et al., 2002).

We observed an increase in BDNF-LI and trkB expression in sensory neurons with electrical stimulation. A similar response was observed in motor neurons (Al-Majed et al., 2000a) and was correlated with an elevated expression of Tα1 tubulin and GAP-43 mRNAs (Al-Majed et al., 2004). BDNF likely plays a role in regeneration in sensory neurons, as it is induced in ~80% of sensory neurons immediately following injury (Karchewski et al., 2002). Antagonizing endogenous BDNF during peripheral nerve repair has been linked to impairment of injury/regeneration-associated gene expression (Geremia et al., 2003, 2004), reduction in axonal sprouting (Streppel et al., 2002) and growth and myelination deficits (Zhang et al., 2000; Geremia et al., 2004). Though electrical stimulation elevated BDNF in the sensory neurons, future studies will determine whether it is the underlying mechanism. Another effect of electrical stimulation is an increase in intracellular cAMP, which can overcome MAG-associated inhibition of axonal growth in the distal stump of an injured peripheral nerve (Cai et al., 2001; Neumann et al., 2002; Qui et al. 2002). However, whether cAMP plays a key

role in altering the cell body response of sensory neurons is less clear (Han et al., 2004; Andersen et al., 2000).

Even though brief stimulation significantly improved the regeneration of both sensory and motor axons, there was a discrepancy between these two populations in response to long-term stimulation. Whereas stimulation for 1 hour, 1 day, 7 days or 14 days was equally effective in increasing the numbers of regenerating motor neurons (Al-Majed et al., 2000b), 1 hour stimulation is optimal for sensory neuron regeneration. Longer periods of stimulation were ineffective in significantly enhancing sensory axonal regeneration. The differential response may be related to trkB expression in response to injury and prolonged exposure to BDNF. Motor neurons respond to injury by elevating trkB expression (Al-Majed et al., 2000a). However, peripheral injury results in an overall decrease in trkB expression in sensory neurons and perineuronal cells (Karchewski et al., 1999) and prolonged exposure to BDNF can downregulate trkB expression in sensory neurons (Geremia et al., 2001). Stimulation for 1 hour resulted in an upregulation of BDNF-immunoreactivity in sensory neurons and trkB expression, while stimulation for 3 hours resulted in enhanced BDNF-immunoreactivity but unchanged sensory axon regeneration and injury/regeneration-associated gene expression. Thus, long-term stimulation could potentially chronically expose these cells to elevated BDNF and lead to desensitization of the sensory neuron to this ligand by downregulation of trkB expression, as was observed.

3.5.4 Significance.

When nerve repair is delayed there are disappointing regenerative outcomes (Fu and Gordon, 1995a, b). A more robust regeneration of injured axons across the repair site would counteract the delay in reinnervation of pathways and enhance functional recovery. The ability of brief electrical stimulation of the nerve to significantly enhance the cell body response, the number of sensory neurons that regenerate, regeneration specificity (Brushart et al., 2005) and the growth of motor axons into the appropriate muscle pathway of the transected femoral nerve (Al-Majed et al., 2000b; Brushart et al.,

2002) has important implications for the design of therapeutic strategies to improve peripheral nerve repair following injury.

4.0 ENDOGENOUS BDNF REGULATES INDUCTION OF THE INTRINSIC NEURONAL GROWTH RESPONSE PROGRAM IN INJURED SENSORY NEURONS.

4.1 Abstract.

Peripheral nerve injury induces a robust regenerative response in sensory neurons that includes expression of injury/regeneration-associated genes. BDNF is the sole neurotrophin that is upregulated in sensory neurons after peripheral nerve injury. Here we show that antagonism of endogenous BDNF at the time of injury by immediate 3 day intrathecal infusion of BDNF antibody decreased expression of the injury/regeneration genes, growth-associated protein-43 and Tα1 tubulin *in vivo* and significantly reduced the intrinsic capacity of these neurons to extend neurites *in vitro*. In contrast, delayed infusion of BDNF antibody had no discernable influence on expression of these regeneration-associated genes. Thus, endogenous BDNF is important for the induction of injury/regeneration-associated genes and the intrinsic growth capabilities of injured sensory neurons, but does not appear necessary for maintaining the cell body response.

4.2 Introduction.

Adult sensory neurons mount a robust cell body response to peripheral nerve injury that is critical for the ability of sensory neurons to regenerate (Richardson and Issa 1984; Hoffmann, 1989; Mohiuddin & Tomlinson, 1997). Signals regulating the alterations in gene expression associated with this response include negative signals, such as disruption in normal retrograde supply of neurotrophins from target tissue (Kashiba et al., 1992; Cougnon-Aptel et al., 1999) and positive signals generated at the ganglia and injured nerve tip (Hirota et al., 1996; Zhong et al., 1999; Cafferty et al., 2001; Cafferty et al., 2004). However, identification of the precise molecular factors,

critical to the induction of a robust regenerative state in sensory neurons remains elusive.

A potential instructive signal for the induction of the regenerative state is the neurotrophin brain-derived neurotrophic factor (BDNF), as it is the sole neurotrophin expressed by the majority of injured sensory neurons, suggesting possible autocrine and/or paracrine roles in the cell body response. Further, BDNF has been shown to be a potent modulator of repair programs (i.e. regeneration-associated gene expression) in motor neurons and the central nervous system (Kobayashi et al., 1997; Mamounas et al., 2000; Kwon et al., 2002; Boyd and Gordon 2002). In sensory neurons, BDNF is expressed in small to medium sized (trkA-positive/NGF responsive) intact dorsal root ganglion (DRG) neurons (Ernfors et al., 1990, Wetmore and Olson, 1995; reviewed in Verge et al., 1996). Following peripheral nerve injury, BDNF is initially upregulated in ~80% of injured sensory neurons, and by 1 week post-injury, its expression is downregulated in trkA-positive neurons while remaining elevated in trkB- and trkC-positive neurons (Michael et al., 1999; Zhou et al., 1999, Karchewski et al., 2002). The initial widespread increase in neuronal BDNF expression coincides with an upregulation in expression of global markers of the injury/regenerating state such as growth-associated protein-43 (GAP-43; Bisby, 1988; Verge et al., 1990; Schreyer and Skene, 1993), Tα1 tubulin (Mohiuddin & Tomlinson, 1997) and the upregulation of the injury marker neuropeptide Y (NPY; Wakisaka et al., 1991) that is expressed in medium-large sized (trkB/BDNF responsive) injured sensory neurons. As well, systemic administration of BDNF antibody after peripheral nerve injury dramatically reduces axonal elongation and myelination of regenerating axons, implicating target and Schwann cell–derived BDNF in sensory neuron regeneration (Zhang et al., 2000). However, the effect of neutralizing ganglionically-derived BDNF on the cell body response of injured sensory neurons is not known.

Here we examine the role of endogenous BDNF in regulation of the cell body response and intrinsic growth capabilities of peripherally injured sensory neurons, by immunoneutralizing BDNF either immediately or 1 week after injury. We find that endogenous BDNF is important for induction of the regenerative response in injured DRG neurons, but is not essential for maintenance of the cell body response.

4.3 Materials and Methods.

4.3.1 Experimental design.

Adult male Wistar rats (300-500g) were anesthetized with sodium pentobarbital (Somnitol 100mg/kg; MTC Pharmaceuticals, Cambridge, ON, Canada). The animals were given subcutaneous injections of buprenorphine (Temgesic, 0.01-0.02 mg/kg) to relieve pre- and post-operative discomfort. The right sciatic nerve was resected at its origin from the L4 and L5 spinal nerves, and a 5-mm segment was transected to prevent regeneration. In one set of experiments, immediately after nerve transection sheep anti-BDNF (1.5μg/μl in sterile distilled water; n=6; Chemicon International, Temecula, CA) was infused intrathecally via a mini osmotic pump (model 2001; Alza Pharmaceuticals. Palo Alto, CA) for 3 days. Controls included intrathecal infusion of a control antibody (sheep IgG; n=6; Sigma Aldrich, Oakville, ON, Canada) or injury alone (n=3). In another set of experiments anti-BDNF (1.5 μg/μl; n=5) was infused intrathecally 7 days after sciatic nerve transection for an additional 3 days. Controls included delayed intrathecal infusion of a control antibody (n=5) or injury alone (n=5). In pump treated animals, a laminectomy was performed and the pump catheter was inserted into the subarchnoid space so the end of the catheter lay in the lumbar region of the intrathecal space delivering solution to the L4-L6 spinal nerves.

All animal procedures were conducted in accordance with the National Institute of Health policy on the use of animals in research and the University of Saskatchewan animal care committee (protocol 19920164).

4.3.2 Tissue Fixation and Preparation.

After 3 or 10 days post-injury, the rats were deeply anesthetized (Somnitol, 100 mg/kg of body weight) and perfused through the left ventricle. Cold PBS (pH 7.4) was followed by cold 4% paraformaldehyde and 1.5 % picric acid in 0.1 M PB (pH 7.4).

The L5 DRGs were dissected, postfixed in 4% paraformaldehyde containing 1.5 % picric acid and cryoprotected in 20-30% sucrose in PBS (pH 7.4) overnight. The DRGs were embedded in OCT compound (Tissue Tek, Miles INC, Elkhart, IN) in a cryomold (Tissue Tek), frozen, and stored at –80°C until further processing. Serial DRG sections were cut at 6 μm using a Microm HM500 cryostat (Zeiss, Canada), thaw mounted onto Probe-On+ slides (Fisher Scientific, Canada) and stored with desiccant at -20°C until hybridization. The DRG contralateral to the injured side were classified as 'intact' DRG neurons.

4.3.3 Penetration of BDNF antibody.

The L5 DRG tissue sections were rinsed 3 X 10 min in PBS, and incubated in biotinylated- anti-sheep IgG (1:150; Vector, Burlington, ON, Canada) for 1 hr at room temperature. To visualize the location of sheep IgGs within the tissue a standard ABC procedure was used (Vector, Canada).

4.3.4 *In situ* hybridization.

In situ hybridization was carried out on tissue using 48 base pair oligonucleotide probes (University Core DNA Services, Calgary, AB, Canada) complementary to and selective for the following mRNAs: GAP-43 [complementary to bases 70-117 (Karns et al., 1987)], NPY [complementary to bases 1671-1714 (Larhammar et al., 1987)],Tα1 tubulin [complementary to bases 1548-1594 (Lemischka et al., 1981)] and trkB (full length) [complementary to bases 1361-1408 (Middlemas et al., 1992)]. All probes were checked against the Genbank database (NIH, Bethesda, MD) to ensure that no greater than 75% homology was found to sequences other than the cognate (www.ncbi.nlm.nih.gov/BLAST/). The probes were labeled at the 3'-end with α-[^{35}S]dATP (New England Nuclear, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham Biosciences, Piscataway, NJ) in a buffer containing 10 mM $CoCl_2$, 1mM dithiothreitol (DTT), 300 mM Tris base, and 1.4 M potassium cacodylate, pH 7.2, and purified through Bio-Spin Disposable

Chromatograph Columns (Bio-Rad Laboratories, Hercules, CA) containing 500mg of Nucleic Acid Purification Resin (DuPont NEN, Boston MA). Dithiothreitol was added to a final concentration of 10 mM. The specific activities ranged from 2.0 to 5.0 X 10^6 cpm/ng for each oligonucleotide.

In situ hybridization was carried out according to published procedures (Dagerlind et al., 1992). Briefly, the sections were hybridized at 43°C for 14-18 hours in a buffer containing 50% formamide (Sigma Aldrich, Oakville, ON, Canada), 4 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1 X Denhardt's solution (0.02% bovine serum albumin and 0.02% Ficoll), 1% sarcosyl (N-laurylsarcosine), 0.02 M phosphate buffer, pH 7.0, 10% dextran sulfate, 500 μg/ml heat-denatured sheared salmon sperm DNA, 200 mM dithiothreitol, and 10^7 cpm/ml of probe. After hybridization, the slides were washed 4 X 15 min in 1 X SSC at 55°C, dehydrated in ascending alcohols, processed for radioautography as per Karchewski et al. (2002), and exposed for 1-42 days before developing in D-19 (Kodak, Rochester, NY).

The specificity of hybridization signal for each probe was ascertained by hybridization of labeled probe, labeled probe with a 1000-fold excess of cold probe (signal abolished), or labeled probe with a 1000-fold excess of a dissimilar cold probe of the same length and similar G-C content (signal unchanged).

4.3.5 *In situ* hybridization quantification and analysis.

Quantitative analysis was performed on DRG sections to detect alterations in the density of GAP-43, Tα1 tubulin, and NPY mRNA hybridization signal over individual neurons. All sections to be compared were processed at the same time and under identical conditions. Representative slides (n=2, animals from control and experimental groups) for each probe were selected for quantitative analysis based on the fact that the findings reflected the trends observed following qualitative analysis of all slides and that all DRG sections mounted on the slide were cut at similar levels and had similar number of neurons. Neurons were considered labeled if they had more than 5 X background level of silver grains, as determined by averaging grain densities over defined areas of neuropil devoid of positively labeled cell bodies. This cut-off

corresponds to the level of hybridization signal that must be present in order to confidently declare the neuron labeled when observed under 63X oil immersion.

4.3.6 Neurite Outgrowth Assay.

A unilateral L4, L5 spinal nerve transection was performed on adult male Wistar rats (300-500 g). BDNF antibody (n=7) was intrathecally infused immediately following the injury in experimental animals while controls included infusion of control antibody (n=7) or injury alone (n=7). After 3 days, the rats were euthanized by exposure to 70 % carbon dioxide and the L4 and L5 DRGs were dissected, rinsed in L15 nutrient media (Gibco, Burlington, ON, Canada) and cleaned of connective tissue. The DRGs were dissociated by incubation in 0.1% collagenase (1 hr, 37°C; Gibco) in L15 followed by trituration. Trypsin (Sigma) was added for a final concentration of 0.02% (15 min, 37°C) and the cells were further triturated. The cells were washed in 10% horse serum in DMEM (Sigma). Cells were plated at a density of 12, 500 cells per well in 35-well plates containing DMEM. All cultures plates were precoated with 100 μg/ml poly-L-lysine (Sigma) and10 μg/ml laminin (Sigma).

After 24 hours, the cultures were immunostained for GAP-43 to analyze neurite length. The cultures were washed with PBS, fixed in cold methanol (30 min, -20°C) and dried under a vacuum (30 min). A blocking solution was added consisting of 0.5% BSA and 2% horse serum for 1 hr at room temperature. A monoclonal antibody, mouse-anti-GAP-43 (9-1E12; gift from DJ Schreyer, Univ. of Saskatchewan, Saskatoon, SK, Canada) was added at a dilution of 1:10 000 overnight at 4°C. The next day, the cultures were incubated in biotinylated anti-mouse IgG (DakoCytomation, Mississauga, ON, Canada) at a dilution of 1:200 for 30 min at room temperature. Standard ABC (VECTASTAIN, Vector) procedures were used to identify GAP-43 immunostained neurons.

For each treatment group 40 fields were taken. This resulted in ~100-150 neurons/group being analyzed. Neurite outgrowth analysis was carried out using Neurobin (Northern Eclipse imaging system, Empix Imaging Inc, Mississauga, ON, Canada). The Kruskal-Wallis nonparametric ANOVA (Prism v.2.01 GraphPad

Software Inc., San Diego, CA) was used to compare the mean number of neurite length for each treatment group. Statistical significance was accepted at the $p < 0.05$ level. All experiments were performed in replicates (n=2).

4.4 Results.

To address the role of BDNF in inducing the cell body response, we antagonized endogenous BDNF by intrathecally infusing function blocking sheep anti-BDNF (1.5 μg/μl/hr) via a mini-osmotic pump for 3 days following a unilateral L4-L5 spinal nerve transection in male Wistar rats. Transection at this levels results in axotomy of greater than 95% of L5 DRG neurons (Swett et al., 1991). In addition, by 3 days postaxotomy, BDNF expression is elevated in 80% of the injured sensory neurons (Michael et al., 1999; Zhou et al., 1999; Karchewski et al, 2002). Control experiments included intrathecal infusion of control antibody (Sheep IgG) or injury alone. The BDNF antibody selectively recognizes BDNF and not other neurotrophins (Zhang et al., 2000; Deng et al., 2000). Dot blot analysis confirmed BDNF antibody was selective for BDNF and that it did not recognize other neurotrophins or cytochrome C (a protein of similar charge and size of BDNF; Fig. Appendix-1).

4.4.1 Ability of intrathecally infused IgGs to effectively penetrate DRG tissue.

Immunohistochemistry was used to reveal the ability of the antibody against BDNF (intrathecally infused for 3 days) to penetrate the DRG tissue. DRG sections stained for sheep IgG revealed immunostaining in the parenchyma and surrounding connective tissue in both the intact (contralateral) and injured (ipsilateral) lumbar DRG. Staining intensity was strongest among the injured DRG, this was likely due to the increased permeability of the blood/nerve barrier that occurs in response to nerve lesion (Bouldin et al., 1991; Harvey et al., 1994; Fig. 4-1).

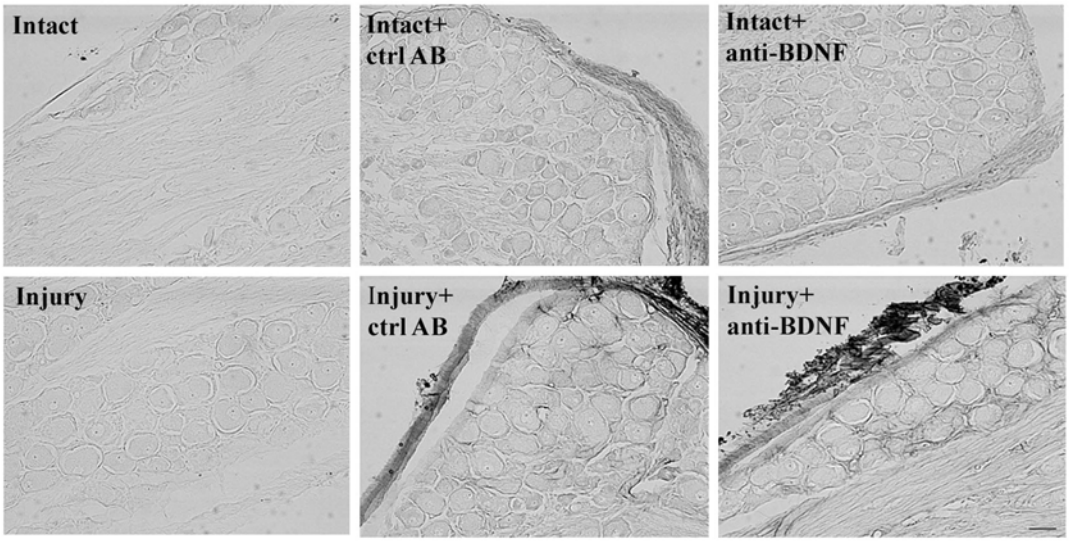

Figure 4-1. Intrathecally delivered anti-BDNF and control IgGs effectively penetrate DRG tissue. Brightfield photomicrographs of L5 DRG (6 μm) sections from L5 DRG having undergone 3 day spinal nerve transection with or without intrathecal infusion of sheep anti- BDNF or control sheep IgGs for the duration of the injury. Scale bar = 40 μm.

4.4.2 Role of endogenous BDNF in regulating injury/regeneration-associated gene expression.

To determine whether endogenous BDNF is involved in the induction of the cell body response in injured sensory neurons, *in situ* hybridization was used to detect the impact of immunoneutralization of BDNF on mRNA expression of the injury/regeneration-associated genes (GAP-43, Tα1 tubulin, and NPY) on L4 and L5 DRG neuron sections. There were no discernable differences in injury/regeneration-associated gene expression between animals treated with intrathecal infusion of control IgG or those with injury alone. Quantification was performed to compare the anti-BDNF and the control antibody groups.

GAP-43 and Tα1 tubulin are both constitutively expressed at low levels in the small-medium size intact DRG neurons. GAP-43 mRNA was expressed at detectable levels in ~51 % (>5X background) of intact DRG neurons, with ~3.2% of sensory neurons expressing moderate to high levels (>20X background). Tα1 tubulin mRNA was expressed at detectable levels in ~82% and at moderate-high levels in ~25% of intact DRG neurons. By 3-days post-injury, both GAP-43 and Tα1 tubulin mRNA expression were upregulated in the majority of injured DRG neurons (Fig. 4-2a & 4-3a). GAP-43 mRNA was expressed in ~94% of injured DRG neurons and ~33% expressed moderate-high levels. As well, Tα1 tubulin mRNA was expressed in ~97% of injured DRG neurons with ~40% expressing moderate-high levels (Fig. 4-2a & Fig. 4-3b). Immunoneutralization of BDNF resulted in a down-regulation of both GAP-43 and Tα1 tubulin mRNA expression in both intact and injured sensory neurons. The effect on intact neurons occurred predominantly in the small-medium sized neurons (Fig. 4-2a and Fig. 4-3a). Anti-BDNF infusion resulted in GAP-43 mRNA detected in ~25% of intact DRG neurons with ~0.45% expressing moderate-high levels. The percentage of neurons expressing detectable Tα1 tubulin mRNA expression was also markedly reduced in response to anti-BDNF infusion with ~39% of intact DRG neurons doing so and ~3.6% at moderate-high levels. The most dramatic influence of intrathecal infusion of anti-BDNF was on GAP-43 and Tα1 tubulin mRNA expression in the injured L4-L5 DRG neurons. Anti-BDNF treatment appeared to influence all sizes of DRG neurons

(Fig. 4-2a & 4-3a), resulting in reduced levels of GAP-43 and Tα1 tubulin mRNA expression, such that expression was detected in ~44% and ~26% of injured DRG neurons, respectively. Of the injured neurons that expressed detectable levels of these injury/regeneration associated genes following anti-BDNF treatment ~6.2% and ~2.1% expressed moderate-high levels of GAP-43 and Tα1 tubulin mRNA, respectively (Fig. 4-2b & 4-3b). It appears that endogenous BDNF regulates the induction of these injury/regeneration-associated genes.

To ascertain whether antagonizing endogenous BDNF simply mediates a global effect on neuronal metabolism such that expression of all genes was downregulated, additional markers of the injury/regenerating state were examined. NPY expression is not detectable in intact sensory neurons, but its expression is up-regulated *de novo* at moderate-high levels in the medium-large sized sensory neurons in response to peripheral injury (Fig. 4-4a; Wakisaka et al., 1991). There was no discernable difference in NPY mRNA expression in injured DRG neurons after intrathecal infusion of anti-BDNF when compared to controls. NPY mRNA was expressed in ~44 % of injured DRG neurons treated with control IgG and ~46% of injured DRG neurons in animals treated with anti-BDNF. Of these neurons, NPY mRNA was expressed at moderate-high levels in ~6.2% of injured control IgG treated DRG neurons and ~5.4% of injured anti-BDNF treated DRG neurons (Fig. 4-4b). Thus, unlike GAP-43 and Tα1 tubulin expression, BDNF does not appear to regulate induction of NPY expression in injured neurons.

4.4.3 Effect of antagonizing endogenous BDNF *in vivo* on the intrinsic growth capacity of sensory neurons.

Injury/regeneration-associated gene expression is associated with the high capacity of neurons to regenerate. The ability of BDNF immunoneutralization to prevent the normal induction of the injury/regeneration-associated genes GAP-43 and Tα1 tubulin following peripheral nerve injury suggests that the neurons may have an impaired ability to extend neurites. We used an *in vitro* neurite outgrowth assay to investigate the impact of *in vivo* manipulations. Enhanced neurite outgrowth *in vitro*

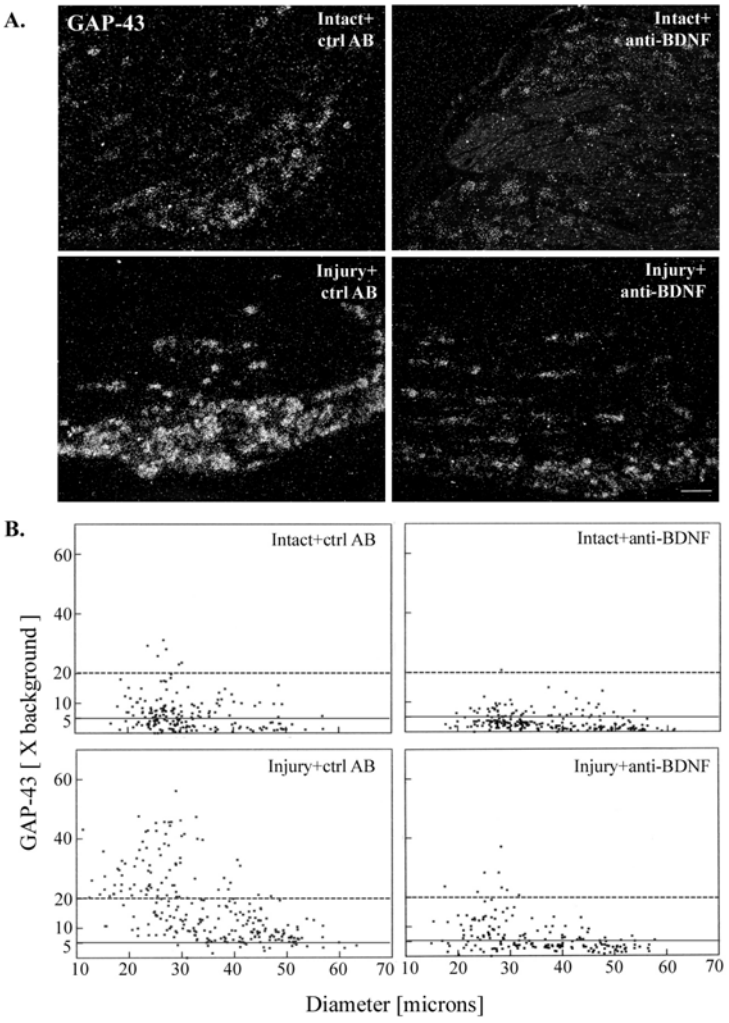

Figure 4-2. Antagonizing endogenous BDNF downregulates GAP-43 mRNA expression in intact and injured DRG neurons. A. Darkfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect relative changes in GAP-43 mRNA expression. Immediately following a unilateral L4, L5 spinal nerve transection, BDNF antibody or control antibody (sheep IgG) was delivered intrathecally via a mini-osmotic pump for 3 days. Scale bar = 100 μm. B. Representative scatterplots depicting relative changes in GAP-43 mRNA hybridization signal over individual neurons as related to cell size. Experimental states are as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations; dashed lines separate moderately labeled from highly labeled.

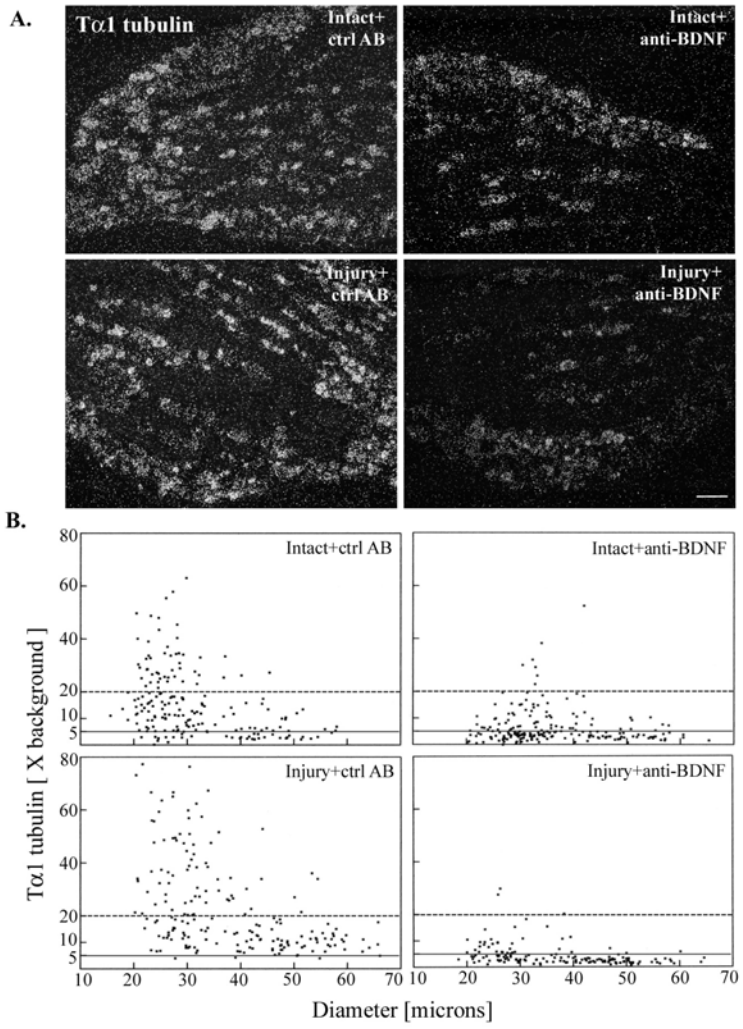

Figure 4-3. Antagonizing endogenous BDNF downregulates Tα1 tubulin mRNA expression in intact and injured DRG neurons. A. Darkfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect relative changes in Tα1 tubulin mRNA expression. Immediately following a unilateral L4, L5 spinal nerve transection, BDNF antibody or control antibody (sheep IgG) was delivered intrathecally via a mini-osmotic pump for 3 days. Scale bar = 100 μm. B. Representative scatterplots depicting relative changes in Tα1 tubulin mRNA hybridization signal over individual neurons as related to cell size. Experimental states are as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations; dashed lines separate moderately labeled from highly labeled.

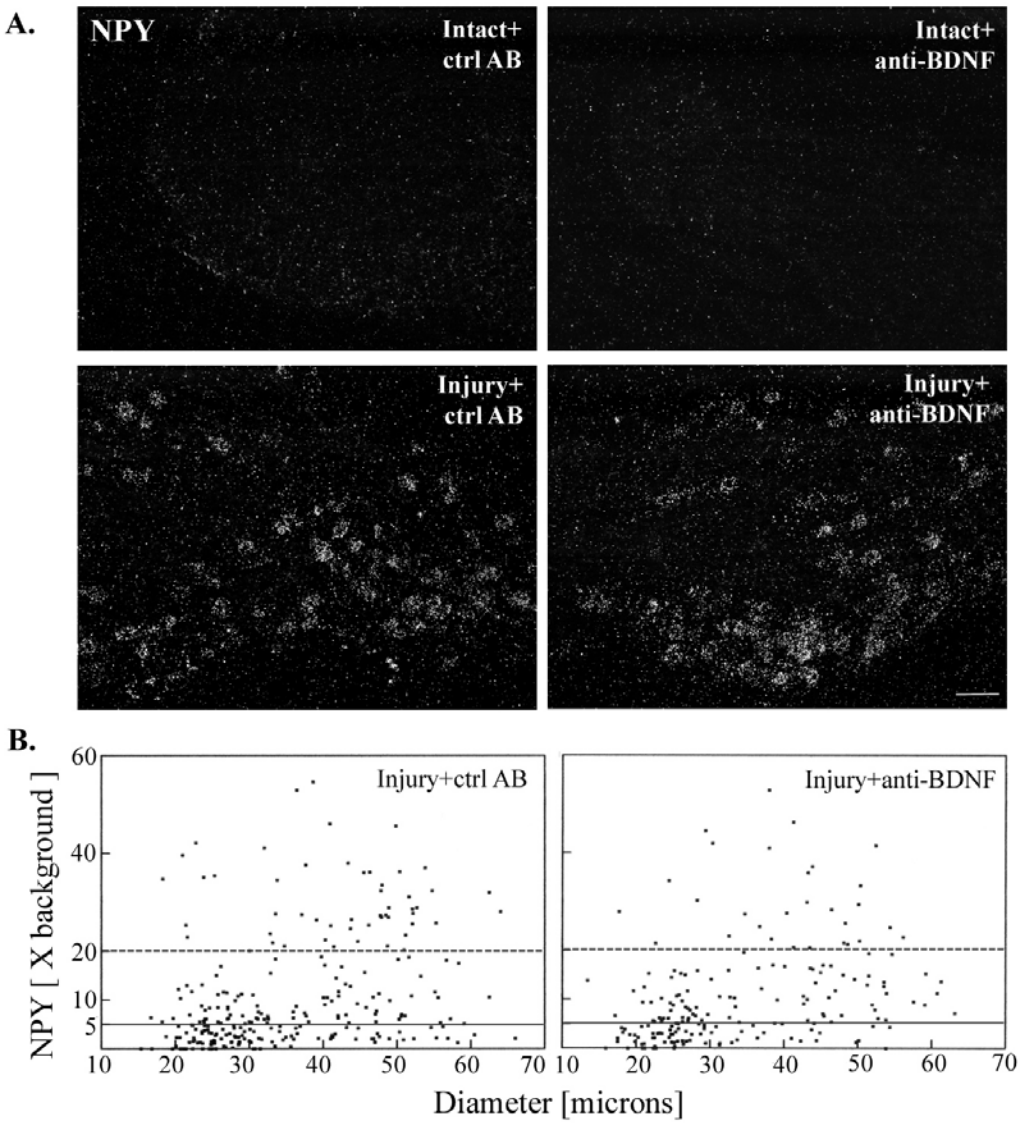

Figure 4-4. Antagonizing endogenous BDNF does not alter NPY mRNA levels in injured DRG neurons. A. Darkfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect relative changes in NPY mRNA expression. Immediately following a unilateral L4, L5 spinal nerve transection, BDNF antibody or control antibody (sheep IgG) was delivered intrathecally via a mini-osmotic pump for 3 days. Scale bar = 100 μm. B. Representative scatterplots depicting relative changes in NPY mRNA hybridization signal over individual neurons as related to cell size. Experimental states are as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations; dashed lines separate moderately labeled from highly labeled.

can be used as an index of growth status induced with *in vivo* manipulations (Smith and Skene, 1997). Following a unilateral L4, L5 spinal nerve transection anti-BDNF was delivered intrathecally for the duration of the 3 day injury. Control antibody (Sheep IgG) infusion or spinal nerve injury alone served as controls. After a 3 day *in vivo* treatment, the L4 and L5 DRG ipsilateral and contralateral to injury were removed and cultured in serum free media for 24 hours. Neurons from intact (contralateral) DRG exhibited limited neurite outgrowth (64 +/- 18 μm SEM). Despite the limited neurite outgrowth, treatment with anti-BDNF *in vivo* reduced the ability of intact sensory neurons to extend neurites (22 +/- 8 μm SEM). Sensory neurons from intact DRG exposed to control IgG had a slightly reduced nonsignificant capacity to extend neurites (47 +/-18 SEM μm; Fig. 4-5). The decrease in neurite outgrowth with anti-BDNF infusion correlated with the decreased GAP-43 and Tα1 tubulin mRNA expression observed in intact sensory neurons.

In contrast to the intact sensory neurons, sensory neurons isolated from the L4, L5 DRG ipsilateral to the lesion demonstrate a robust intrinsic capacity to extend neurites. DRG neurons from the injury alone group displayed extensive neurite outgrowth (907 +/- 85 μm SEM) and did not differ significantly from the injury + control IgG infused (913 +/- μm 92 SEM). Intrathecal infusion of anti-BDNF *in vivo* resulted in reduced neurite outgrowth *in vitro* (415 +/-45 μm SEM) when compared to controls, though there was the occasional neuron that displayed high levels of neurite outgrowth (Fig. 4-5). Thus, endogenous BDNF appears to regulate the ability of injured neurons to grow likely through the induction of injury/regeneration-associated gene expression, such as GAP-43 and Tα1 tubulin.

4.4.4 Role of endogenous BDNF in maintaining the cell body response.

Next we tested whether endogenous BDNF is responsible for maintaining the cell body response. As before, a spinal nerve transection was performed. However instead of immediate infusion, anti-BDNF or control IgG infusion was delayed by 1 week. *In situ* hybridization detected changes in GAP-43 and Tα1 tubulin mRNA expression in L4 or L5 DRG sections following anti-BDNF infusion, control antibody

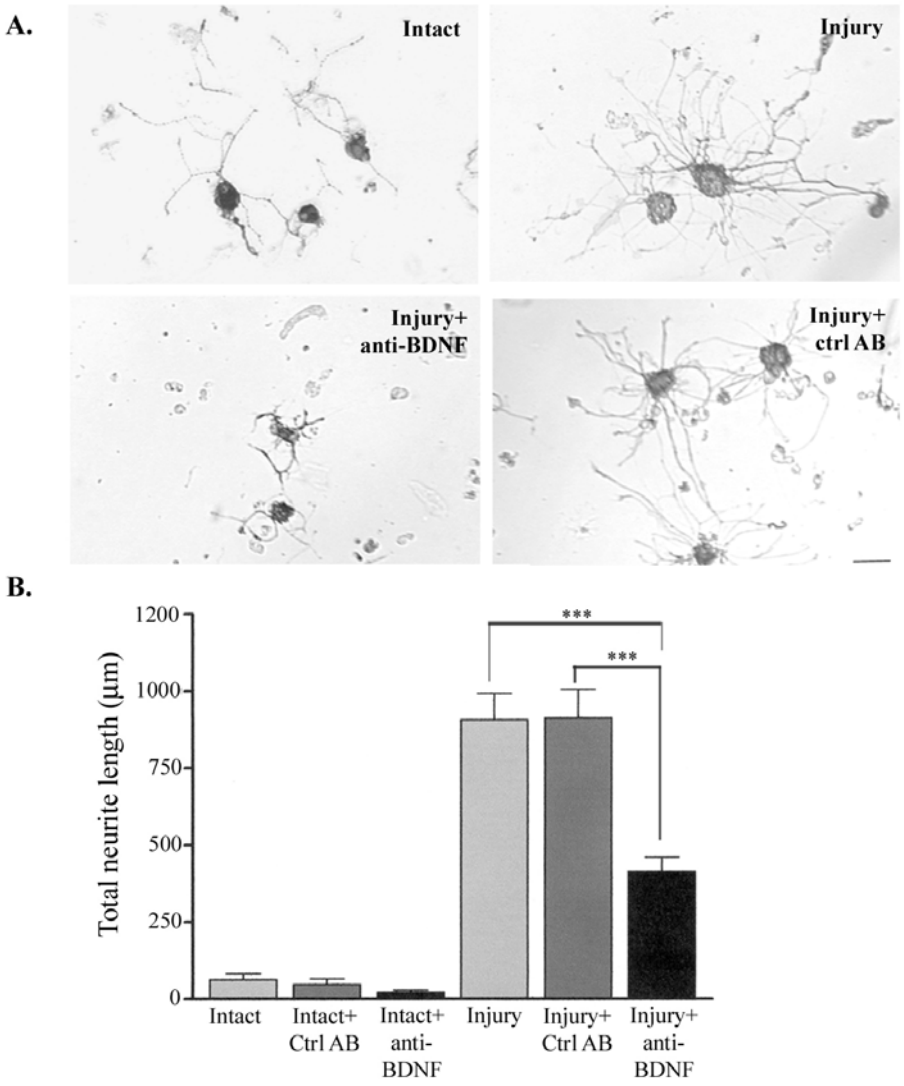

Figure 4-5. Treatment with BDNF antibody *in vivo* significantly reduces neurite outgrowth of DRG neurons *in vitro.* A. Brightfield photomicrographs depicting L4 and L5 DRG neurons cultured under serum-free conditions for 24 hours on poly-L-lysine and laminin coated plates. L4 and L5 DRG were removed from animals infused with anti-BDNF or ctrl AB (sheep IgG) for 3 days following an L4, L5 unilateral spinal nerve transection. Scale bar = 50 µm. B. The mean (+/- SEM) total neurite length of intact and injured DRG neurons following *in vivo* treatments as indicated (***$p<0.001$). Note: There was no significant difference between injury and injury + ctrl AB).

treatment or injury alone (Fig. 4-6, -7). As with immediate infusion, there were no discernable differences in injury/regeneration-associated gene expression between control IgG infusion or injury alone groups. As a result only control IgG and anti-BDNF groups were quantitatively analyzed. Similar to immediate infusion, delayed infusion of anti -BDNF had a modest effect on GAP-43 and Tα1 tubulin mRNA expression in the intact sensory neurons. Delayed infusion of control antibody resulted in ~71% of intact DRG neurons expressing detectable (>5X background) levels of Tα1 tubulin and ~49% of intact DRG neurons expressed GAP-43. Of these neurons only ~7.5% and ~1.1% of DRG neurons expressed moderate-high levels (>20X background) of Tα1 tubulin and GAP-43 mRNA, respectively. Infusion of anti-BDNF resulted in a decrease in Tα1 tubulin expression in intact DRG neurons, with ~66% DRG neurons expressing detectable levels and ~1.3% doing so at moderate-high levels. As well, infusion of anti-BDNF decreased GAP-43 expression in intact DRG neurons, such that only ~37% DRG neurons expressed detectable levels and none expressed moderate-high levels.

At 11 days postaxotomy, GAP-43 and Tα1 tubulin mRNA expression is similar to the pattern found with a 3 day injury, with their expression being elevated in most injured DRG neurons. In injured DRG neurons infused with control IgG, ~89% and ~94% of DRG neurons expressed detectable levels of Tα1 tubulin and GAP-43 mRNA, respectively. Of these neurons, ~22% and~ 44% expressed moderate-high levels of Tα1 tubulin and GAP-43 mRNA, respectively. However unlike immediate infusion of anti-BDNF, delayed infusion of anti-BDNF had no discernible effect on GAP-43 or Tα1 tubulin mRNA expression in injured DRG neurons when compared to controls. Tα1 tubulin expression was detected in ~84% of injured DRG neurons infused with anti-BDNF and ~24% of these neurons expressed moderate-high levels of Tα1 tubulin. After delayed infusion of anti-BDNF, GAP-43 expression was detected in 91% of DRG neurons and ~41% of these neurons expressed moderate-high levels of GAP-43 mRNA (Fig. 4-6, -7). These findings suggest that maintenance of the intrinsic cell body response is independent of endogenous BDNF.

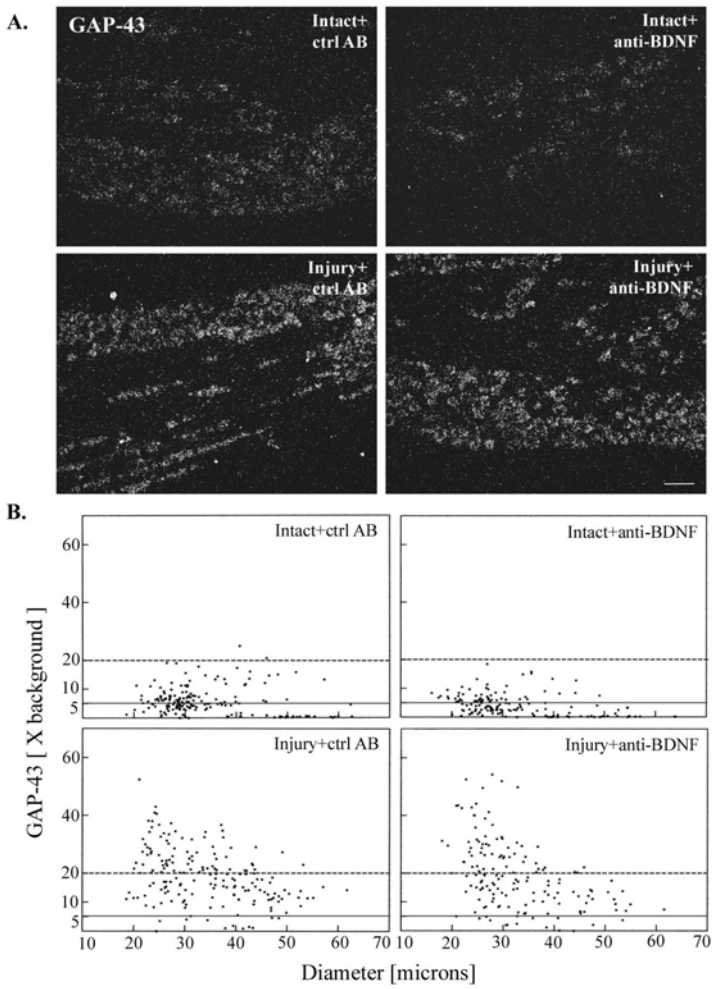

Figure 4-6. Delayed infusion of BDNF antibody has no discernable effect on GAP-43 mRNA expression in injured DRG neurons. A. Darkfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect GAP-43 mRNA expression. Seven days, after an L4, L5 spinal nerve transection, BDNF antibody or control antibody (sheep IgG) was delivered intrathecally via a mini-osmotic pump for 3 days. Scale bar = 100 μm. B. Representative scatterplots depicting relative changes in GAP-43 mRNA hybridization signal over individual neurons as related to cell size. Experimental states are as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations; dashed lines separate moderately labeled from highly labeled.

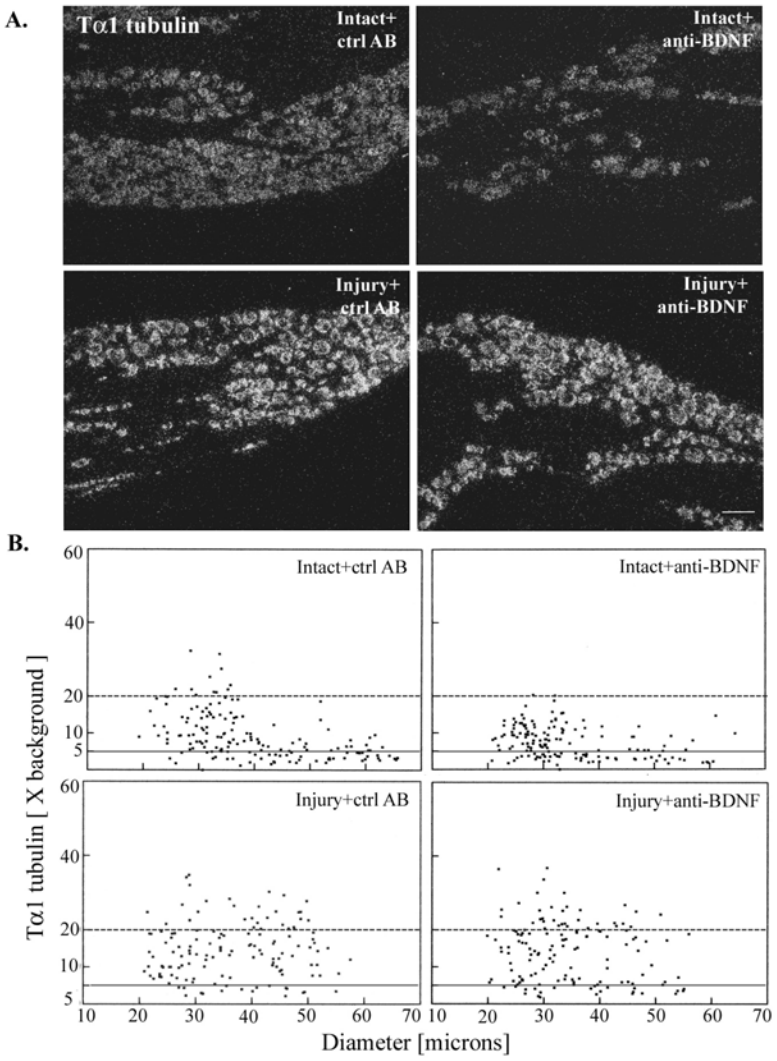

Figure 4-7. Delayed infusion of BDNF antibody has no discernable effect on Tα1 tubulin mRNA expression in injured DRG neurons. A. Darkfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect Tα1 tubulin mRNA expression. Seven days after an L4, L5 spinal nerve transection, BDNF antibody or control antibody (sheep IgG) was delivered intrathecally via a mini-osmotic pump for 3 days. Scale bar = 100 μm. B. Representative scatterplots depicting relative changes in Tα1 tubulin mRNA hybridization signal over individual neurons as related to cell size. Experimental states are as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations; dashed lines separate moderately labeled from highly labeled.

4.5 Discussion.

Our findings implicate ganglion-produced BDNF in the induction of the robust cell body response in DRG neurons observed following peripheral nerve injury. Endogenous BDNF appears to be critically linked to the ability of an injured sensory neuron to induce robust regeneration-associated gene expression and the intrinsic ability of these neurons to extend neurites. However, because the delayed infusion of BDNF antibody did not alter injury/regeneration-associated gene expression, this suggests that endogenous BDNF is not essential for the maintenance of injury/regeneration-associated gene expression. At the time point of the delayed infusion (one week postaxotomy), BDNF expression decreased in the small (trkA-positive) DRG neurons and remained elevated in the medium-large (trkB- and trkC- positive) neurons (Karchewski et al., 2002). Antagonizing endogenous BDNF at this time point did not alter expression of the injury/regeneration-associated genes examined, not even in the trkB subpopulation of sensory neurons. This implicates other factors in the maintenance of injury/regeneration-associated gene expression.

4.5.1 The role of BDNF in the injured/regenerating sensory neuron.

BDNF is similar to other neurotrophins, in that it is produced by target tissues (Funakoshi et al., 1993; Zhao et al., 2004). However, BDNF is unlike the other neurotrophins in that it is also synthesized by the DRG neurons and is the sole neurotrophin that is upregulated following nerve injury. A possible role for the upregulation of BDNF is to promote sensory neuronal survival. Neuronal survival was reduced in isolated sensory neuron cultures when BDNF synthesis was inhibited by antisense oligonucleotides to BDNF (Acheson et al., 1995). However, recent evidence from ganglionic explant cultures suggests BDNF may promote apoptosis in a p75 dependent manner (Zhou et al., 2005). In our *in vivo* model, the short term infusion of anti-BDNF did not appear to induce sensory neuron death, as there were no apparent changes in the number of DRG sections, ganglion size, nor markers of apoptosis (ie. caspase 3 and PARP) among treatment groups. As well, the use of BDNF function

blocking antibodies did not have a global impact on cell metabolism as other markers of the regenerative state such as NPY, a marker of the injured state, was unchanged by the treatment. The lack of influence of endogenous BDNF on NPY expression in our study confirmed previous studies demonstrating a lack of effect by either endogenous or exogenous BDNF on injury-associated NPY expression (Li et al., 1999; Kerekes et al., 2000).

Our findings suggest an additional role for ganglion-derived BDNF. We found that BDNF is important in switching the mode of sensory neurons from homeostatic to a regenerative state in response to nerve lesion. Our work is the first to demonstrate a significant role for ganglion-derived BDNF in the neuronal cell body response to injury and supports a role for BDNF in inducing this response, but not for maintaining this response. At later time points following the inductive phase, alternative sources of BDNF may play a critical role in the regenerative process. For example, Schwann cell-derived BDNF may influence later events in the regeneration process as BDNF is upregulated within the distal sump at 4 days post-injury (Meyer et al., 1992, Zhang et al., 2000; Sheu et al., 2000). Zhang et al. (2000) found that systemically antagonizing BDNF for two weeks reduced myelination and peripheral nerve regeneration. It is unlikely that Schwann cell-derived BDNF is regulating the induction of injury/regeneration-associated gene expression as its expression is only elevated at a time point following the inductive phase. It is also unlikely that BDNF is the sole molecule critical to this inductive phase, as there were a small percentage of injured neurons that expressed moderate-high levels of GAP-43 and Tα1 tubulin mRNA levels and a few neurons that displayed extensive neurite outgrowth *in vitro* in the anti-BDNF treated group. This is not surprising since there is a subset of the small and medium - sized DRG neurons that are not responsive to neurotrophins but respond to glial cell-line derived neurotrophic factor, a member of the transforming growth factor-β superfamily (Silverman et al., 1990; Bennett et al., 2000).

Other trophic molecules that are upregulated in DRG neurons in response to transection are members of the neuropoeitic cytokine family, such as leukocyte inhibitory factor (LIF) and interleukin-6 (IL-6). These cytokines may compensate for the loss of target-derived neurotrophins. LIF is rapidly upregulated in the proximal and

distal stump in response to peripheral nerve injury and transported to the small-medium sized DRG neurons (Banner and Patterson, 1994; Curtis et al., 1994; Thompson et al., 1997). LIF knockout mice failed to increase the intrinsic growth capacity in response to a conditioning lesion and the addition of exogenous LIF enhanced growth of the small-medium sensory neurons *in vitro* of the LIF knockout mice (Cafferty et al., 2001). Whether endogenous BDNF regulates expression of LIF is not known. IL-6 is upregulated in medium-large sized injured DRG neurons in response to injury at 1 day postinjury and maximally increased at 2-4 days (Murphy et al., 1995). IL-6 is able to modulate neuronal growth so that in IL-6 deficient mice, sensory neuron regeneration was impaired and GAP-43 expression failed to up-regulate in axotomized neurons (Zhong et al., 1999; Cafferty et al., 2004). IL-6 may be necessary for the induction of BDNF, as BDNF was attenuated in IL-6 knockout mice and BDNF gene expression is regulated by downstream transcription factor (STAT3) activated by IL-6 (Murphy et al., 2000). Though other molecules may be involved in the regenerative response BDNF may lie downstream or upstream of these molecules and is critically linked to the ability of these neurons to induce regeneration-associated gene expression.

4.5.2 Potential mechanism of action of endogenous BDNF on injured sensory neurons.

Antagonizing endogenous BDNF resulted in a down-regulation of injury/regeneration-associated gene expression in ~80% of sensory neurons. However, only ~30% of intact DRG neurons express the receptor for BDNF, trkB and this expression is reduced following axotomy (Karchewski et al., 1999). Thus, it is unlikely that BDNF is influencing injury/regeneration-associated gene expression by direct signaling through neuronal trkB receptors. Alternatively, BDNF may be acting through the common neurotrophin receptor p75 that is expressed by ~80% of sensory neurons (Karchewski et al., 1999). Even though neuronal expression of this receptor is downregulated following axotomy, expression is still detectable within the time frame critical for the induction of the cell body response (Verge et al., 1992; Sebert and Shooter, 1993; Zhou et al., 1996).

Finally, BDNF may regulate the induction of injury/regeneration-associated gene expression in neurons indirectly by signaling through truncated trkB receptors expressed on satellite cells (Wetmore and Olson, 1995). The two truncated trkB isoforms (trkB. T1 and trkB. T2) express the same extracellular and transmembrane domain as the full-length trkB receptor but lack the cytoplasmic tyrosine -kinase domain (Middlemas, et al., 1991). Despite the lack of the catalytic domain, trkB. T1 and trkB. T2 receptors are capable of mediating signal transduction events that are dependent on ligand binding (Baxter et al., 1997). TrkB. T1 has been found to regulate distal dendritic growth of cortical neurons (Yacoubian et al., 2000). The binding of BDNF to truncated trkB may indirectly regulate injury/regeneration-associated gene expression via a perineuronal -neuronal interaction.

4.5.3 Endogenous BDNF may also regulate 'growth' of intact neurons.

Some DRG neurons have a high capacity for growth. The small-medium sized DRG neurons constitutively express low levels of GAP-43 and Tα1 tubulin and have a high propensity for growth. We found a small percentage of intact sensory neurons were capable of neurite outgrowth *in vitro*; in agreement with previous findings (Smith and Skene, 1997). The growth of these neurites *in vitro* is reminiscent of collateral sprouting. Collateral sprouting is associated with increased GAP-43 expression (Mearow et al., 1994) and is NGF dependent (Diamond et al., 1992b), while regenerative growth is likely the consequence of the loss of target-derived neurotrophins (NGF and NT-3) and is independent of NGF (Diamond et al., 1992a). Here, immediate infusion of the BDNF antibody resulted in a down-regulation of GAP-43 and Tα1 tubulin mRNA expression in the small-medium intact neurons and a corresponding slight decrease in neurite outgrowth. Thus, endogenous BDNF may be involved in collateral sprouting in the intact sensory neurons and the propensity of the intact neurons to grow *in vitro*.

4.5.4 Conclusion.

Endogenous BDNF is critical for induction of the robust regenerative response of sensory neurons following peripheral nerve injury. Altering BDNF expression could be a potential therapeutic treatment to enhance axon regeneration following peripheral nerve injury and may possibly stimulate regeneration of BDNF-responsive neurons of the central nervous system.

5.0 EXOGENOUS BRAIN-DERIVED NEUROTROPHIC FACTOR ENHANCES THE INTRINSIC CELL BODY RESPONSE OF INJURED SENSORY NEURONS.

5.1 Abstract.

We previously found that endogenous BDNF influences the initial upregulation of injury/regeneration-associated genes in the dorsal root ganglia DRG of the sensory neurons after peripheral nerve injury (Geremia et al. 2003, 2004). To determine whether exogenous BDNF can enhance the intrinsic regenerative response of the sensory neurons, intrathecal BDNF was infused at a low dose (200 ng/μl/hr) and a high dose (800 ng/μl/hr) immediately following spinal nerve transection for 3 days. *In situ* hybridization was used to detect mRNA levels of injury/regeneration-associated genes (i.e. GAP-43, Tα1 tubulin, HSP-27 and the BDNF receptor trkB. Infusion of a low dose of BDNF enhanced injury/regeneration-associated gene expression most evident in small-medium size injured DRG neurons but had no apparent influence on trkB mRNA expression. Infusion of a high dose of BDNF further enhanced GAP-43, Tα1 tubulin and trkB mRNA expression. Acute 3 day BDNF infusion had no discernable effect on intact neurons. Even when a high dose of BDNF was infused intrathecally for 7 days in naïve animals, there was no apparent difference in GAP-43 and HSP-27 mRNA expression when compared to controls. However, a 7 day chronic exposure to a high dose of BDNF downregulated trkB mRNA levels. Thus, exogenous BDNF is capable of enhancing injury/regeneration associated gene expression in injured sensory neurons but is not sufficient to induce an injury phenotype in intact sensory neurons.

5.2 Introduction.

Injured axons in the adult peripheral nervous system (PNS) undergo a robust regenerative response unlike those of the central nervous system (CNS). One reason for successful regeneration is the intrinsic response to axonal injury (reviewed in Plunet et al., 2002). Following peripheral nerve injury, the neurons undergo a characteristic

change in gene expression. In the dorsal root ganglia (DRG), axotomy results in an increase in growth-associated protein-43 (GAP-43; Bisby, 1988; Verge et al., 1990; Schreyer and Skene, 1993), Tα1 tubulin (Mohiuddin and Tomlinson, 1997) and heat shock protein-27 (HSP-27; Costigan et al., 1998). These genes are referred to as injury/regeneration-associated genes and their expression is correlated with axonal growth (Hoffman, 1989; Mohiuddin and Tomlinson, 1997).

Despite the robust regenerative response of injured peripheral neurons, functional recovery often fails to occur (reviewed in Sunderland, 1978; reviewed in Kline and Hudson, 1995). Often regenerating axons need to grow long distances and their growth rate is slow so by the time they enter the distal nerve stump the growth potential of the axotomized neurons has deteriorated (Fu and Gordon, 1995a, b; Whitworth et al., 1996). Strategies aimed at enhancing neuronal growth states might therefore be directed at enhancing regeneration-associated gene expression. Enhancing the intrinsic regenerative response can promote CNS axonal regeneration (Richardson and Verge, 1984; 1987; Kobayashi et al., 1997; Neumann and Woolf, 1999; Bomze et al., 2002; reviewed in Plunet et al., 2002) and peripheral nerve regeneration (McQuarrie and Grafstein, 1973; 1977; Al-Majed et al., 2004; Geremia et al., 2005).

We previously found that endogenous brain-derived neurotrophic factor (BDNF) has a critical role in the initial upregulation of regeneration-associated genes in DRG neurons after injury (Geremia et al., 2003; Geremia et al., 2004). As well, neutralizing antibodies to BDNF reduced myelination and peripheral nerve regeneration (Zhang et al., 2000). BDNF, a member of the neurotrophin family, is expressed in small to medium sized (trkA-positive) intact DRG neurons (Ernfors et al., 1990b, Wetmore and Olson, 1995; reviewed in Verge et al., 1996). After peripheral nerve injury, BDNF is initially upregulated in ~80% of injured sensory neurons but by 1 week post-injury its expression is downregulated in trkA-positive neurons while remaining elevated in trkB- and trkC- positive neurons (Michael et al., 1999; Zhou et al., 1999, Karchewski et al., 2002). BDNF exerts its effect through two types of receptors, the p75 receptor which binds all members of neurotrophin family (Johnson et al., 1986; Radeke et al., 1987; Ernfors et al., 1990a; Rodriguez-Tebar et al., 1990) and via the tropomyosin related

kinase B (trkB) which binds BDNF with high affinity (Klein et al., 1989; Middelmas et al., 1991; reviewed in Barbacid, 1994).

Since BDNF is important in inducing regeneration-associated gene expression in injured DRG neurons (Geremia et al., 2003; Geremia et al., 2004), treatment of axotomized peripheral neurons with exogenous BDNF may enhance regeneration. In this study we examined the effect of intrathecal infusion of low and high dose of BDNF in intact and injured DRG neurons on injury/regeneration-associated genes and trkB mRNA expression. We found that exogenous BDNF was able to enhance the expression of injury/regeneration-associated genes following spinal nerve transection in a dose dependent manner, while having no apparent effect on the expression of these genes in intact neurons.

5.3 Materials and Methods.

5.3.1 Experimental Design

Adult male Wistar rats (300-500 g) were anesthetized with sodium pentobarbital (Somnitol 100mg/kg; MTC Pharmaceuticals, Cambridge, ON, Canada) and given subcutaneous injections of buprenorphine (Temgesic, 0.01-0.02 mg/kg) to relieve pre- and post-operative discomfort. In one experimental group, the right sciatic nerve was resected at its origin from the L4 and L5 spinal nerves, and a 5-mm segment was retransected to prevent regeneration. Immediately after nerve transection a low dose of BDNF (200 ng/µl/hr; n=4) or a high dose of BDNF (800 ng/µl/hr; n=6) was infused intrathecally via a mini osmotic pump (model 2001; Alza Pharmaceuticals. Palo Alto, CA) for 3 days. BDNF was generously supplied by Regeneron Pharmaceuticals, Tarrytown, NY. Controls included intrathecal infusion of a vehicle containing 1mg/ml rat serum albumen (Sigma Chemicals, USA) and 100 U/ml penicillin/streptomycin (Gibco BRL, Grand Island, NY) in PBS (n=3) or injury alone (n=7). In another set of experiments in intact (naïve) animals, a high dose of BDNF (800 ng/µl/hr; n=6) was infused intrathecally via a mini osmotic pump for 7 days. Controls included intrathecal infusion of a vehicle (n=3) or naïve animals (n=6). In pump treated animals, a

laminectomy was performed at the lumbar/sacral junction and the pump catheter was inserted into the subarachnoid space so the end of the catheter lay in the lumbar region of the intrathecal space delivering solution to the L4-L6 spinal nerves.

All animal procedures were conducted in accordance with the National Institute of Health policy on the use of animals in research and the University of Saskatchewan animal care committee (protocol 19920164).

5.3.2 Tissue Fixation and Preparation.

After 3 or 7 days, the rats were deeply anesthetized (Somnitol, 100 mg/kg of body weight) and perfused through the left ventricle. Cold PBS (pH 7.4) was followed by cold 4% paraformaldehyde and 1.5 % picric acid in 0.1 M PB (pH 7.4). The L5 DRGs were dissected and postfixed in 4% paraformaldehyde and 1.5 % picric acid (1-1.5 hours) and cryoprotected in 20-30% sucrose in PBS (pH 7.4) overnight. The L5 DRG contralateral to the injury was referred to as the intact DRG. The DRG were embedded in OCT compound (Tissue Tek, Miles INC, Elkhart, IN) in a cryomold (Tissue Tek), frozen, and stored at –80°C until further processing. Serial DRG sections were cut at 6 μm using a Microm HM500 cryostat (Zeiss, Canada), thaw mounted onto Probe-On+ slides (Fisher Scientific, Canada) and stored with desiccant at -20°C until hybridization.

5.3.3 In situ hybridization.

In situ hybridization was carried out on tissue using 48 base pair oligonucleotide probes (University Core DNA Services, Calgary, AB, Canada) complementary to and selective for the following mRNAs: GAP-43 [complementary to bases 70-117 (Karns et al., 1987)], HSP-27 [complementary to bases 408-455 (Uoshima et al., 1993)], Tα1 tubulin [complementary to bases 1548-1594 (Lemischka et al., 1981)] and trkB (full length) [complementary to bases 1361-1408 (Middlemas et al., 1992)]. All probes were checked against the GenBank database (NIH, Bethesda, MD) to ensure that no greater than 75% homology was found to sequences other than the cognate

(www.ncbi.nlm.nih.gov/BLAST/). The probes were labeled at the 3'-end with α-[^{35}S]dATP (New England Nuclear, Boston, MA) using terminal deoxyucleotidyl-transferase (Amersham Biosciences, Piscataway, NJ) in a buffer containing 10 mM $CoCl_2$, 1mM dithiothreitol (DTT), 300 mM Tris base, and 1.4 M potassium cacodylate, pH 7.2, and purified through Bio-Spin Disposable Chromatograph Columns (Bio-Rad Laboratories, Hercules, CA) containing 500mg of NENSORB PREP Nucleic Acid Purification Resin (DuPont NEN, Boston MA). Dithiothreitol was added to a final concentration of 10 nM. The specific activities ranged from 2.0 to 5.0 X 10^6 cpm/ng for each oligonucleotide.

In situ hybridization was carried out according to published procedures (Dagerlind et al., 1992). Briefly, the sections were hybridized at 43°C for 14-18 hours in a buffer containing 50% formamide (Sigma Aldrich, Oakville, ONT, Canada), 4 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1 X Denhardt's solution (0.02% bovine serum albumin and 0.02% Ficoll), 1% sarcosyl (N-laurylsarcosine), 0.02 M phosphate buffer, pH 7.0, 10% dextran sulfate, 500 μg/ml heat-denatured sheared salmon sperm DNA, 200 mM dithiothreitol, and 10^7 cpm/ml of probe. After hybridization, the slides were washed 4 X 15 min in 1 X SSC at 55°C, dehydrated in ascending alcohols, processed for radioautography according to Karchewski et al. (2002), and exposed for 1-42 days before developing in D-19 (Kodak, Rochester, NY).

The specificity of hybridization signal for each probe was ascertained by hybridization of labeled probe, labeled probe with a 1000-fold excess of cold probe (signal abolished), or labeled probe with a 1000-fold excess of a dissimilar cold probe of the same length and similar G-C content (signal unchanged).

5.3.4 In situ hybridization quantification and analysis.

Quantitative analysis was performed on DRG sections to detect the density of GAP-43, Tα1 tubulin, trkB, and HSP-27 mRNA hybridization signal. All sections to be compared were processed at the same time and under identical conditions. Representative slides (n=2) for each probe were selected for quantitative analysis based on the fact that the findings reflected the trends observed following qualitative analysis

of all slides and that all DRG sections mounted on the slide were cut at similar levels and had similar number of neurons. Neurons were considered labeled if they had more than 5 X background level of silver grains, as determined by averaging grain densities over defined areas of neuropil devoid of positively labeled cell bodies. This cut-off corresponds to the level of hybridization signal that must be present in order to confidently declare the neuron labeled when observed under 63X oil immersion.

5.4 Results.

5.4.1 Exogenous BDNF upregulates GAP-43 and Tα1 tubulin mRNA in a dose dependent manner.

A low (200 ng/μl/hr) or high (800 ng/μl/hr/) dose of BDNF was delivered intrathecally immediately following an L4, L5 spinal nerve transection for 3 days. At this time point endogenous BDNF is upregulated in ~80% of DRG neurons (Karchewski et al, 2002) and appears to play a role in the induction of injury/regeneration associated genes (Geremia et al, 2003; Geremia et al., 2004). Low and high doses of BDNF were chosen because in other systems exposure to high dose BDNF downregulates trkB expression and the functional response of this neurotrophin (Carter et al, 1995; Frank et al, 1996; Knusel et al, 1997; Sommerfeld et al, 2000). Control experiments included infusion of a vehicle or injury alone. There was no qualitative difference between vehicle infused and injury alone groups with regards to the expression of the phenotypic markers examined in this study (data not shown). To detect the effect of exogenous BDNF on GAP-43 and Tα1 tubulin mRNA expression, *in situ* hybridization was performed on L5 DRG sections.

In intact (contralateral) DRG neurons, GAP-43 and Tα1 tubulin mRNA is constitutively expressed in small-medium sized neurons, as well as some medium-large sized neurons. Infusion of either a low dose or a high dose of BDNF had no apparent influence on GAP-43 and Tα1 tubulin mRNA expression in intact DRG neurons when compared to control groups (Fig. 5-1, -2). In injured sensory neurons, GAP-43 and Tα1 tubulin mRNA expression is upregulated across all size ranges of DRG neurons.

Infusion of low dose BDNF upregulated both GAP-43 and Tα1 tubulin mRNA mostly in the small-medium sized neurons. Infusion of high dose BDNF further elevated GAP-43 and Tα1 tubulin mRNA expression in all size ranges of neurons, including the medium-large sized neurons when compared to injury alone (Fig. 5-1a, -2a).

Quantitative analysis confirmed the qualitative observations. After injury, ~82% of small-medium sized neurons (<35 μm) and ~2.5% of medium-large size neurons (>35 μm) express moderate- high levels of GAP-43 expression (>20X background). Infusion of low dose BDNF increased GAP-43 mRNA expression in the injured neurons such that ~90% of small-medium sized neurons and ~21% of medium-large sized neurons expressed GAP-43 mRNA. Infusion of high dose BDNF further elevated GAP-43 mRNA expression in all injured neurons, with ~98% of small-medium sized neurons and ~46% of medium-large neurons expressing GAP-43 at moderate-high levels (Fig. 5-1b). A similar response was found for Tα1 tubulin mRNA expression. After injury, ~58% of small-medium sized neurons and 38% of medium-large sized neurons were found to express moderate-high levels of Tα1 tubulin mRNA. Infusion of low dose BDNF increased Tα1 tubulin mRNA levels when compared to control, with ~86% of small-medium sized neurons and ~58% of medium-large sized neurons expressing moderate-high levels Tα1 tubulin mRNA. Infusion of high dose BDNF elevated Tα1 tubulin mRNA levels when compared to infusion of low dose BDNF. Infusion of high dose BDNF resulted in ~98% small-medium sized neurons and ~96% of medium-large sized neurons expressing moderate-high levels of hybridization signal for Tα1 tubulin mRNA (Fig. 5-2b).

5.4.2 High dose of BDNF elevates trkB mRNA expression in injured neurons.

Next, we determined whether there was a corresponding influence of exogenous BDNF on trkB mRNA expression. BDNF binds with high affinity to the trkB receptor. In intact DRG neurons, trkB mRNA hybridization signal was detected in ~25% of DRG

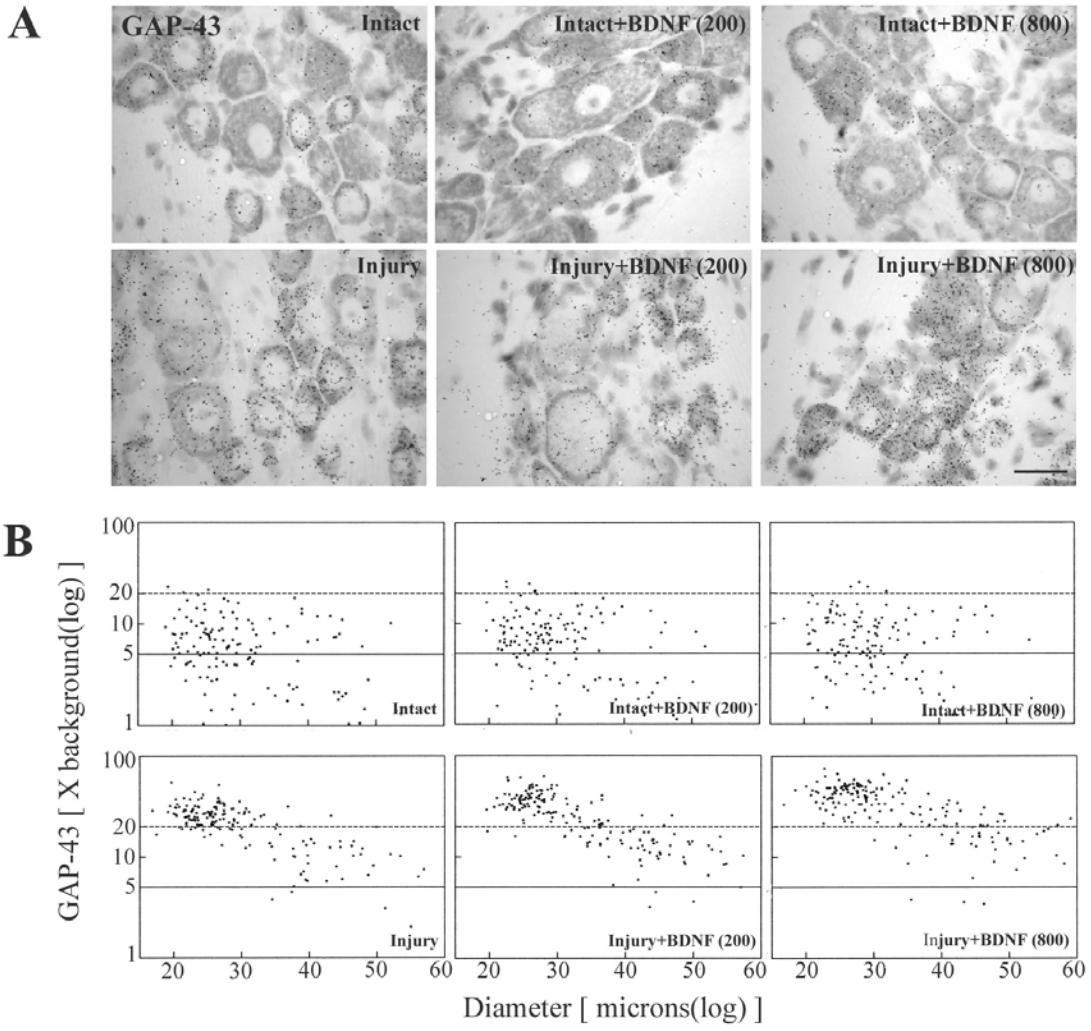

Figure 5-1. Exogenous BDNF influences GAP-43 expression in injured DRG neurons. A. Brightfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect GAP-43 mRNA expression. Immediately following an L4, L5 spinal nerve transection, exogenous BDNF was delivered in a dose dependent manner (low dose-200 ng/μl/hr; high dose-800 ng/μl/hr) intrathecally via a mini-osmotic pump for 3 days. Scale bar = 30 μm. B. Representative scatterplots depicting relative changes in GAP-43 mRNA hybridization signal over individual neurons as related to cell size. Experimental states as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations; dashed lines separate moderately labeled from highly labeled.

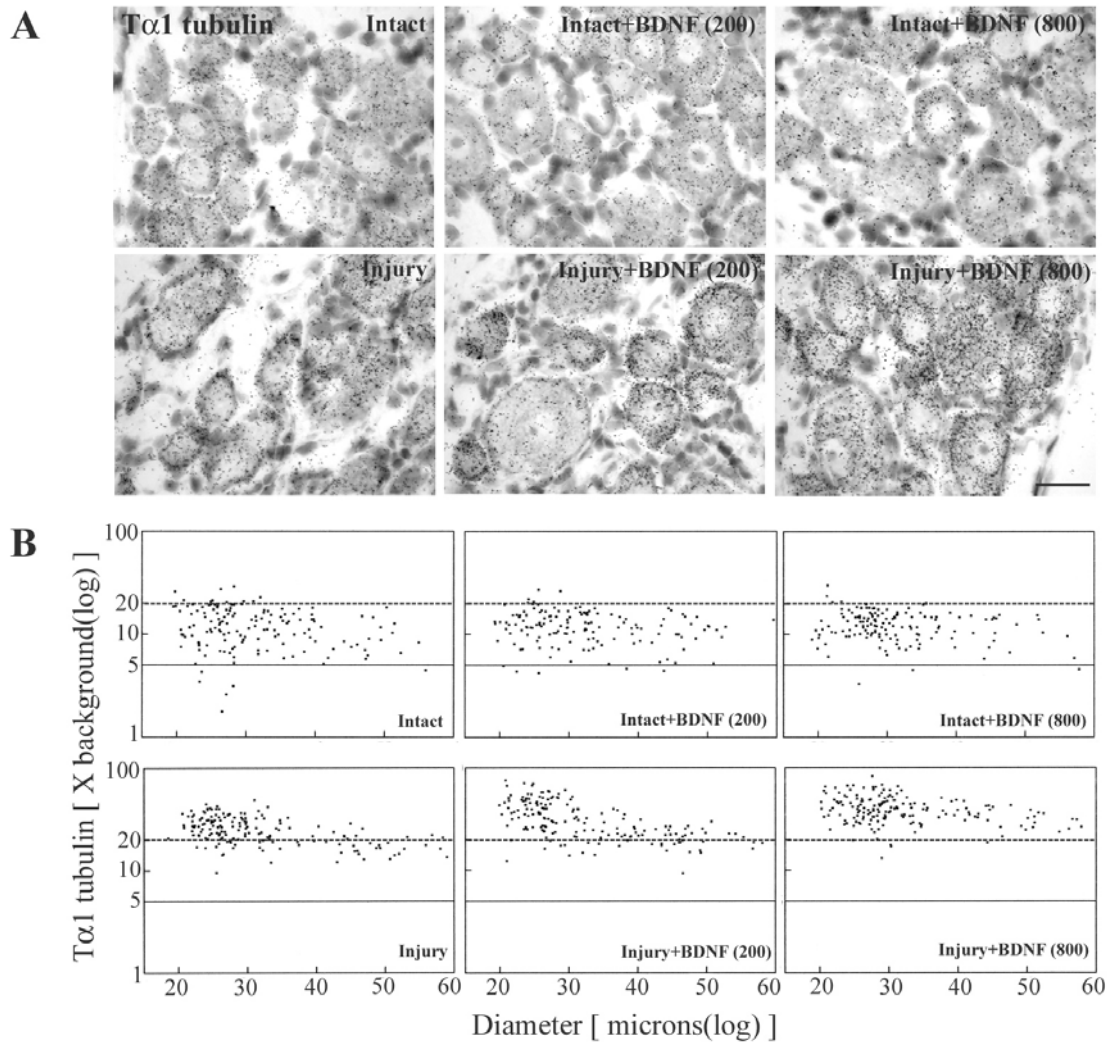

Figure 5-2. Exogenous BDNF influences Tα1 tubulin expression in injured DRG neurons. A. Brightfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect Tα1 tubulin mRNA expression. Immediately following an L4, L5 spinal nerve transection, exogenous BDNF was delivered in a dose dependent manner (low dose-200 ng/μl/hr; high dose-800 ng/μl/hr) intrathecally via a mini-osmotic pump for 3 days. Scale bar = 30 μm. B. Representative scatterplots depicting relative changes in Tα1 tubulin mRNA hybridization signal over individual neurons as related to cell size. Experimental states as indicated. Solid lines (5X) divide the plots

into labeled and unlabeled populations; dashed lines separate moderately labeled from highly labeled.

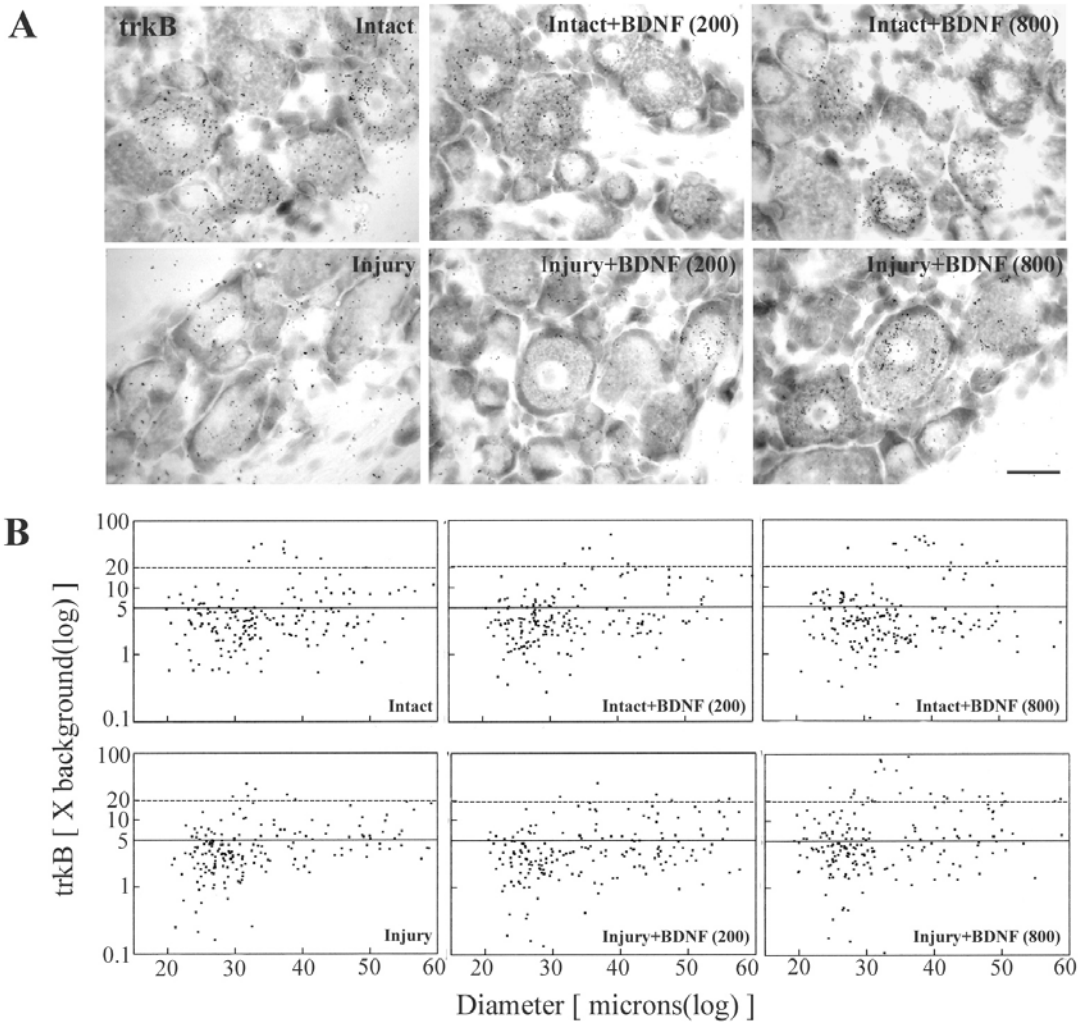

Figure 5-3. Exogenous BDNF influences trkB expression in injured DRG neurons. A. Brightfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect trkB mRNA expression. Immediately following an L4, L5 spinal nerve transection, exogenous BDNF was delivered in a dose dependent manner (low dose-200 ng/μl/hr; high dose-800 ng/μl/hr) intrathecally via a mini-osmotic pump for 3 days. Scale bar = 30 μm. B. Representative scatterplots depicting relative changes in trkB mRNA hybridization signal over individual neurons as related to cell size. Experimental

states as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations; dashed lines separate moderately labeled from highly labeled. neurons, as previously shown in other studies (McMahon et al., 1994; Wetmore and Olson, 1995; Karchewski et al., 1999). Infusion of low dose or high dose BDNF for 3 days following a spinal nerve transection did not alter the levels of trkB mRNA expression in intact sensory neurons with ~26% and ~25% of neurons expressing detectable levels of trkB after infusion of low or high dose of BDNF, respectively (Fig. 5-3b). Following injury, neuronal trkB mRNA expression was downregulated (Karchewski et al., 1999; 2002). While ~30% of injured neurons still express detectable levels of trkB after a 3 day injury, there was a decrease of the number of DRG neurons that express moderate to high levels of trkB mRNA expression with only ~1.5% of DRG neurons expressing these levels (Fig. 5-3). Infusion of low dose BDNF did not alter trkB mRNA expression when compared to injury alone; ~32 % of injured sensory neurons expressed trk B mRNA (>5X background) and ~2.6 % expressed trkB mRNA at moderate-high levels (Fig. 5-3). However, infusion of high dose BDNF resulted in a notable increase in the number of neurons expressing detectable trkB mRNA with ~43% of injured neurons expressing detectable levels of trkB mRNA and ~11% of DRG neurons express moderate-high levels of hybridization signal for trkB mRNA (Fig. 5-3).

5.4.3 Chronic exposure of intact DRG neurons to BDNF does not alter expression of injury markers.

The ability of high dose of BDNF to enhance GAP-43 and Tα1 tubulin in injured sensory neurons suggests it may induce an injury phenotype in intact sensory neurons. Since a high dose of exogenous BDNF did not induce any changes in intact sensory neurons after a 3 day intrathecal infusion, a chronic (7 day) high dose of BDNF was intrathecally infused in intact (naïve) animals. *In situ* hybridization was used to detect levels of GAP-43 mRNA and HSP-27 mRNA in DRG neurons. Like GAP-43, HSP-27 is constitutively expressed in small-medium sized DRG neurons. Infusion of exogenous BDNF for 7 days had no apparent influence on the expression of these

markers in DRG neurons (Fig. 5-4, -5). In intact sensory neurons ~61% expressed detectable levels (>5X background) of GAP-43 mRNA and ~79% expressed HSP-27 mRNA. Infusion of high dose BDNF in naïve animals did not alter markedly either

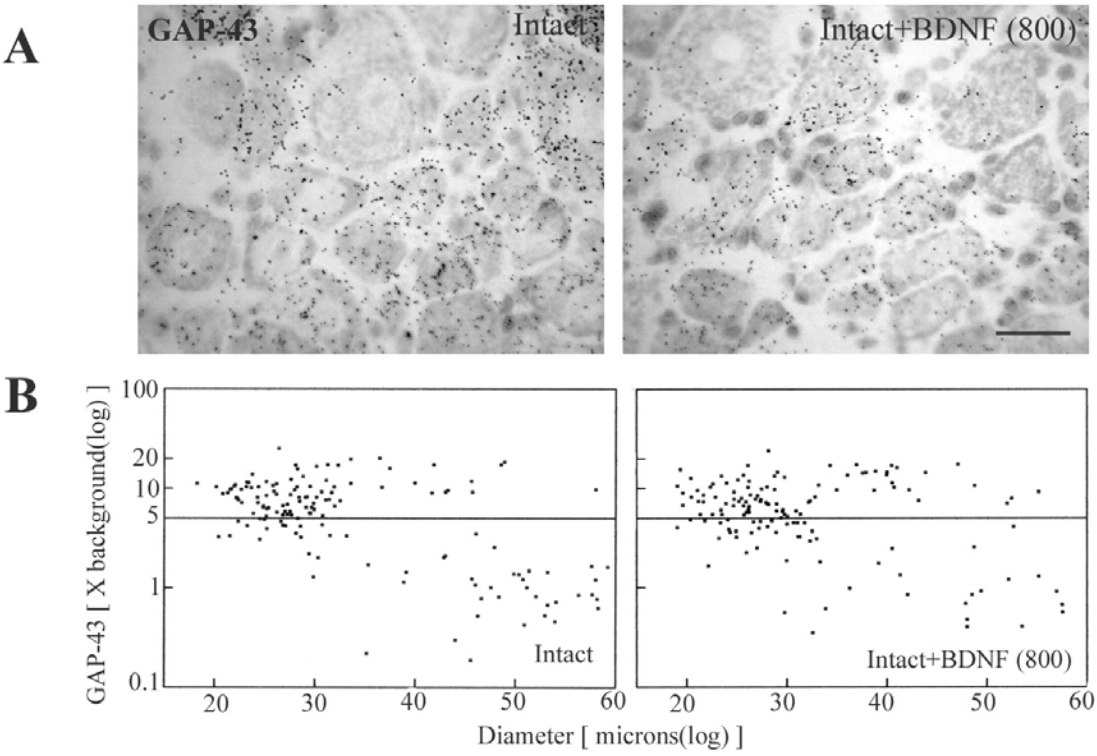

Figure 5-4. Chronic exposure of exogenous BDNF does not alter GAP-43 mRNA expression in intact DRG neurons. A. Brightfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect GAP-43 mRNA expression. A high dose of exogenous BDNF (800 ng/μl/hr) was delivered intrathecally via a mini-osmotic pump for 7 days in naïve rats. Scale bar = 30 μm. B. Representative scatterplots depicting relative changes in GAP-43 mRNA hybridization signal over individual neurons as related to cell size. Experimental states as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations.

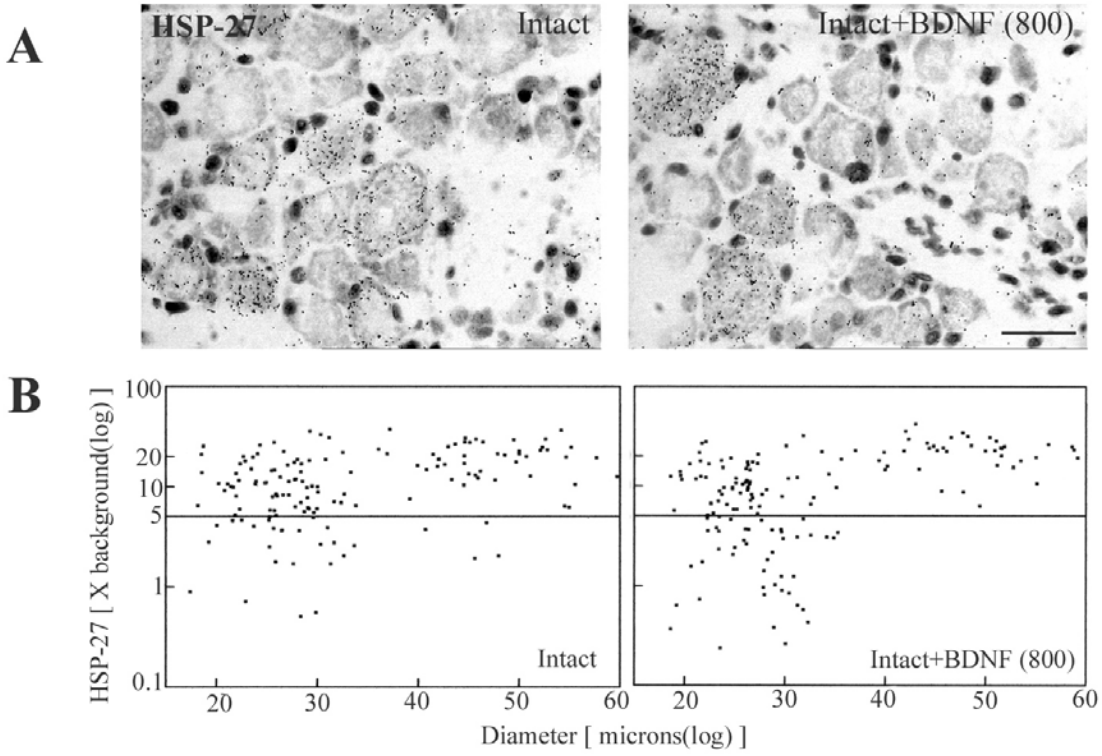

Figure 5-5. Chronic exposure of exogenous BDNF does not alter HSP-27 mRNA expression in intact DRG neurons. A. Brightfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect HSP-27 mRNA expression. A high dose of exogenous BDNF (800 ng/μl/hr) was delivered intrathecally via a mini-osmotic pump for 7 days in naïve rats. Scale bar = 30 μm. B. Representative scatterplots depicting relative changes in HSP-27 mRNA hybridization signal over individual neurons as related to cell size. Experimental states as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations.

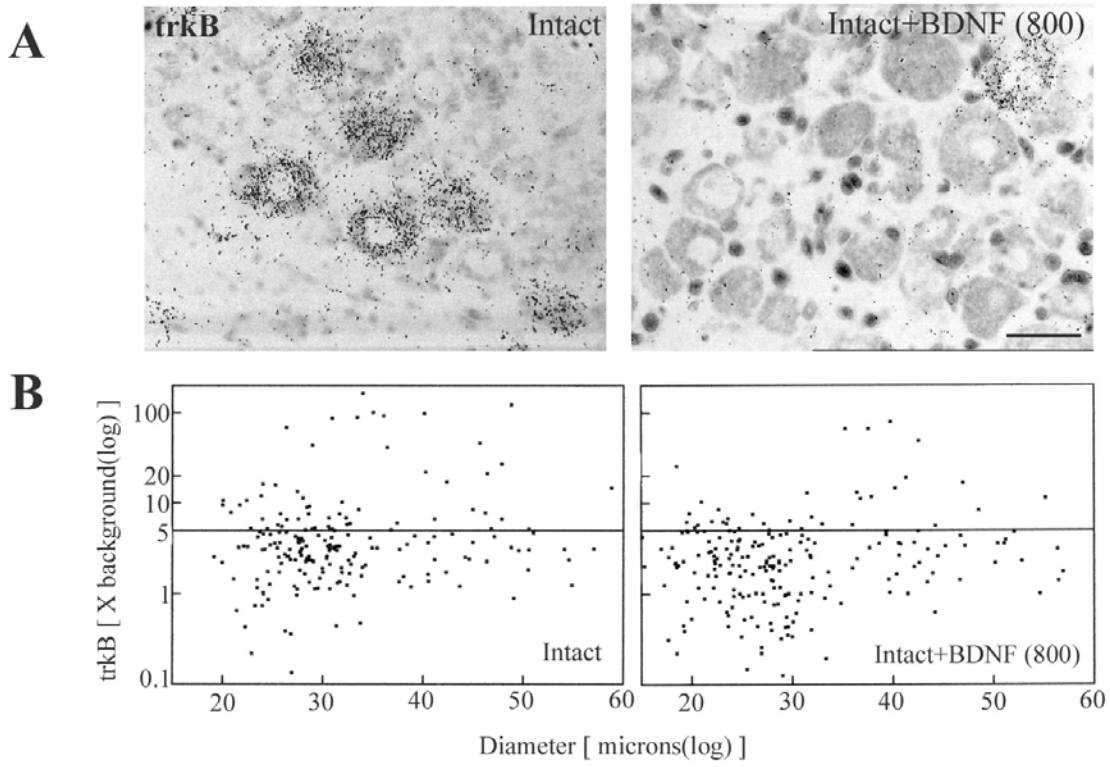

Figure 5-6. Chronic exposure of exogenous BDNF downregulates trkB mRNA expression in intact DRG neurons. A. Brightfield photomicrographs of L5 DRG (6 μm) processed for *in situ* hybridization to detect trkB mRNA expression. A high dose of exogenous BDNF (800 ng/μl/hr) was delivered intrathecally via a mini-osmotic pump for 7 days in naïve rats. Scale bar = 30 μm. B. Representative scatterplots depicting relative changes in trkB mRNA hybridization signal over individual neurons as related to cell size. Experimental states as indicated. Solid lines (5X) divide the plots into labeled and unlabeled populations.

GAP-43 or HSP-27 mRNA expression, with ~59% of neurons expressing detectable levels of GAP-43 mRNA and ~70% of neurons expressing detectable levels of HSP-27 mRNA (Fig. 5-4b, -5b).

5.4.4 trkB mRNA expression is downregulated in intact DRG neurons in response to chronic exposure of BDNF.

Acute infusion of high dose BDNF had no apparent influence on trkB expression in intact DRG neurons. However chronic exposure of high dose BDNF downregulated trkB mRNA expression (Fig. 5-6a). Consistent with other studies, trkB mRNA was detected in ~30% of DRG neurons (McMahon et al., 1994; Wetmore and Olson, 1995; Karchewski et al., 1999). Chronic infusion of BDNF downregulated the incidence of trkB mRNA expression, such that only ~9.6% of DRG neurons expressed detectable levels of trkB mRNA (Fig. 5-6).

5.5 Discussion.

This study demonstrates that exogenous BDNF is capable of enhancing regeneration-associated gene expression in injured sensory neurons in a dose dependent manner. Low dose of BDNF resulted in elevated injury/regeneration-associated gene expression with the most apparent changes observed in the small-medium sized DRG neurons. A high dose BDNF enhanced the regeneration-associated genes and the BDNF receptor trkB in medium-large DRG neurons in addition to its positive influence on small-medium sized neurons. However, exogenous BDNF was not able to induce a regeneration phenotype in intact sensory neurons after either acute (3 day) or chronic (7 day) exposure. Instead, chronic exposure of intact DRG neurons to intrathecal infusion of exogenous BDNF resulted in a downregulation of trkB mRNA, appearing to have the potential to desensitize neurons to BDNF. Thus, BDNF alone could not induce an injury

phenotype in intact adult sensory neurons, but has the capacity to enhance the intrinsic regenerative response in a dose dependent manner in injured sensory neurons.

5.5.1 Exogenous BDNF can enhance the intrinsic cell body response to injury, but is not sufficient to induce an injury phenotype.

The ability of exogenous BDNF to enhance injury/regeneration- associated gene expression of DRG neurons suggests BDNF is an important modulator of sensory neuron regeneration. Systemic administration of BDNF/CNTF (Cheng et al, 1998) or infusion at the repair site (Lewin et al, 1997) following sciatic nerve injury improved the rate and extent of sciatic functional recovery after nerve repair. BDNF covalently linked to collagen matrix used at the repair site also improved sciatic nerve regeneration (Utley et al, 1996). However, in these previous studies the influence of BDNF on regeneration was examined in only motor neurons. We are the first to demonstrate the positive influence of exogenous BDNF on the cell body response of sensory neurons to injury of the peripheral nerve. It is unknown if the observed increase in injury/regeneration-associated gene expression effected by exogenous BDNF is correlated with enhanced sensory axonal growth and future studies are required. However, elevated GAP-43 and Tα1 tubulin mRNA expression are markers of the regenerative state in sensory neurons and increased expression of these genes is correlated with enhanced regenerative growth in other studies (Hoffman, 1989; reviewed in Plunet et al., 2002).

Exogenous BDNF has been shown to enhance injury/regeneration-associated genes and influence the regeneration of injured neurons in other systems. In rubrospinal neurons, infusion of BDNF enhanced GAP-43 and Tα1 tubulin mRNA and maintained the level of trkB mRNA which normally decreases in response to injury (Kobayshi et al, 1997). In axotomized retinal ganglion cells, BDNF enhances GAP-43 expression but does not alter the expression of Tα1 tubulin (Fournier et al., 1997; Fournier and McKerracher, 1997). Thus, BDNF regulation of injury/regeneration-associated genes may be system dependent.

In the current study, exposure of intact sensory neurons to elevated levels of BDNF was not sufficient to induce the injury/regeneration phenotype despite endogenous BDNF playing a critical role in this process in injured neurons (Geremia et al., 2003; 2004). Thus, it is likely that additional injury induced signals are required for these actions of BDNF.

5.5.2 Potential mechanisms of action.

Most known effects of BDNF are mediated by BDNF binding to trkB, which is expressed in ~30% of sensory neurons and/or by binding to the common neurotrophin receptor p75 receptor, which is expressed in ~80% of sensory neurons (Zhou et al., 1993; 1996; Wetmore and Olson, 1995; Karchewski et al., 1999). In our study, a low dose of BDNF was found to enhance injury/regeneration-associated genes in the small-medium sized injured DRG neurons. However, trkB is expressed mainly in the medium-large sized DRG neurons. Thus it seems likely that if BDNF is mediating its effect directly on the small-medium sized neurons it is doing so via another receptor, possibly through the p75 neurotrophin receptor.

The role of p75 mediated signaling at the cell body to regulate regeneration in DRG neurons is not known. The p75 receptor and BDNF interaction on the growing axon after peripheral nerve injury have been implicated in myelination and axonal regeneration (Zhang et al, 1999; Cosgaya et al, 2002; Tolwani et al, 2004). However this interaction is at the growing axon and not at the cell body. p75 signaling at the cell body has been associated with cell death in the absence of trk activation (reviewed in Rabizadeh and Bredesen; 2003; Friedman and Greene, 1999). It is unlikely that BDNF is binding to p75 and inducing cell death as this requires either an absence of trk and or elevated p75 expression such as that observed in development when these neurons are susceptible to BDNF induced apoptotic death via p75 (Zhou et al., 2005). In adult sensory neurons the expression of p75 is greatly reduced along with trkB receptor expression in response to peripheral nerve injury (Verge et al., 1992; Sebert and Shooter, 1993; Karchewski et al., 1999).

A high dose of exogenous BDNF was able to further enhance regeneration-associated gene expression to include an effect on the medium-large sized DRG neurons when compared to infusion of a low dose of BDNF or injury alone. The enhanced expression of GAP-43 and Tα1 tubulin in the medium-large sized sensory neurons may be the result of BDNF interaction with trkB. Unlike a low dose of BDNF, infusion of a high dose of BDNF also elevated trkB mRNA expression in the medium-large DRG neurons. BDNF may be able to upregulate trkB mRNA expression by signaling through downstream pathways and activating CREB. TrkB is a target for CREB signaling since two CRE sites are located within the promoter of the trkB gene (Deogracias et al, 2004). BDNF can activate CREB through two pathways, one that involves activation of CaMKIV and another that involves activation of the Ras-ERK pathway (Finkbeiner et al, 1997). However the exact mechanism of how trkB mRNA is upregulated in response to a high dose of BDNF or if CREB is elevated in response to BDNF is beyond the scope of this study.

In intact sensory neurons, acute exposure to BDNF did not alter expression of trkB mRNA or the regeneration-associated genes, while chronic exposure of BDNF led to a ligand-induced downregulation of trkB. It appears that too much BDNF is detrimental. Chronic treatment of BDNF in other systems has also resulted in reduced trkB expression (Knusel et al, 1997; Frank et al, 1996; Sommerfeld et al, 2000). As well, chronic exposure to BDNF in chick embryonic DRG reduced neurotrophin response likely through desensitization of trkB (Carter et al, 1995). Though a high dose of BDNF enhanced trkB and regeneration-associated genes in injured sensory neurons, a longer duration or a higher dose of BDNF may inhibit the response. In a previous experiment, axonal growth of sensory neurons was enhanced in response to brief electrical stimulation. The increase in axon growth was correlated with enhanced levels of BDNF, trkB and regeneration-associated genes. Stimulation of longer periods further enhanced BDNF but resulted in no increase of axonal growth and downregulation of trkB, GAP-43 and Tα1 tubulin mRNA (Geremia, 2005). Thus, dose and duration of infusion of BDNF is critical when attempting to enhance the regenerative response. Further studies are necessary to determine if the high dose of BDNF used in this study

is the optimal dose or an even higher dose can further upregulate injury/regeneration associated genes without downregulating the trkB receptor.

5.5.3 Conclusion.

Peripheral nerve injury interferes with the retrograde flow of signals from the target. There is a loss of a target-derived neurotrophins available to the DRG neurons and this likely contributes to the series of biochemical changes observed in sensory neurons (Verge et al., 1989; Raivich et al, 1991; reviewed in Verge et al., 1996). BDNF is the sole neurotrophin that is upregulated in response to injury in sensory neurons and appears critically linked to the cell body response of sensory neurons to injury. Here we show that this role can be amplified in a dose dependent manner by acute application of exogenous BDNF. We also found that the elevated levels of BDNF alone are insufficient to induce this response in intact neurons. The exact molecular and physiological signals involved in sensing axonal injury and inducing repair programs are beginning to be elucidated. However BDNF is not the sole molecule involved in eliciting the cell body response of sensory neurons to injury, it can augment this response in injured neurons. Exogenous BDNF may have a potential role in enhancing sensory axonal regeneration following peripheral nerve injury.

6.0 GENERAL DISCUSSION.

6.1 Summary of findings.

My results demonstrate the following: (1) (a) brief electrical stimulation can enhance the number of regenerating sensory axons following femoral nerve injury. (b) brief electrical stimulation promotes elevated expression of injury/regeneration-associated genes (i.e. GAP-43, Tα1 tubulin, trkB, and BDNF); (2) endogenous BDNF is important in inducing the intrinsic regenerative response but is not necessary for maintaining this response in DRG neurons at longer survival times; (3) exogenous BDNF can enhance the levels of injury/regeneration-associated gene expression in a dose dependent manner; (4) exogenous BDNF is not sufficient to induce an injury phenotype in intact DRG neurons; instead chronic infusion of exogenous BDNF downregulates trkB mRNA expression.

6.2 The role of BDNF in the injured/regenerating sensory neuron.

BDNF is the only neurotrophin synthesized by adult DRG neurons in both the intact and injured sensory neuron. In the intact DRG neurons, BDNF is expressed in a subpopulation of neurons (trkA-positive) and rarely overlaps with expression of its receptor trkB (Ernfors et al., 1990b; Wetmore and Olson, 1995; reviewed in Verge et al., 1996). The trkA-positive neurons are functionally classified as nociceptive neurons and the role of BDNF in nociception has been well established. In the injured DRG neuron, BDNF expression rapidly increases and is initially present in ~80% of sensory neurons (Michael et al., 1999; Zhou et al., 1999; Karchewski et al., 2002). The functional role of this neurotrophin in injured DRG neurons remains unknown. Acheson

et al. (1995) demonstrated an important role of BDNF in supporting the survival of ~35 % of neurons via a paracrine/autocrine mechanism. My research suggests an additional role for BDNF. I postulate that BDNF is important for inducing the intrinsic regenerative response of sensory neurons. Antagonizing endogenous BDNF reduced injury/regeneration-associated gene expression (chapter 4), while increasing exogenous BDNF by intrathecal infusion (and perhaps by electrical stimulation) resulted in an increase in injury/regeneration-associated gene expression in injured neurons (chapter 3 and 5). Thus, the neurotrophic role of this molecule is beyond that of a survival factor and is important for the intrinsic regenerative response.

Altering the cell body response is often associated with increased regeneration and likely underlies the beneficial effect of a conditioning lesion, FK506 treatment, or electrical stimulation in motor neurons (see 1.3.2.). The ability of BDNF to alter the levels of expression of injury/regeneration-associated genes, such as GAP-43 and Tα1 tubulin is another example of the importance of the cell body response in enhancing regeneration. It should be noted that it is unknown if the enhanced intrinsic response observed with intrathecal infusion of exogenous BDNF is correlated with increased sensory axonal regeneration. However reduced injury/regeneration-associated gene expression is correlated with reduced neurite outgrowth *in vitro* and the upregulation of injury/regeneration-associated gene expression by electrical stimulation is associated with increased sensory axonal regeneration. Thus, altering the intrinsic regenerative response by BDNF likely influences sensory axon regeneration.

Though not examined in this thesis, BDNF may also enhance regeneration by altering the local growth environment. Priming of DRG neurons *in vitro* with BDNF, GDNF or NGF can overcome the inhibition of MAG and myelin through a cAMP – dependent mechanism (Cai et al., 1999). BDNF is able to upregulate cAMP levels by binding to trkB and activating a downstream signaling pathway, the RAS-extracellular-signal-regulated kinase (ERK) pathway (reviewed in Grewal et al., 1999; reviewed in Kaplan and Miller et al., 2000). Activated ERK inhibits phosphodiesterase 4 activity, the enzyme that hydrolyzes cAMP, and allows an increase in cAMP levels (Hoffman et al., 1999; Baillie et al., 2000; MacKenzie et al., 2000; Gao et al., 2003). Inhibition of ERK only allows cAMP to overcome low levels of MAG concentration but not high

levels of MAG concentration. The presence of BDNF is necessary to raise the concentration of cAMP to overcome high levels of MAG concentration; overcoming MAG inhibition requires crosstalk in neurons between ERK and cAMP signaling pathways (Gao et al., 2003). Thus, BDNF is not only capable of enhancing regeneration-associated gene expression but may also help the axon to overcome inhibitors in the local growth environment producing a maximal regeneration response.

It appears that BDNF is an important modulator of regeneration in injured DRG neurons. The upregulation of BDNF with electrical stimulation is associated with an increased number of regenerating sensory axons. Increasing the number of regenerating sensory axons likely increases the chance that axons will cross the repair site and reinnervate their appropriate targets. Brief electrical stimulation may also reduce asynchronous (staggered) regeneration. It is unknown if electrical stimulation of the femoral nerve increases the rate of regeneration of sensory axons or if the regeneration program has been modified to facilitate crossing the repair site. The ability of regenerating axons to enter the distal stump in a shorter amount of time enhances the likelihood of functional recovery as regenerating axons will enter an environment that is still permissive for growth and their target organs have not degraded. The increase in axonal regeneration may also result in improved functional recovery. Recent work by Brushart et al. (2005) found that brief electrical stimulation using the same electrical stimulation protocol used in this thesis increased the specificity of regenerating sensory axons of the femoral nerve.

6.3. Site of Action of BDNF.

The findings in this thesis show that BDNF could influence a large population of DRG neurons much larger than the population of DRG neurons that express the trkB receptor. In intact DRG neurons, trkB is mainly localized to medium DRG neurons and is present in ~30% of DRG neurons (McMahon et al., 1994; Wetmore and Olson, 1995; Karchewski et al., 1999). After injury the number of DRG neurons expressing high levels of trkB downregulate (Karchewski et al., 1999; 2002). It is possible that BDNF is exerting its effect on the injury/regeneration-associated genes by upregulating its trkB

receptor. Upregulation of trkB mRNA expression was correlated with enhanced GAP-43 and Tα1 tubulin mRNA expression following electrical stimulation and infusion of exogenous BDNF. The interaction of BDNF with trkB receptor has been shown to enhance injury/regeneration-associated gene expression and regeneration in motor neurons (Al-Majed et al., 2000a; reviewed in Boyd and Gordon, 2001), rubrospinal neurons (Kobayashi et al., 1997; Kwon et al., 2002; Kwon et al., 2004), serotonergic neurons (Mamounas et al., 2000) and retinal ganglion neurons (Hirsch et al., 2000; Avwenagha et al., 2003). As well, overexpression of trkB in the mouse brain induced GAP-43 expression in the hippocampus and parietal cortex (Koponen et al., 2004). The pathways that BDNF activates via trkB to promote regeneration are not clear (reviewed in Goldberg and Barres, 2000). As well, changes in injury/regeneration-associated genes occurred in the small-medium sized DRG and large DRG neurons that do not express trkB. Thus, interaction of BDNF with trkB cannot explain the observed changes in non-trkB expressing population of DRG neurons.

A likely mode of action is BDNF acting through the p75 neurotrophin receptor. As mentioned previously, the p75 neurotrophin receptor is present in ~80% of DRG neurons (Zhou et al., 1993; 1996; Wetmore and Olson, 1995; Karchewski et al., 1999). The role of p75 mediated signaling at the cell body in the regulation of regeneration in DRG neurons is not known. Interactions of the p75 neurotrophin receptor upregulated at the injury site have been associated with axonal regeneration and myelination (Zhang et al, 1999; Cosgaya et al, 2002; Tolwani et al, 2004). However, the role of p75 in mediating the cell body response is not known. A well-studied role of p75 mediated cell signaling is cell death. p75 mediated cell death often occurs in the absence of trk receptor (Davies et al 1993; Yoon et al., 1998). However in the DRG neurons p75 is rarely detected in the absence of a trk receptor (Verge et al., 1992; Karchewski et al., 1999). BDNF may also interact with p75 to reduce the affinity with which NGF binds to trkA of the DRG neurons. After peripheral nerve injury, NGF is produced at low levels at the proximal stump (Heumann et al., 1987). BDNF can bind to p75 and induce ceramide-mediated serine phosphorylation of trkA intracellular domain attenuating the trkA signaling (MacPhee and Barker, 1997). Repressing NGF may prevent the repression of injury/regeneration-associated genes resulting in a more robust response.

However, the downregulation of p75 neurotrophin receptor is more dramatic than the downregulation of the trkB receptor (Zhou et al., 2005) and evidence in p75 knockout mice suggests p75 is not important in the regenerative response. In p75 knockout mice with a deletion of exon III, the DRG neurons of the p75 knockout mice display a robust response to injury when compared to wildtype, including an increase in both BDNF and GAP-43 mRNA expression (Karchewski et al., 2000). A similar response was found in motor neurons, survival and regeneration of motor neurons was improved in p75 knockout mice when compared to wildtype mice (Ferri et al., 1998; Gordon and Boyd, 2001). However, peripheral neurons may compensate for the loss of the gene product in the p75 knockout mice during development.

The mechanism by which BDNF elicits changes in injury/regeneration-associated gene expression is currently unknown, and might not be direct. Rather, BDNF, acting through either trkB or p75, could stimulate the production and exocytosis of intermediate factors, and these could act on a large population of DRG neurons through a different receptor resulting in the expression of regeneration-associated genes. Also, there is evidence that BDNF locally applied to the DRG axon can increase levels of cytoskeletal mRNAs into the axonal compartment (Willis et al., 2005). These proteins synthesized in the axon could be retrogradely transported and initiate regeneration-associated gene expression in the cell body (reviewed in Perlson et al., 2003). In order for BDNF to act on the growing axon tip at the 3-day regeneration period time point (see Chapter 4 or 5), BDNF is likely being anterogradely transported (Tonra et al., 1998) to the growing axon rather than produced by the local environment. BDNF is not detected in the distal stump until 3-4 days postaxotomy (Heumann et al., 1987). Another possibility is that BDNF may directly enhance injury regeneration-associated gene expression through a third as of yet unidentified receptor.

Though my work emphasized the role of neuron-derived BDNF, there are other sources of non-target-derived BDNF and they may contribute to the results observed in this thesis. BDNF is produced by platelets from the blood (Yamamoto and Gurney, 1990), local immune cells (Schulte-Herbruggen et al., 2005) and satellite cells (Wetmore and Olsen, 1995; Deng et al., 2000). The role of these cells in the injured DRG neurons is not known. However satellite cells in the DRG might influence the

regenerative response as activation of these cells is associated with enhanced dorsal root regeneration (Liu and Richardson, 1991). The satellite cells surround individual DRG neurons and synthesize not only BDNF but truncated trkB (McMahon et al., 1994; Wetmore and Olsen, 1995). After nerve damage the satellite cells undergo morphological and biochemical changes. The satellite cells become active and upregulate p75 (Lu and Richardson 1991; Zhou et al., 1996). The satellite cells couple to other satellite cells that surround other neurons by forming gap junctions with each other. Gap junctions allow the passage of small ions and molecules. Satellite cells may enhance propagation of signals from the DRG neurons via a pathway involving the gap junctions of satellite cells (Hanani et al., 2002). As well, BDNF may signal through the truncated trkB or p75. Thus, the ability of BDNF to influence a large proportion of DRG neurons may be complex and may involve interaction with satellite or other nonneuronal cells.

6.4 Modulators of BDNF expression.

The initial upregulation of BDNF in the injured DRG neurons induces the intrinsic regenerative response. BDNF is unable to induce this response in naïve animals. Understanding the regulators of BDNF is important in understanding the cellular and molecular pathways involved in regeneration following peripheral nerve injury of the sensory neurons.

6.4.1 Axotomy.

Intrathecal infusion of exogenous BDNF into naïve animals was unable to induce an injury phenotype. BDNF is not the only molecule involved in the injury response, but appears to be an essential one as it is critically linked to induction of this response and brief exposure of immediate injured sensory neurons can augment injury/regeneration-associated gene expression. The exact physiological and molecular signals that act as sensor of axonal injury and induce BDNF are not known.

Following axotomy there is the loss of retrograde supply of transported trophic factors (ie. NGF and NT-3) and the activation of proteins from the injury site. These are referred to as 'negative' and 'positive' signals, respectively (reviewed in Perlson et al., 2003) and they act on the cell body to elicit injury/regeneration-associated gene expression. Interruption of retrograde transport by disrupting axonal microtubules can induce the expression of injury/regeneration-associated genes (Kashiba et al., 1992; Cougnon-Aptel et al., 1999). As well, there are some injury/regeneration-associated genes whose expression is dependent on the distance of the axotomy site from the DRGs (Kenney and Kocsis, 1998) and their activation appears to be dependent on retrogradely transported signals. Functional studies have shown that axonal protein synthesis contributes to generation of retrograde signaling complexes (Hanz et al., 2003). The identity of these proteins synthesized at the axon from the injury site is not known. The induction of BDNF may require both negative and/or positive signals.

As mentioned previously, BDNF is initially upregulated in ~80% of DRG neurons. Within 1 week there is a decrease in BDNF in the small-medium DRG neurons whereas BDNF expression remains elevated in the medium-large DRG neurons. This bimodal phenotypic change in BDNF expression following injury may be the result of interruption of retrograde transport of neurotrophins. The initial upregulation of BDNF in the small-medium sized DRGs, within the first few days following injury, likely occurs through the transient upregulation of NGF in the injured nerve (Heumann et al., 1987). NGF is an important modulator of BDNF expression. Injection of NGF results in a significant increase in BDNF expression (Apfel et al., 1996; reviewed in Verge et al., 1996). As well, inflammation, where NGF synthesis is increased, results in an upregulation of BDNF (Cho et al., 1997). NGF is upregulated within 2 hours postaxotomy and may briefly compensate for the loss of target-derived NGF (Heumann et al., 1987). The loss of target-derived NGF likely correlates with the loss of BDNF synthesis in the small-medium sized DRG neurons as observed 1 week postaxotomy NGF.

The upregulation of BDNF in the medium-large DRG neurons may be a response to the loss of target-derived NT-3. In the homeostatic state expression of BDNF in medium-large DRG neurons is suppressed by the presence of NT-3. NT-3

intrathecal infusion results in a decrease in BDNF expression in intact and injured DRG neurons. This decrease is apparent in both trkC and non-trkC expressing populations (Karchewski et al., 2002). In addition to activating its cognate receptor trkC, NT-3 can exert its effect via an isoform of trkA, which is believed to confer enhanced responsiveness to NT-3 (Barker et al., 1993; Clary & Reichardt, 1994; Karchewski et al., 1999). The loss of target-derived NT-3 may result in the upregulation of BDNF in medium-large DRG neurons and possibly the transient increase in BDNF in the small-medium sized DRG neurons. Without NT-3 binding to the trkA isoforms it may no longer interfere with NGF binding and it may allow the trkA -positive neurons to become more sensitive to NGF produced by the injured nerve.

Alternatively, BDNF synthesis may be regulated by cytokines. The neuropoeitic cytokines, LIF and IL-6 are important molecules involved in the intrinsic regenerative response and knockout of these molecules impairs sciatic nerve regeneration (Zhong et al., 1999) and the conditioning response (Cafferty et al., 2001; Cafferty et al., 2004 see 1.2.3.2.1 and 1.2.3.2.2). LIF is produced at the site of injury and retrogradely transported to the small-medium sized DRG neurons, in addition to being synthesized by nonneuronal cells in the DRG (Banner and Patterson, 1994; Curtis et al., 1994; Sugiura et al., 2000). IL-6 is synthesized in the medium-large DRG neurons following peripheral nerve injury and requires a 'positive' signal from the injury site (Gadient and Otten, 1996). LIF may regulate BDNF synthesis in the small-medium sized DRG neurons and IL-6 may regulate BDNF synthesis in the medium-large size DRG neurons. Exogenous IL-6 induces synthesis of BDNF *in vitro* and endogenous IL-6 is required for axotomy induced synthesis of BDNF *in vivo* as IL-6 knockout mice attenuate BDNF expression in the medium-large DRG neurons (Murphy et al., 2000). Both LIF and IL-6 activate signal transducer and activator of transcription 3 (STAT3). STAT3 has been shown to regulate BDNF synthesis and STAT3 response elements are likely present in the sequence 5' to exon IV (Murphy et al., 2000). LIF may also upregulate the transient upregulation of BDNF in the small-medium sized DRG neurons through activation of STAT3. However, the upregulation of LIF lasts for ~ 2 weeks (Banner and Patterson, 1994; Curtis et al., 1994) and is not transient like the upregulation of BDNF in the small-medium sized DRG neurons. Thus, it is unlikely that LIF is involved in the

transient elevation of BDNF expression in these DRG neurons and is likely the result of the transient increase of NGF expression in the proximal nerve (see above). NT-3 may also alter expression of IL-6 upon which BDNF expression in the medium-large DRG neurons is likely dependent. NT-3 can mitigate the upregulation of IL-6 (Verge and Johnston, 2000), possibly through altering a positive signal which regulates IL-6 postaxotomy (Murphy et al., 1999).

6.4.2 Electrical stimulation.

Brief electrical stimulation applied to the repaired femoral nerve was found to upregulate BDNF mRNA expression in DRG neurons (chapter 3). Electrical stimulation is capable of upregulating BDNF in DRG neurons above the levels normally observed following axotomy. As mentioned previously, BDNF responds to stimulation possibly via a calcium-dependent mechanism (chapter 1.3.2.4). Depolarization of the axon membrane results in an elevation of intracellular calcium and cAMP (Ming et al., 2001). Intracellular calcium can elevate cAMP by activating a calcium-dependent adenylyl cyclase (Xia et al, 1991; Meyer-Franke et al., 1995; reviewed in Xia and Storm, 1997). Both calcium and cAMP can upregulate BDNF expression by binding to a calcium response element site and a cAMP response element sites located in the promoter region of the BDNF gene, respectively (Nibuya et al., 1996; Shieh et al., 1998; Tao et al., 1998; chapter 1.3.2.4). As previously mentioned, BDNF is capable of increasing cAMP levels by inhibiting phosphodiesterase 4 (chapter 6.2). In the CNS, inhibition of phosphodiesterase 4 can also enhance induction of BDNF mRNA in the rat hippocampus (Fujimaki et al., 2000). Thus, it is possible that both cAMP and BDNF can regulate each other's expression and that electrical stimulation can promote sensory axonal regeneration by elevating the synthesis of these two molecules.

It should be noted that my work is unable to relate BDNF as the mechanism underlying the cell body response to electrical stimulation and future work such as anti-BDNF studies in conjunction with electrical stimulation or a time course of BDNF upregulation in relation with injury/regeneration-associated genes is required to delineate the role of BDNF. However, the ability of endogenous BDNF to induce an

acute injury response and the ability of exogenous BDNF to enhance the level of injury/regeneration-associated genes suggest that the upregulation of BDNF in response to electrical stimulation is responsible for the observed cell body response.

6.5 The potential therapeutic role of BDNF.

BDNF may have a potential therapeutic role in improving regeneration of the sensory neurons of the peripheral nervous system. The proper dose and method of administration that provide the best regenerative response without negative effects have to be determined before BDNF is used in a clinical setting.

Our work found that BDNF was able to improve the intrinsic regenerative response in a dose dependent manner. A low dose of BDNF was found to enhance injury/regeneration-associated genes when compared to controls, but a high dose of BDNF further upregulated injury/regeneration-associated genes including trkB (chapter 4). A similar response was found after brief electrical stimulation applied immediately following repair of the injured femoral nerve. Stimulation for 1 hour upregulated BDNF and this was correlated with enhanced injury/regeneration-associated gene expression including trkB. Whereas stimulation for longer than 1 hour can further upregulate BDNF, it is correlated with a downregulation of injury/regeneration-associated genes including trkB. BDNF is similar to many extracellular signaling molecules, in that its upregulation is associated with downregulation of its receptor (chapter 1.3.2.4). A chronic infusion (7 days) of a high dose of BDNF in intact sensory neurons also reduces trkB mRNA expression. Thus, a dose of BDNF that maximally upregulates injury/regeneration-associated genes but does not decrease trkB expression is optimal.

Administration protocols are also important in regulating the effect of BDNF on the injured/regenerating sensory neurons. Infusion of BDNF onto the proximal stump or into the intrathecal space may elicit a different response, as infusion of BDNF directly onto the cut nerve is acting on the growing sensory axon rather than directly onto the cell body and perineuronal environment. As well, electrical stimulation upregulation of BDNF will likely induce a different response than intrathecal infusion of exogenous

BDNF, as electrical stimulation is not only altering BDNF expression but a variety of different molecules. Different administration protocols of BDNF may allow a higher dose of BDNF to be chronically infused without the downregulation of trkB. In the CNS, increased endogenous BDNF levels can lead to increased seizure activity. However continuous infusion of BDNF inhibits epileptogenesis likely because high levels of BDNF decrease trkB (Xu et al., 2004). Administration of BDNF via a bolus injection rather than continuous infusion in the hippocampus is able to accelerate kindling development and prevents the decrease of trkB levels (Xu et al., 2004). Though intrathecal infusion of 800ng/μl/hr of BDNF may be sufficient to induce a robust regenerative response in injured sensory neurons it would be interesting to determine if alterations in administration programs can further enhance the intrinsic regenerative response and sensory axonal regeneration.

Before BDNF can be used to improve the intrinsic regenerative response, it is necessary to determine whether exogenous BDNF is inducing neuropathic pain via the injured DRG neurons. In intact sensory neurons, BDNF plays a neuromodulatory role in pain transduction (chapter 1.1.3.3). Following nerve injury, anterograde transport of BDNF is increased after peripheral nerve injury (Tonra et al., 1998). The initial upregulation of BDNF in the small-medium nociceptive neurons has been associated with allodynia and was attenuated by antibodies to BDNF during the early phase of injury (Zhou et al., 2000). As time progresses after peripheral nerve injury, BDNF expression decreases in the small-medium size neurons and the transport of this molecule to their central projections is decreased. As BDNF expression increases in the medium-large DRG neurons there is an increase in the transport of BDNF to their target in the spinal cord and gracile nuclei (Cho et al., 1998; Michael et al., 1999; Zhou et al., 1999). The upregulation of BDNF in these medium-large diameter neurons that generally subserve transmission of innocuous stimuli suggests that BDNF is unlikely to be involved in pain transmission in these neurons. The administration of exogenous BDNF may regulate neuropathic pain by augmenting sympathetic sprouting. Following nerve injury, sprouting sympathetic axons surround and form arborizations (baskets) mostly around the medium-large DRG neurons (McLachlan et al., 1993; Chung et al., 1993). As sympathetic activity of these baskets is triggered reflexively there is a chance

that neuropathic pain states can develop likely through becoming electrically coupled to sensory neurons (Chung et al., 1993; reviewed in Ramer et al., 1999). The upregulation of BDNF in DRG neurons and surrounding satellite cells increases sympathetic sprouting. The delivery of BDNF antibody or antisense oligomers to injured DRGs decreases sprouting of sympathetic nerves (Deng et al., 2000). Administration of exogenous BDNF may mediate neuropathic pain through transport of the small-medium sized DRG neurons to their central projections and/or by increasing sympathetic sprouting. Future work is required to determine the role of exogenous BDNF in mediating neuropathic pain following peripheral nerve injury before it is used to enhance the intrinsic regenerative response in the clinical setting.

BDNF was able to induce a robust injury response in injured DRG neurons when infused immediately following nerve transection. However, in the clinical situation, immediate repair is not always possible. It is unknown whether a delayed exogenous administration of BDNF or electrical stimulation can further upregulate injury/regeneration-associated genes. The delayed intrathecal infusion of BDNF antibody had no influence on the injury/regeneration association genes, thus it seems unlikely that additional BDNF can modify the regenerative response once it is induced. However, systemic injection of BDNF antibody for two weeks following injury has been linked to impaired peripheral nerve regeneration and myelination by interfering with Schwann-cell derived BDNF (Zhang et al., 2000). Thus, after chronic axotomy, the levels of Schwann cell synthesized BDNF, might be an important regulator of regeneration rather than neuronal-derived BDNF. There is evidence that BDNF can enhance regeneration following chronic peripheral nerve regeneration when administered at the site of injury, though the results are variable. Both immediate and delayed (two weeks after injury) injection of BDNF at the site of nerve repair are capable of improving axonal diameter of the sciatic nerve during functional recovery, however the improvements are not significant when compared to controls (Moir et al., 2000). When BDNF was infused directly onto the proximal stump of the injured tibial nerve immediately after repair for 28 days, there was no improvement in motor neuron regeneration with a low dose of BDNF. However, after 2 month chronic injury, a low dose of BDNF infused directly onto the proximal stump reversed the negative effects of

chronic axotomy and improved motor neuron regeneration. An infusion of a high dose of BDNF profoundly reduced motor neuron regeneration either after immediate or delayed infusion when compared to saline control (Boyd and Gordon, 2002). It is difficult to predict the role of exogenous BDNF on sensory neuron regeneration after chronic axotomy from motor neuron regeneration experiments as motor neurons and sensory neurons respond differently to axotomy. However, it is apparent that dose and method of application need to be considered in order to use BDNF to improve peripheral nerve regeneration.

Finally, the effect of BDNF administration on both motor neurons and sensory neurons need to be considered for peripheral nerve regeneration to be successful. Though BDNF has been shown to enhance regeneration in both motor and sensory neurons it is necessary to determine the optimal dose and mode of application that will result in optimal regeneration. At this time, brief electrical stimulation may hold tremendous clinical implications to improve peripheral nerve repair. Electrical stimulation for 1 hour was capable of improving the number of sensory neurons that regenerate (chapter 3), the specificity of sensory neuron regeneration (Brushart et al., 2005), and was capable of maximizing the appropriate growth of regenerating motor neurons of the transected femoral nerve (Al-Majed et al., 2000b; Brushart et al., 2002).

6.6 Conclusion.

Axotomy of the peripheral nerve initiates a series of well-defined patterns of cellular and molecular changes within the cell body and at the injured nerve. The upregulation of BDNF in the cell bodies of the injured sensory neurons is an example of one of the changes that occur. The use of brief electrical stimulation enhanced sensory axonal regeneration through the elevation of injury/regeneration-associated genes including the upregulation of BDNF. This thesis found that BDNF is an important inducer of the acute intrinsic regenerative response and can augment this response to hopefully promote axonal regeneration.

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APPENDIX: METHODS

1.1 Animal Surgery.

The use of animals was conducted in accordance with the NIH policy on the use of animals in research and the animal care committee guidelines of the University of Saskatchewan (protocol 19920164).

1.1.2 Spinal nerve transection.

Adult male Wistar rats (300-350 g; Charles River Laboratories, Wilmington, MA) were anesthetized with Somnitol 100 mg/kg by intraperitoneal injection. The absence of the tail flick response was used to ensure adequate level of anesthesia. The tail flick response was elicited by pinching the distal tip of the tail. Once it was ensured that the rat was adequately anesthetized, the right hindlimb was shaved and the area was swabbed with 70% alcohol followed by sterile PBS (137 mM NaCl, 2.7mM KCl, 10 mM NaH_2PO_4, 2 mM KH_2PO_4; pH 7.4). An incision was made lateral to the midline of the vertebral column and a L4 and L5 spinal nerve were unilaterally transected at their origin. A 5 mm segment was resected to prevent nerve regeneration. Animals were given a pre- and post-operative subcutaneous injection of buprenorphine (Temgesic, 0.01 mg/kg). After surgery, the rats were placed in separate cages and placed under a heat lamp to maintain body temperature until they awoke from the anesthetic.

1.2 Mini-Osmotic Pumps.

1.2.1 Preparation of osmotic pumps.

Silicone tubing (Dow Corning Corp., Midland, MI) was assembled to create a catheter that delivers solution from the body of the osmotic pump to the site of delivery. Silicone tubing of a small diameter (0.305 mm inner diameter X 0.635 mm outer diameter) measured and cut to 3 cm length was inserted into silicone tubing of a larger diameter (0.635 mm inner diameter X 1.194 mm outer diameter) cut to 0.8 cm length. The two pieces of tubing were joined with silicone glue (Dow Corning Corp) and autoclaved.

The pumps were filled and assembled under sterile conditions in a laminar flow hood or biosafety cabinet. Mini-osmotic pumps (Alzet model 2001, Alza Pharmaceuticals, Palo Alto, CA) output 1 μl/hr for 7 days and each pump requires about 300 μl of solution (i.e. BDNF antibody (chapter 4) or BDNF (chapter 4, 5). The pump was filled with the solution and the sterile silicone tubing was placed over the stainless tube on the top of the osmotic pump cap and secured with sterile 4-0 silk. The pumps were assembled ~18-20 hours prior to surgery and stored in a falcon tube at 4°C with sufficient amount of sterile PBS to cover the pump bodies.

1.2.2 Pump implantation.

Pumps were either implanted immediately after spinal nerve transection or after a delay. After the rats were anesthetized, their backs were shaved and swabbed with 70% ethanol followed by sterile PBS (pH 7.4). An incision was made rostro-caudal over the lumbar and sacral regions of the spinal cord and the skin was separated from the muscle with blunt scissors to create a space for the body of the pump. A scalpel was used to cut through the muscles on either side of the vertebral column and a laminectomy was performed. Using the sacrum as a landmark, the L6 spiny processes were removed with rogeurs to expose the dura and the #5 forceps were used to separate and open the dura mater. The silicone tubing was inserted into the opening of the dura

mater so that 1.5 cm portion of the tubing was located into the subarachnoid space, delivering the contents of the pump at the level of the L5 DRG. The body of the pump was secured to the muscle with 3 sutures (2 sutures around the body of the pump and one around the stainless steel tube; 4-0 silk). The overlying skin was sutured closed (4-0 silk) and the rats underwent postoperative treatment as previously described.

1.3 Tissue Preparation.

1.3.1 Perfusion.

After the required time postaxotomy, animals were adequately anesthetized as described above. The chest and abdominal area were shaved. The chest cavity was opened using blunt tip scissors and the pericardium was gently teased away from the heart. A syringe containing 50 ml of cold PBS (pH 7.4) was inserted into the heart via the left ventricle, a hole was cut into the right atrium with microscissors, and cold (4°C) PBS was slowly pushed into the circulatory system followed by 300 ml of fixative of 4% paraformaldehyde and 1.5% picric acid in 0.1M PB.

1.3.2 Dissection of lumbar ganglia.

Following the perfusion, the L4 and L5 DRG were removed. During dissection the proper position of the silicone tubing was ensured and any sign of infection was noted. Deep incisions were made into the skin rostro-caudally and both lateral to the spinal cord. Perpendicular cuts were made along the spiny processes. Along the vertebral column the muscle and spiny processes were removed. The iliac crest of the pelvis was used as a landmark to locate the L5 vertebrae. The #5 forceps were used to expose and gently tease away the DRG. The DRG were cut free from their roots using microscissors and postfixed (1-1.5 hrs) in fixative used for perfusion. The DRG were then placed in 20% sucrose overnight and any excess sucrose on the DRG was blotted on filter paper prior to placement in cryomolds (Tissue Tek, Miles Labs., Elkhart, IN).

The mold was filled with OCT compound (Tissue Tek) and the tissue was frozen rapidly by placing in a beaker of isopentane (2-methylbutane) cooled in a slurry of dry ice and acetone. The cryomolds were covered in tin foil and stored in ziplock bags in the -80°C freezer until processed.

1.4.1 Sectioning of lumbar DRG.

Blocks containing the DRG were removed from -80°C freezer and placed in the cryostat (Microm HM5000 cryostat; Zeiss Canada) and were left to equalize to the temperature of -22°C. Sections were cut at 6μm and thaw mounted on Probe-ON+ slides (Fischer Scientific, Canada). The slides were stored in slide boxes containing cold desiccant and kept at -20°C until tissue was processed for *in situ* hybridization or stored at -80°C if tissue was to be processed for both *in situ* hybridization and immunohistochemistry. To count the number of backlabeled sensory neurons, sections were cut at 10 μm and stored at -20°C.

2.0 *In situ* Hybridization.

Unless otherwise noted all solutions were made in DEPC- H_2O and prepared in RNase- free conditions.

2.1 Probe labeling.

In situ hybridization was carried out using 48 base pair oligonucleotide probes (University Core DNA services, University of Calgary, Calgary, AB, Canada) complementary to the selective mRNA. All probes were checked against Genbank databases (www.ncbi.nlm.nih.gov/BLAST/) to ensure no greater than 75% homology with any other transcript. The probes were resuspended with DEPC-H_2O for a stock

solution of 400 ng/ml or a working concentration of 40 ng/ml. The probes were stored at -20°C freezer.

The 3' ends of the probes were labeled with α-[^{35}S]dATP nucleotidyltransferase (Dupont New England Nuclear, Boston, MA). To label the oligonucleotide probe, 100 μCi α-[^{35}S]dATP was added to a buffer containing 50 mM sodium cacodylate (pH 7.2), 1 mM $CoCl_2$ and 0.1 mM 2-mercaptoethanol. For each reaction 80 ng of oligonucleotide probe was added to the buffer and the isotope was bound to the 3'end with the use of terminal deoxynucleotidyltransferase (39 units of terminal deoxynucleotidyltransferase in 7.2 mM potassium phosphate (pH 7.2), 18 mM KCl, 0.12 mM 2-mercapoethanol, 6% glycerol; Amersham Pharmacia Biotech Inc., Baie d'Urfe, PQ). After the addition of the enzyme, the tubes were placed in the 37°C water bath for 1.5-2 hours. The reaction was stopped by adding 0.1 M Tris-HCl (pH 8.0).

To purify the probe 0.5 g of resin (Nucleic Acid Purification Resin; DuPont NEN, Boston, MA) was placed into 2 ml polystyrene columns (Biolynx, Brockville, ON, Canada). The resin was prewetted in the column by adding 3 ml of methanol, followed by 3 ml of 0.1 M Tris-HCL (pH 8.0). Gently applying pressure to an air-filled syringe was used to push solutions through the columns so only 1 drop/2 sec was pushed through and the resin bed was not allowed to dry. The sample containing the probe was added to the column and was rinsed with 1.5 ml of 0.1 M Tris-HCL (pH 8.0) to remove unbound probe. Finally, 0.5 ml of 30% methanol was added to elute the radiolabeled oligonucleotides from the column and drops 25-40 were collected. Additional 0.1M Tris-HCl (pH 8.0) was added to the collected sample to bring the final volume to ~400ul and 0.1 mM dithiothreitol dissolved in 0.01 M sodium acetate (pH 5.2) was added to prevent the aggregation of ^{35}S.

Probes can also be purified using spin columns (CHROMA spin columns, Clonetech, BD Biosciences, Mississauga, ON, Canada). Probes were labeled as before and the enzyme reaction was stopped using 0.1 M Tris-HCL (pH 8.0). The columns were spun at 700 X g for 5 min. Prior to the addition of probe reaction, 0.05 μg of sheared salmon sperm DNA was added. The columns were spun at 700 X g for 5 min. The purified sample is found at the bottom of the collection tube and additional 0.1M

Tris-HCL (pH 8.0) was added to bring the final volume to 400 µl and 0.1 M dithiothreitol (pH 5.2)

The radioactivity of the labeled probe was determined using a scintillation counter (LS 6500 Liquid Scintillation System; Beckman Instruments Inc., Fullerton, CA). The specific activities used for *in situ* hybridization studies normally use counts ~ 400 000 cpm/µl. Probes with radioactivity of <100 000 cpm/µl were not used.

2.2 Pretreatment of fixed tissue.

Tissue that was fixed underwent a pretreatment prior to *in situ* hybridization so that the mRNA was accessible to the oligonucleotide probe. The slides were allowed to equilibrate to room temperature (~20 minutes). Slides were postfixed in 4% paraformaldehyde for 20 minutes and then washed 3 X 10 minutes in PBS (pH 7.4). The slides were then treated with proteinase K (20 µg/ml proteinase K, 0.05 M Tris-HCl (pH 7.6), 5 mM EDTA) for 6 minutes. Slides were rinsed in PBS for 5 minutes and refixed in 4% paraformaldehyde for 5 minutes. Finally, slides were rinsed in 2 X 5 minutes in PBS (pH 7.4), 1 X 5 minutes in DEPC-H_2O and dehydrated in ascending alcohols.

2.3 Hybridization.

In situ hybridization was performed according to procedures previously described by Dagerlind et al. (1992).

Slides were covered with hybridization buffer solution consisting of 50% formamide (Fischer Scientific, Canada), 4 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1 X Denhardt's solution (0.02% BSA, 0.02% Ficoll and 0.02% plyvinylpyrollindone), 1 % sarcosyl, 0.02 M PB (pH 7.0), 10% dextran sulfate, 500 mg/ml of heat-denatured sheared salmon sperm DNA, 200 mM DTT and 10^7 cpm/ml of

^{35}S-labeled oligonucleotide probe. Using a hinge technique, another Probe-ON+ slide was place on top of the other slide covered in hybridization solution, avoiding air bubbles. Slides were placed in a hybridization box that contained filter papers wetted with 1X SSC and taped shut to maintain a humidified chamber. The box was placed in the hybridization oven at 43°C for 14-18 hours.

The next day, the slides were separated by being immersed in heated (55°C) 1 X SSC. The slides were washed 4 X 15 minutes in 1 X SSC at 55 °C and then brought to room temperature (~30 minutes). The slides were rinsed in 2 X dH_2O and dehydrated in ascending alcohols. Slides were left to dry.

In situ hybridization control experiments were performed on adjacent sections to ascertain the specificity of hybridization signal of each probe. Sections were hybridized with labeled probe with a 1000-fold excess of dissimilar unlabeled probe of the same length and guanine and cytosine content. If probe binding was specific there was no change in hybridization. As well, sections were hybridized with labeled probe with a 1000-fold excess of unlabeled probe. The excess unlabeled probe abolished the binding of labeled probe.

2.3.1 Radioautography.

Radioautographic processes were performed in a dark room, and if necessary sodium vapor lamps were used (turned-on ~20 minutes prior to use). NTB-2 nuclear track emulsion (Kodak, Eastman Kodak Co, Rochester, NY) was diluted 1:1 with distilled water and melted at 39-43°C in a water bath for ~30 minutes. Slides were dipped in the emulsion and left on slide drying racks lined with moistened lint-free towels for ~2.5 hours. Slides were placed in appropriate slide boxes (sorted according to probe) containing desiccant in light-proof bags and stored at 4°C until they were developed. Optimal time of exposure was determined by developing control slides. Slides were considered ready for developing if silver grain density was maximal but the grain overlap was minimal so neurons exhibiting low levels of labeling could be identified. To develop the emulsion- coated slides, the slides were brought to room

temperature (~20 minutes) and placed in D-19 developer (18-20°C, Kodak) for 2.5-3 minutes. The slides were rinsed in H_2O and fixed in Rapid Fix (Kodak) for 5-6 minutes. Finally, the slides were rinsed in tap water (20°C) for 20 minutes and excess emulsion was removed from the back of the slide with a razor blade. The slides were dried and stored in a dust-free environment. The silver grains were viewed under a darkfield condenser (Darklite darkfield stage adaptor Micro Video Instruments Inc, Avon, MA).

2.4 Quantification of relative levels of mRNA hybridization signal.

To visualize the cells under a light microscope the sections were lightly stained with filtered 0.5% Toludine blue (BDH Chemicals, Toronto, ON, Canada;) in 0.01 M acetate buffer (pH 4-4.5). The slides were dehydrated, cleared in xylene and coverslipped using Permount (Fischer Biotech, Fair Lawn, NJ). Slides were visualized under both darkfield and brightfield conditions. Slides (n=2) were chosen for quantification for each probe whose sections contained similar numbers and reflected overall changes in label as determined through qualitative analysis of all sections processed with the probe. Montages were made for each section on the slide to keep track of the neurons that were analyzed, ~100-200 neurons were quantified for each DRG. Using 63X oil immersion light microscopy with an interactive computer-assisted image analysis system (software developed by W.G Tatton, Dalhousie University, Halifax, Canada) the relative silver grain densities and cross-sectional area of individual neurons for each ganglia was determined. To maintain the constant label of silver grains, the density threshold was adjusted interactively for each image. To determine the label for each neuron the density of silver grains over the cell was subtracted from the background label. Background labeling was determined by averaging the levels of silver grains over 5 areas of neuropil devoid of positive label. Neurons were considered labeled if they had more than 5 X background levels of silver grains. This cut-off corresponds to the level of hybridization signal that must be present in order to

confidently declare the neuron labeled when observed under 63X oil immersion light microscope. The relative level of label was only compared among ganglia located on the same slide as these ganglia were processed under identical conditions. Differences in section thickness, hybridization and wash conditions, and emulsion thickness can cause variability of silver grain densities that can exist from one slide to the next.

3.1 Immunohistochemistry.

Prior to all immunohistochemistry procedures the slides were removed from the freezer and warmed to room temperature (~20-30 minutes). Control sections were included for each immunohistochemistry procedure described below and in these sections the primary antibody was replaced by antibody diluent, block solution or undiluted rabbit serum.

3.1.1 BDNF Immunohistochemistry.

To detect BDNF protein expression, the slides were washed at 3 X 10 minutes in PBS (pH 7.4). The sections on each slide were covered with blocking solution (4% horse serum, 1.5% BSA and 0.01% triton X-100 in PBS) for 1 hour at room temperature. The blocking solution was replaced with rabbit anti-BDNF (gift from Cindy Wetmore, Mayo Clinic, Rochester, MN; Wetmore and Olsen, 1995) diluted at 1:500 in antibody diluent (2% horse serum, 0.5% BSA and 0.01% triton X-100 in PBS)and was left on the tissue overnight at 4°C. The next day, the slides were washed in 3 X 10 minutes of PBS (pH 7.4). The sections were incubated in secondary antibody Cy3-conjugated donkey anti-rabbit IgG (Jackson, Westgrove, PA) diluted 1:300 in antibody diluent for 1 hour in the dark at room temperature. The excess secondary antibody was removed in 3 X 10 minutes of PBS (pH 7.4). The slides were coverslipped with glycerol/PBS and sealed with nail polish. Care was taken to limit exposure of the slides to the light and the slides were stored in light-proof bags at -20°C.

3.1.2 ATF3 Immunohistochemistry.

Slides were washed in 3 X 10 minutes in TBS (20 mM Tris, 0.5 M NaCl; pH 7.4), and sections were blocked in 10% goat serum in TBS (pH 7.4) for 1 hour at room temperature. The block solution was removed by suctioning and replaced by rabbit anti-ATF3 (Santa Cruz Biotech Inc, Santa Cruz, CA) diluted 1:300 in TBS (pH 7.4) and was left on the tissue overnight at 4°C. After 14-18 hours, the sections were washed in 3 X 10 minutes in TBS (pH 7.4) and then incubated in secondary antibody biotinylated anti-rabbit IgG (Vector, Burlington, ON, Canada) diluted 1:300 in TBS (pH 7.4). The slides were washed in 3 X 10 minutes in TBS (pH 7.4) and standard ABC (Vector) procedure was used to visualize immunostaining of ATF3. The sections were incubated in ABC solution (VECTASTAIN ABC kit, Vector) diluted 1:50 in TBS (pH 7.4) for 1 hour at room temperature. The slides were washed in 2 X 10 minutes in TBS (pH 7.4) and the final rinse was in 1 X 10 minutes in Tris (0.1 M; pH 7.4). Peroxidase activity was localized using 0.067% DAB and 0.024% H_2O_2 in Tris (0.1 M; pH 7.4). Reaction times may vary from 30s – 5 minutes and were stopped before background levels were too high. The reaction was stopped by rinsing the slide in Tris (0.1 M; pH 7.4). The slides were dehydrated in ascending alcohols, cleared in xylene, and coverslipped using Permount mounting medium.

3.1.3 Sheep IgG immunohistochemistry.

Immunohistochemistry was used to identify the penetration of sheep IgG after intrathecal infusion of sheep-anti BDNF or control antibody (sheep IgG). The slides were washed in 3 X 10 minutes in PBS (pH 7.4) and incubated in a secondary antibody biotinylated anti-sheep (Vector) diluted 1:150 in antibody diluent (2% horse serum, 0.1% triton X-100 in PBS) for 1 hour at room temperature. The slides were then rinsed at 3 X 10 minutes in PBS (pH 7.4) and standard ABC procedures were used to visualize binding of secondary antibody (see Appendix 3.2).

4.0 Immunocytochemistry.

Cells in culture were immunostained with GAP-43 antibody. The media was removed and the cells were rinsed 2 X 10 minutes PBS (pH 7.4). The cells were fixed in cold 100% methanol (-20°C) for 30 minutes and dried under a vacuum for 30 minutes. A blocking solution (0.5% BSA and 2% horse serum in PBS) was added to the wells for 1 hour at room temperature. A monoclonal antibody, mouse-anti-GAP-43 (9-1E12; gift from DJ Schreyer, Univ. of Saskatchewan, Saskatoon, SK, Canada) was added at a dilution of 1:10 000 overnight at 4°C. The next day, the cells were washed in 3 X 5 minutes 0.05% Tween 20 in PBS (pH 7.4) and the cells were incubated in secondary antibody biotinylated anti-mouse IgG (DakoCytomation, Mississauga, ON, Canada) diluted 1:200 in blocking solution for 30 min at room temperature. The cells were rinsed in 3 X 5 minutes 0.05% Tween 20 in PBS (pH 7.4) and incubated in ABC solution (VECTASTAIN ABC kit, Vector) diluted 1:50 in PBS (pH 7.4) for 1 hour at room temperature. The slides were washed in 3 X 5 minutes washes in 0.05% Tween 20 in PBS (pH 7.4) and the substrate reaction was visualized with 0.025% DAB and 0.05% H_2O_2 in PBS for 5-20 minutes. The cells were rinsed in PBS (pH 7.4) and stored in PBS at 4°C until analyzed.

5.0 Neurite Outgrowth Analysis.

Neurite outgrowth analysis was carried out using Neurobin (Northern Eclipse imaging system, Empix Imaging Inc, Mississauga, ON, Canada). The neurons encountered along 40 fields on a defined track across the plate were used for quantification (~100-180 neurons). To measure total neurite length per neuron, the neurites were highlighted with the threshold function and the background and cell bodies were erased manually. The remaining neurites were skeletonized and the total skeletal neurite length was determined by the program. The number of branch points for each neuron was counted manually.

6.0 Dot Blot.

To ascertain the specificity of BDNF antibody (sheep anti-BDNF; Chemicon International, Temecula, CA), a dot blot was performed. Nitrocellulose membrane (Sigma Aldrich, Oakville, ON, Canada) and two pieces of filter paper were presoaked in 20% methanol for 10 minutes. The nitrocellulose membrane was placed on top of the two pieces of filter paper. Different amounts (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng and 0ng) of NGF (Cedarlane Laboratories LTD, Hornby, ON, Canada), NT-3 (Amgen, Thousand Oaks, CA), BDNF (Alomone Laboratories LTD, Jerusalem, Israel) and cytochrome C (cytochrome C has a similar weight and charge as BDNF) were blotted onto the nitrocellulose membrane and dried onto the membrane under a vacuum (~40 minutes). The membrane was rinsed in 1 X 10 minutes in PBS (pH 7.4) and incubated in blocking solution (20% horse serum in PBS) for 1 hour at room temperature. The blocking solution was replaced with sheep anti-BDNF (Chemicon) diluted 1.5μg/μl (1:2000) in diluent (1% horse serum and 0.3% Triton X-100 in PBS) for overnight at 4°C. The next day the membrane was rinsed in 3 X 10 minutes 0.5% Tween 20 in PBS (pH 7.4) and incubated in secondary antibody biotinylated anti-sheep (Vector, Burlington, ON, Canada) diluted to 1:200 in diluent (1% horse serum in PBS) for 1 hour at room temperature. After several washes the membrane was incubated in ABC solution (VECTASTAIN, Vector) at a dilution of 1:100 in PBS for 45 minutes at room temperature. The membrane was rinsed in 2 X 10 minutes TBS (pH 7.4) and 1 X 10 minutes in Tris (0.1 M; pH 7.4). The membrane was developed in 0.05% DAB and 0.006% H_2O_2 for 4 minutes. The reaction was stopped by placing the membrane in dH_2O.

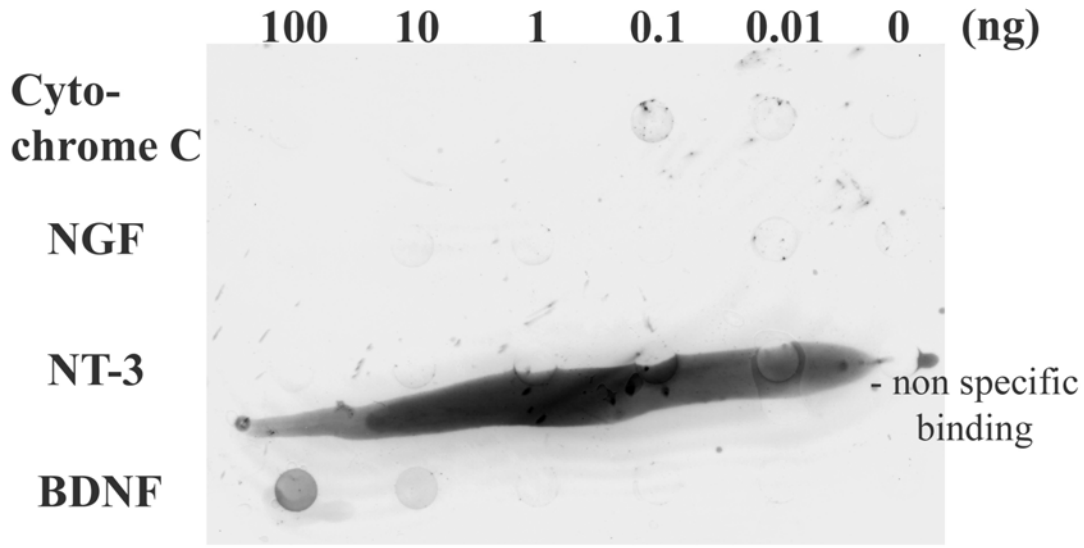

Figure Appendix-1. Dot Blot analysis to determine specificity of BDNF antibody. Nitrocellulose membrane was dotted with protein concentrations of neurotrophins and cytochrome C as indicated. The BDNF antibody recognized 100 ng and 10 ng of BDNF protein, but not the other proteins.