MECHANISMS CONTROLLING THE CELL BODY RESPONSE TO AXON

INUURY IN DORSAL ROOT GANGLION NEURONS

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ABSTRACT

Successful axon regeneration appears to depend on the development of an injury response. Dorsal root ganglion neurons exemplify the necessity of this injury response in a unique way. Peripheral nerve transection leads to development of an injury response and successful regeneration whereas central root transection does neither. The injury response may involve extracellular and intracellular pathways. To investigate the extraneuronal influences, we performed nerve transection of either the central or peripheral axon branches and studied the expression of GAP-43, a key growth associated protein, and the transcription factors ATF3, c-Jun, and STAT3. Our results show that the responses to peripheral versus central nerve transection are fundamentally different. Peripheral but not central nerve transection increases GAP-43, ATF3, and c-Jun expression. STAT3, however, is upregulated as a result of central but not peripheral nerve transection. To investigate potential intracellular signalling pathways, we applied FGF-2, an extracellular mitogen, or an analog of cAMP, an intracellular second messenger to the cut end of the peripheral axon. Our results indicate that FGF-2 and cAMP act as activators of GAP-43 expression. On the other hand, FGF-2 and cAMP act to downregulate the expression of ATF3. FGF-2 upregulates c-Jun and the activated form of STAT3. Paradoxically, the regulation of GAP-43 expression by cAMP or by FGF-2 *in vivo* shows opposing results from the previously reported *in vitro* studies. Our present results suggest that the peripheral nerve injury response may be governed by at least three different signalling pathways.

ACKNOWLEDGMENTS

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ADF   actin-depolymerizing factor

Akt   protein kinase B

AMPA   α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

AP-1   activator protein 1

ATF/CREB   activating transcription factor / cyclic AMP response element binding protein

ATF3   Activating transcription factor 3

ATP   adenosine-5'-triphosphate

B2   bradykinin receptor

BDNF   brain-derived neurotrophic factor

CaMKIV   calcium/calmodulin-dependent kinase IV

cAMP   Cyclic 3', 5'-adenosine monophosphate

CAP-23   cortical cytoskeleton-associated and calmodulin binding protein

CB1   cannabanoid 1 receptor

C/EBPbeta   CCAAT enhancer binding protein-beta

cGMP   guanosine 3',5'-monophosphate

CGRP   calcitonin gene related peptide

CMAK2   calcium/calmodulin-dependent kinase 2

CNS   central nervous system

CNTF   ciliary neurotrophic factor

CNTFRa   ciliary neurotrophic factor receptor a

CREB   cAMP response element binding protein

CREM   cAMP-responsive element modulator

dbcAMP   dibutyryl cyclic AMP

DC	direct current
DOR	δ opioid receptor
DREZ	dorsal root entry zone
DRG	dorsal root ganglion/dorsal root ganglia
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular regulated kinase
Erk5	extracellular regulated kinase 5
ETA	endothelin A receptor
ET1	endothelin 1
FG	FluorGgold®
FGF-2	fibroblast growth factor 2
FGFR1-3	fibroblast growth factor 2 receptors 1-3
gadd153/Chop10	Growth arrest and DNA damaging/ C/EBP-homologous protein 10
GAL1, 2	galanin receptor 1, 2
GAP-43	growth-associated protein 43
GDNF	glial cell line-derived neurotrophic factor
GFRα	glial cell line-derived neurotrophic factor receptor alpha
GLUR1, and 5	glutamate receptor1 or 5
gp130	glycoprotein-130
H1	histamine receptor
HCN1,2	hyperpolarization-activated cyclic nucleotide-gated potassium channel 1,2
HSPGs	heparan sulfate proteoglycans
HSPs	heat shock proteins

Hsp-27	heat shock protein-27
IB4	isolectin B4
IGF-1 and 2	insulin-like growth factors 1 and 2
IGFR	insulin-like growth factor receptor
IHC	Immunohistochemistry
IL-1β	interleukin-1 beta
IL-6	interleukin-6
JAK/STAT	anus kinase/signal transducer and activator of transcription
JNK	c-Jun-N-terminal kinase
JNK/SAPK	c-Jun-N-terminal kinase/stress-activated protein kinase
KOR	κ opioid receptor
LIF	leukemia inhibitory factor
LIF-R	leukemia inhibitory factor receptor
LL	large light neurons
LRF-1	liver regenerating factor-1
MAG	myelin-associated glycoprotein
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemotactic protein-1
MIP-1α	macrophage inflammatory protein-1alpha
MOR	GDNF family coreceptor, μ opioid receptor
NF200	neurofilament 200
NCAM	neural cell adhesion molecule
NF-κB	nuclear factor kappa light chain enhancer of activated B cells
NGF	nerve growth factor
NgR	Nogo receptor

NK1	neurokinin-1 receptor
NLS	nuclear localization signal
nmj	neuromuscular junction
NPY	neuropeptide protein Y
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
OMgp	oligodendrocyte-myelin glycoprotein
ORL1	opioid-like receptor 1
PACAP	pituitary adenylate cyclase-activating polypeptide
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PKCε	epsilon isoform of protein kinase C
PKA	protein kinase A
PLC	phospholipase C
PLD	phospholipase D
PNS	peripheral nervous system
PPT	preprotachykinin
pSTAT3	phosphorylated signal transducer and activator of transcription 3
P2X(3)	purinergic receptor 2X(3)
P2Y1	purigenic receptor 2Y1
p75NTR	p75 neurotrophin receptor
PSCs	perisynaptic Schwann cells
Rap1	Ras-proximate-1
RET	rearranged during transfection receptor
RGCs	retinal ganglion cells

RNA	ribonucleic acid
STATs	signal transducers and activators of transcription
SD	small dark
SSTR2a	somatostatin receptor
SOX11	sex determining region Y-box 11
SP	substance P
Sp1	specificity protein-1
STAT3	signal transducer and activator of transcription 3
TGF-α	transforming growth factor alpha
TGFβ1	transforming growth factor-beta 1
TNF-α	tumor necrosis factor alpha
TNFR	tumor necrosis family receptor
tPA	tissue plasminogen activator
trk (A, B, C)	tropomyosin-related kinase
TRP	transient receptor potential cation channels
TTX	tetrodotoxin
VIP	vasoactive intestinal polypeptide

CHAPTER 1
1.0 INTRODUCTION

Axonal injury as a result of axotomy, crush, chronic constriction ligation, or inflammation induces biochemical and molecular alterations in the neuron collectively referred to as the injury response (Stoll and Muller, 1999). This type of response occurs readily in mammalian peripheral nervous system (PNS) neurons (Bosse et al., 2006; Bosse et al., 2001), but to a much lesser extent in central nervous system (CNS) neurons (Bovolenta et al., 1992; Hagg, 2007). PNS neuronal response to injury is robust and leads to successful repair and regeneration (Monaco et al., 1992); whereas the injury response that occurs in CNS neurons is weak or absent, a fact that may contribute to the weak or absent regenerative capacity of CNS neurons (Fawcett, 1992; Tatagiba et al., 1997; Teng and Tang, 2006). A comprehensive understanding of the nature of the PNS injury response is yet to be attained. It is hoped that increased knowledge of the cellular mechanisms that mediate the injury response and lead to successful PNS regeneration will lead to unlocking the mysteries of poor regenerative response in the CNS.

1.1 Peripheral nerves.

The PNS includes all nerves that lie peripheral to and outside the pial covering of the CNS. Peripheral nerves contain motor, sensory and autonomic axons. Peripheral spinal nerves are covered by an outer connective tissue covering called the epineurium. Inside the epineurium, the collagenous perineurium covers bundles of individual fibers. Axons are covered by an intrafascicular connective tissue called the endoneurium. The endoneurium contains fibroblasts, macrophages and mast cells. Peripheral nerves are richly supplied by blood vessels. The presence of macrophages in the endoneurium and

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the rich blood supply are indispensable components of the regenerative response to injury

of the peripheral axon branch (Michael-Titus et al., 2010).

Axons within the nerve are intimately associated with Schwann cells along their

entire length. These Schwann cells enclose the axons either with a multi-layer myelin

sheath, or with a simpler ensheathing arrangement. Schwann cells also produce an

organized extracellular matrix in the form of a basal lamina (Kandel and Jessel, 2000).

1.1.1 Structure and organization of the dorsal root ganglion.

Dorsal root ganglia (DRG) are aggregates of sensory neurons located dorsally on

either side of the spinal cord. These sensory neurons are unique in that they are devoid of

dendrites typically seen in multipolar neurons, but instead have a single axon that

bifurcates to form two axon branches at a short distance from the cell body. The longer

branch extends into the periphery and forms the sensory endings in the skin, muscle,

viscera, tendons, and joints and other organs, and the shorter branch enters the spinal cord

through the dorsal root entry zone (DREZ) to form synapses on CNS neurons (Mescher,

2009).

These two axonal branches, though they originate from the same neuronal cell

body, respond to injury in dramatically different ways. The peripheral branch has an

intrinsic capacity to regenerate readily following injury, resulting in functional recovery.

However, the central branch appears to have a lower capacity to regenerate if it is injured.

This difference in regenerative capacity appears to be largely intrinsic (discussed below),

but the cellular mechanisms responsible for this difference are poorly understood.   The

fact that DRG neurons have two axon branches with fundamentally different injury

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response properties provides a unique opportunity to perform insightful experiments. Each axon branch can be manipulated individually, simultaneously, or at a spaced time interval. Likewise, changes in regenerative ability can be examined independently in each axon branch (Kandel and Jessel, 2000).

Early in embryonic development the DRG neurons start out with a bipolar morphology. Later, they undergo a fundamental morphological change, termed pseudo-unipolarization, to assume their mature pseudounipolar form. Specifically, on the fourteenth day of rat gestation, the vast majority of neurons are bipolar and spindle-shaped. As development proceeds, the initial portions of the central and peripheral neuronal processes gradually approach each other and fuse to form a common initial portion. Finally, at about the eighteenth day of gestation, the cytoplasm of this common initial portion becomes thinner and elongates to form the stem process of the mature pseudounipolar neuron (Matsuda et al., 1996; Matsuda et al., 2000; Matsuda and Uehara, 1984; Pannese, 1974). Little is understood about the developmental events that confer distinct identities on the common initial segment of the axon, or on the peripheral and central axon branches.

Morphometric analysis reveals that mature DRG neurons are endowed with small perikaryal projections that are enveloped by satellite cells (Pannese, 1981) a non-neuronal cell type found in ganglia, but not in the nerves. These projections are more numerous on the surface of the mature than on that of premature bipolar neurons and are believed to increase in number as the neuronal cell bodies grow larger. The perikaryal projections increase the neuronal surface area and neuron-satellite cell interface and improve the efficiency of metabolic exchange between these two cell types (Matsuda et al., 2000).

Under the light microscope, DRG neuronal cell bodies, devoid of dendrites, appear as large cells with large, centrally located nuclei displaying prominent nucleoli. Within a given DRG, there is heterogeneity in the size of the neurons. In the rat, DRG neurons can range between 15 – 100 μ in diameter. The two main morphological types of sensory neurons, large light (LL) and small dark (SD) neurons are intermixed within the ganglion (Hanani, 2005; Lawson, 1992; Price, 1985; Zochodne, 2008). According to the presence of Nissl substance (rough endoplasmic reticulum) LL neurons appear to be large and have less Nissl substance and thus appear and are classified as light. On the other hand, SD neurons appear to be small with higher concentrations of Nissl substance, and thus appear and are classified as dark. LL/SD neurons can also be subdivided on the basis of conduction properties. The axons of SD neurons are classified as C fibers (non-myelinated, slow conducting), whereas the axons of LL neurons are classified as A types (myelinated, fast conducting). Neurons within the A type are further classified as Aα, Aβ, and Aδ (Millan, 1999). Many of the SD cells are nociceptive and they function mainly in thermo- and mechanoreception, whereas LL neurons are low threshold mechanoreceptors (Lawson, 1992).

1.1.2 Biochemical markers of DRG neurons.

The previous LL and SD classification of DRG neurons is based on size and morphological appearance. However, with the advent of molecular biological techniques, a more current classification of DRG neurons has been in use based on specific markers. DRG neurons do not receive synapses; they are, however, endowed with receptors for numerous neurotransmitters (Devor, 1999; Julius and Basbaum, 2001).

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Additionally, DRG neurons express a diverse array of peptides, factors and specific biochemical markers. The expression of these factors set the stage for the biochemical classification of DRG neurons. Based on neurochemistry, morphology, trophic requirements and sensory modalities, DRG neurons are thus classified into three major types: small, medium, and large neurons.

Small DRG neurons are associated with C and Aδ axons. These small neurons selectively express peptides such as endothelin 1 (ET1), galanin, nociceptin, somatostatin, Substance P (SP), pituitary adenylate cyclase activating polypeptide (PACAP) , and vasoactive intestinal polypeptide (VIP). Small DRG neurons also express specific receptors such as the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)- and kainate-type glutamate receptors, bradykinin receptor (B2), histamine receptor (H1), epidermal growth factor receptor (EGF), fibroblast growth factor 2 (FGF-2) receptor, neurokinin-1 receptor (NK1), somatostatin receptor (SSTR2a), purigenic receptor (P2X3), galanin receptor (GAL2), endothelin A receptor (ETA), and glial cell line-derived neurotrophic factor (GDNF) family receptor (GFRα3). Small DRG neurons express ion channels including bradykinin, calcium-activated potassium channel, L and N-type calcium channels, potassium channel ($K_v1.4$), sodium channel ($Na_v1.9$), Naβ3, and members of the transient receptor potential cation channels including TRPA1, TRPM8, TRPV1, TRPV4, TRPV3 for a review please see (Zochodne, 2008).

Medium DRG neurons express calcitonin gene-related peptide (CGRP). The specific receptors expressed by this class of neurons are tropomyosin-related kinase A (trkA), p75, GFRα1, GFRα2, and rearranged during transfection receptor (RET). Other receptors are also include a GDNF family coreceptor, μ opioid receptor (MOR), δ opioid

receptor (DOR), κ opioid receptor (KOR), and opioid-like receptor 1 (ORL1) and P2X2/3. Small-to-medium neurons also express sodium channel proteins Na_v 1.7, 1.8 reviewed in (Zochodne, 2008).

Small diameter DRG sensory neurons that are nociceptors can be divided into two populations based on neurochmistry: isolectin B4 IB4-positive nonpeptidergic neurons, and IB4-negative peptidergic neurons (Stucky and Lewin, 1999). IB4-positive neurons depend on glial-derived neurotrophic factor (GDNF), whereas IB4-negative neurons depend on NGF for survival during postnatal development (Molliver et al., 1997). IB4-positive neurons are characterized by longer-duration action potentials than IB4-negative neurons. This difference in electrophysiological properties expressed by IB4-positive and IB4-negative small neurons may contribute to their distinct functions especially in neuronal response to injury (Fang et al., 2006; Stucky and Lewin, 1999).

Most of the neurons in the small and medium size groups are peptidergic and respond to nerve growth factor (NGF). Some of the calcitonin gene related peptide (CGRP) positive neurons also respond to GDNF (Nagy and Hunt, 1982).

Large DRG neurons express TRPV2, cannabanoid 1 receptor (CB1), GAL1, GLUR2/3, ganglioside receptor 1 (GM1), purigenic receptor (P2Y1), and trkC. This class of DRG neurons also expresses channels Naβ1, K_v 1.1, K_v β2.1, and hyperpolarization-activated cyclic nucleotide-gated potassium channel 1,2 (HCN1,2) (Devor, 2009; Kovalsky et al., 2009; Maher et al., 2009; Wickenden et al., 2009).

Interestingly, between 30-40% of the lumbar DRG neurons are grouped among the large and medium diameter neurons, and identified based on the expression of the heavy chain neurofilament, NF200. This subpopulation of neurons typically has large

myelinated axons and functions as mechanoreceptors and proprioceptors. The neurons

that express NF200 usually express trkA and trkC, as well as the p75 neurotrophin

receptor (p75NTR) (Kai-Kai, 1989), and reviewed in (Zochodne, 2008).

Approximately 70% of the neurons express one or more of the high affinity

neurotrophin receptors trkA, trkB or trkC (Lindsay, 1996a; McMahon et al., 1994;

Wright and Snider, 1995). While the trk family of receptors act +as high affinity

receptors for the neurotrophins (NGF, BDNF and neurotrophin 3 (NT3)), the GDNF

family of trophic factors exerts its actions by binding to two different receptors: a high

affinity receptor GFR-α, and a low affinity RET (Tucker and Mearow, 2008).

1.1.3 Anatomy of the rat sciatic nerve.

The peripheral axon branches of sensory neurons in the DRG contribute to the

formation of peripheral nerves. The sciatic nerve of the rat originates from the DRG at

lumbar spinal segments 4 to lumbar segment 6 (L4-L6). The L4 and L5 DRG contribute

almost all of their peripheral axons, along with a partial contribution from L6. The

sciatic nerve in the minor pelvis runs in the deep groove between the dorsal side of the

ischium and the sacral bone. It passes on the ventral side of the piriformis muscle after

leaving the sciatic notch. It courses over the quadratus femoris muscle obliquely to the

thigh region caudal to the piriformis muscle, where a small branch innervates the biceps

femoris, semi-tendinosus and semi-membraneous muscles. Then at the knee joint, it

terminates into its two main branches: posterior tibial and peroneal (fibular) nerves. The

tibial portion gives rise to the tibial and the sural nerves, and the peroneal portion gives

rise to the peroneal nerve and a cutaneous branch that perforates the lateral hamstring

muscles to innervate the proximolateral face of the calf (Schmalbruch, 1986; Uysal et al., 2009). Anatomical variations exist as to the origin of the rat sciatic nerve. The rat sciatic nerve in the Sprague-Dawley strain originates from L3 to L6. The major components are L4 and L5, since the contribution of both L3 and L6 nerves to the sciatic nerve is small (Asato et al., 2000). The first sacral segment (S1) has also been reported to contribute to the sciatic nerve (Uysal et al., 2009). Despite these anatomical variations, 98 to 99% of all sciatic DRG perikarya reside in the L4 and L5 DRG (Swett et al., 1991). The anatomy of the rat sciatic nerve enables the experimenter to easily access the nerve on the dorsal aspect of the thigh, allowing for various surgical manipulations that can be perfomed peripherally at any point along the course of the nerve.

DRG central branches extend through the dorsal roots and into the spinal cord through the DREZ. Fibers either terminate within a short distance or, travel up (the large and myelinated fibers) the dorsal columns via the fasicularis gracilis and fasicularis cuneatus to project to the dorsal column nuclei in the caudal medulla. Some sensory afferent fibers enter dorsal horn grey matter immediately to synapse with interneurons, projection neurons, or motoneurons (Zochodne, 2008).

1.1.4 Satellite cells.

Each DRG neuronal perikaryon is enveloped by its own satellite cell sheath forming a distinct morphological and functional unit (Hanani, 2005). The distance between satellite cells and the neuronal membranes is very small, about 20 nm (Pannese, 1981). DRG neurons invaginate microvilli-like structures into the surrounding satellite cells allowing for possible cell-cell communication between the two cell types (Pannese,

2002). The number of satellite cells per DRG neuron is size-dependant: the larger the neuron the more satellite cells that are present in the perikaryal area (Ledda et al., 2004).

Following axotomy (Humbertson et al., 1969; Shinder et al., 1999), or inflammation (Lu and Richardson, 1991), satellite cells proliferate, and increase in number around the damaged DRG neurons (Hanani et al., 2002; Shinder et al., 1999), indicating a possible role for satellite cells in the injury response. A function of phagocytosis of neuronal debris has also been ascribed to satellite cells (Pannese, 1978). Activated satellite cells have been found to upregulate expression of a number of growth and neurotrophic factors such as transforming growth factor alpha (TGF-α) (Xian and Zhou, 1999), FGF-2 (Grothe et al., 1997), and GDNF (Hammarberg et al., 1996), nerve growth factor (NGF) (Zhou et al., 1999), and neurotrophin-3 (NT-3) (Zhou et al., 1999), providing additional evidence that satellite cells are part of the overall injury response mechanism within the DRG.

1.1.5 Schwann cells.

Schwann cells provide support to both myelinated and unmyelinated axons throughout the PNS. For larger axons, Schwann cells form a multilayered lipid myelin sheath around the axons that can be several micrometers in thickness. The myelin sheath is not longitudinally continuous. The gaps devoid of myelin between adjacent Schwann cells are called the nodes of Ranvier. The myelin sheath insulates the axon and decreases its membrane capacitance, thereby increasing the conduction velocity of the nerve impulse (Berta et al., 2008; Renganathan et al., 2001). The presence of voltage gated sodium channels located preferentially at the nodes of Ranvier facilitates action potential

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conduction through what is called saltatory conduction. This is achieved by re-propagation of the impulse at the nodes of Ranvier, thereby increasing the fidelity and velocity of conduction.

Myelinating Schwann cells form the myelin sheath during late development and work by wrapping around and ensheathing the axon. Schwann cells are also essential for the maintenance of healthy axons. They produce a variety of supporting factors, including neurotrophins, and may also provide nutritive support and maintenance of ionic balance in the extracellular space (discussed below).

Schwann cells can also provide support to smaller PNS axons by ensheathing them, without forming myelin. Non-myelinating Schwann cells maintain axons and are crucial for neuronal survival. Some non-myelinating Schwann cells surround small C-fiber axons that are in close proximity and form what is termed as a Remak bundle. A Schwann cell in this bundle keeps the small axons from touching each other by interposing its cytoplasm between the axons, thus providing both structural and metabolic support (Andres et al., 1985; Griffin and Thompson, 2008; Murinson et al., 2005; Pannese et al., 1988a; Pannese et al., 1988b; Pannese et al., 1988c; Peyronnard et al., 1975). In addition, non-myelinating perisynaptic Schwann cells (PSCs) have distinct roles in the neuromuscular junction. PSCs cover the neuromuscular synapses and activate neurotransmitter receptors (Auld and Robitaille, 2003). The location of these perisynaptic Schwann cells at the synapse supports the idea of a role in the formation and maintenance of synaptic connections and transmission. Perisynaptic Schwann cells have purinergic and muscarinic receptors that can cause release of internal stores of calcium to generate calcium waves upon binding of purines and acetylcholine (Jahromi et al., 1992).

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A number of neurotrophins have been implicated in maintenance of the neuromuscular junction synapse. For example, BDNF and CNTF may act cooperatively to modulate and maintain proper functioning of synapses (Stoop and Poo, 1996a; b). In addition to CNTF and BDNF, NT-3 present at the synapse modulate the Ca^{++} release and thus differentially regulate perisynaptic Schwann cell functions through modulating the purinergic or cholinergic signalling pathways (Todd et al., 2007). Furthermore, PSCs maintain synaptic growth, and repair (Griffin and Thompson, 2008).

During early neurogenesis, Schwann cells do not survive without signals originating from the developing axons such as neuregulins and endothelins (Jessen and Mirsky, 2002; Mirsky et al., 2002). Schwann cells may carry out other specialized functions in growth, maintenance, and repair of peripheral nerves as well (Clarke and Richardson, 1994). Schwann cells play an important role in early peripheral nerve formation, and provide tropic and trophic support (Bray et al., 1981; Lundborg et al., 1994). Tropic support is provided by extracellular matrix molecules (ECM) that are involved both in cell adhesion and migration. It is well established that the expression of laminin, neuronal cell adhesion molecule (NCAM), fibronectin and tenascin are all increased on the surface of Schwann cells during peripheral nerve regeneration process (Bailey et al., 1993; Martini, 1994). Trophic support is provided by a number of growth factors such as NGF, and the transforming growth factor β family (TGFβ) (Scherer et al., 1993). By providing both structural and biochemical support, Schwann cells are considered an integral part of the integrity and function of the peripheral nervous system in both normal and injured states.

1.1.6 Macrophages.

In the peripheral nervous system, macrophages are present in normal DRG tissues (Arvidson, 1977; Hamburger and Levi-Montalcini, 1949; Pannese, 1978; Perry and Gordon, 1988; Scaravilli et al., 1991; Stevens et al., 1989). Macrophages are found primarily perineuronally in contact with the neuron/satellite cell complex. A few exist in the perivascular regions, while others exist in interstitial position not in apparent contact with either blood vessels or neurons (Lu and Richardson, 1993).

Peripheral nerve trauma and the ensuing injury-induced inflammation, together with the increase in macrophages, play important roles in neuronal survival and axonal regeneration after injury (Richardson and Lu, 1994). The number of macrophages in DRG increases 2-4 days after sciatic nerve axotomy and remains elevated for four weeks. It has been proposed that injury-induced recruitment and/or proliferation of macrophages in the injured DRG contribute to the survival or regeneration of axotomized neurons (Lu and Richardson, 1993), potentially supplying neurotrophic support to nerve cell bodies (Richardson and Lu, 1994). Macrophage proliferation and recruitment in injured DRG also increases expression of certain neuronal mRNAs (Lu and Richardson, 1995). Macrophages produce cytokines and may play a role in the generation of neuropathic pain (Hu and McLachlan, 2002).

In injured peripheral nerves, resident and recruited macrophages function in the removal of debris of degenerating nerves, and to induce Schwann cell proliferation (Beuche and Friede, 1986). Direct injection of isogenous macrophages into the DRG enhances sciatic nerve regeneration *in vivo* (Lu and Richardson, 1991), and neurite growth *in vitro* by cultured DRG neurons (Hikawa et al., 1993; Luk et al., 2003).

Macrophages therefore appear to be key players in the mounting of a successful regenerative response in the peripheral nervous system.

Macrophages and Schwann cells interact during the injury response, particularly in Wallerian degeneration. Wallerian degeneration is part of the neuronal response to injury. In particular, peripheral nerves undergo a process of degeneration of distal aspects of a nerve axon following injury to the cell body or proximal portion of the axon. The process is also characterized by fragmentation of the axon and its myelin sheath, resulting in atrophy and destruction of the axon. A detailed description of Wallerian degeneration process is given below (section 1.2.3).

When the proximal stump of a transected nerve is separated from the distal stump, Schwann cells proliferate. Proliferating Schwann cells then co-migrate with regrowing axons and guide bundles of regrowing axons across interstump gaps (Hall, 1989).

Processes such as initiation of myelin breakdown, subsequent macrophage recruitment, and activation and myelin phagocytosis could not be achieved without the proper molecular communication and interaction between the Schwann cells and macrophages (Martini et al., 2008). A Phospholipase A_2 signaling cascade appears to play a very important role in both cell types, initiating the breakdown of compact myelin, and inducing chemokine and cytokine expression. Cytokines such as monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1α), and Interleukin-1 beta (IL-1β) are essential activators of subsequent macrophage recruitment and myelin phagocytosis (Martini et al., 2008). Thus, the interaction between Schwann cells and macrophages with the cellular and extracellular components in the PNS may

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determine the degree of tissue inflammation and repair processes such as remyelination associated with neuronal growth and nerve regeneration.

1.1.7 Neurotrophins.

Neurotrophins are a family of highly conserved extracellular ligands that affect the differentiation, survival and biological function of neuronal cells in vertebrates. The family includes NGF, BDNF, NT-3 and neurotrophin-4/5 (NT-4/5) (Gotz and Schartl, 1994). Neurotrophins enhance neuronal cell survival and growth during the development of DRG neurons. They influence the proliferation and differentiation of neuron progenitor cells and regulate the expression of several differentiated traits of neurons in early development and throughout life (Davies, 1994a; b). In the PNS, a rapid neuronal cell body reaction and nuclear responses to distally-derived neurotrophins require retrograde transport of ligand-receptor complex (Watson et al., 1999). The survival of the developing neuron is promoted by and depends upon neurotrophins primarily synthesized and released by target tissues. All members of the neurotrophin family bind a common p75 receptor with low affinity. However, there are also high affinity tropomyosin-related kinase (trk) receptors which more selectively recognize each of the individual neurotrophins. Thus, NGF binds to trkA, BDNF and NT-4/5 bind to trkB, and NT-3 binds to trkC receptors. NT-3 promiscuously binds to the other trk receptors at a lower affinity (Zochodne, 2008). When neurotrophins bind trk receptors at the nerve terminals, the receptor/ligand complexes are endocytosed and trafficked through the axon back to the cell body. Endocytosed neurotrophins/trk receptor complexes activate the extracellular signal-related protein kinase 5 (Erk5) pathway, causing nuclear translocation

of Erk5, and the phosphorylation of cAMP response element binding protein (CREB). Therefore, activation of the Erk5 pathway plays an important role in retrograde signaling, leading to enhanced neuronal survival (Watson et al., 2001). Because of the molecular and biological specificity of the mechanisms of action of neurotrophins, especially in nerve regeneration, interest into designing derivatives and analogues as potential therapeutic agents has been an active area of research in pharmacology (Ibanez, 1995; Lindsay, 1996b).

The importance of retrograde transport comes from experiments where dynein-based transport is inhibited. Under these conditions, neurotrophin stimulation of axon terminals will not support survival. This indicates that defects in dynein-based retrograde transport obstructs the positive survival effects of target-derived trophic factors, leading to degeneration of target-dependent neurons (Heerssen et al., 2004). Receptor mediated retrograde axonal transport of neurotrophic factors is increased after peripheral nerve injury (DiStefano and Curtis, 1994). The neurotrophins NGF, BDNF, and NT-3 display distinct patterns of retrograde axonal transport in peripheral neurons (DiStefano et al., 1992). In addition to peripheral targets, Schwann cells and macrophages themselves have also been identified as potential sources for supplying neurotrophic factors to DRG neuronal cell bodies following injury (Richardson and Lu, 1994).

Sources of neurotrophic factors not only can be from the target, but also from surrounding glial cells or the neuron itself and mediate their effects via receptor-mediated uptake and specific retrograde transport or via an autocrine or paracrine mechanisms (Korsching, 1993).

1.1.7.1 Nerve growth factor (NGF).

In contrast to other typical growth factors, NGF is not a mitogen. NGF is a neurotrophic molecule essential for the survival, development and maintenance of function of specific populations of peripheral and central neurons (Thoenen et al., 1985). NGF was initially discovered as a survival and outgrowth-promoting factor by Levi-Montalcini (Angeletti et al., 1968; Levi-Montalcini, 1964; 1975; 1976; 1979; 1987; Levi-Montalcini and Angeletti, 1968; Levi-Montalcini and Calissano, 1979; Varon, 1968). NGF selectively stimulates the growth of sympathetic and embryonic spinal sensory ganglia. Target-derived NGF promotes the phenotypic maintenance of DRG nociceptive neurons (Delcroix et al., 2003).

As a result of crush injury to rat sciatic nerve, NGF accumulates at the distal side of the crush site, whereas no NGF is detectable proximal to the crush (Korsching and Thoenen, 1983). Two-site enzyme immunoassay for NGF shows that NGF is present in the peripheral but not the central axonal projections. It is thus concluded that NGF supply comes exclusively from peripheral and not central axon branches (Korsching and Thoenen, 1985). Following peripheral denervation NGF level in the target increases (Korsching and Thoenen, 1985). This supports the role of target-derived growth factors in controlling target organ re-innervation. Although NGF is required for DRG neuron survival during early development, by adulthood, not all DRG sensory neurons require NGF for survival (Levi-Montalcini, 1987; Lindsay, 1988). Some subpopulations of sensory neurons that do not require NGF for survival, respond instead to other neurotrophic factors such as BDNF or NT-3 for prolonged survival (Vogel, 1993).

NGF is also produced by cells other than target organs such as Schwann cells, macrophages, or the DRG neurons themselves (Ernfors et al., 1990; Schecterson and Bothwell, 1992), suggesting an autocrine, and/or paracrine (Lindsay, 1996a) function of NGF in adult DRG.

NGF exerts its effects mainly by interaction with its high affinity specific receptor trkA. This leads to the activation of several intracellular signaling pathways. One such effect is an increase in intracellular calcium levels. Calcium and calmodulin are both necessary for the acute activation of extracellular signal-regulated kinases (ERKs) after trkA stimulation (Egea et al., 2000). Therefore, NGF activation of the ERK pathway is facilitated by Ca^{+2} ions and calmodulin.

Studies provide evidence for the presence of endosomes within DRG neurons. The endosomes contain NGF, activated trkA, and signaling proteins of the Ras-proximate-1 Rap1/Erk1/2, p38MAPK, and PI3K/Akt pathways, and are retrogradely transported in the isolated sciatic nerve *in vitro* (Delcroix et al., 2003; Grimes et al., 1996; Tsui-Pierchala et al., 2000; Wu et al., 2001). NGF injection in the peripheral target of DRG neurons increased the retrograde transport of the endosomes. Conversely, NGF antibody injections decreased the retrograde transport of the endosomes, indicating that signaling endosomes convey NGF signals from the target of nociceptive neurons to their cell bodies (Delcroix et al., 2003). NGF trophic signaling may also be facilitated by the RET coreceptor. Therefore, NGF may promote phosphorylation of a number of signaling proteins resulting in augmented growth, metabolism, and gene expression associated with sensory neuron function (Tsui-Pierchala et al., 2002).

It has been shown that retrograde survival signals can be carried by a mechanism activated by NGF at the axon terminal surface and travels to the cell body without the transport of NGF endosomes (Campenot and MacInnis, 2004). This mechanism of retrograde signaling adds to the complexity of retrograde signaling mechanisms.

1.1.7.2 Brain-derived neurotrophic factor (BDNF).

BDNF was first isolated from pig brain (Barde et al., 1982). The BDNF gene was later cloned and characterized (Leibrock et al., 1989; Thoenen, 1991; Thoenen et al., 1991) and the deduced amino acid sequence of BDNF revealed a high degree of homology to NGF. BDNF protein is 120 AAs long and shares about 54% homology to NGF, particularly in the cysteine residues and in the sequences flanking these residues. Moreover, like NGF, BDNF rescues a subset of sensory neurons from naturally occurring cell death. Despite their structural resemblance, there are differences between BDNF and NGF in their biological activity and neuronal specificity. They both are target-derived neurotrophic factors that play important roles in the development and maintenance of multiple neuronal types of both the CNS and PNS, but especially primary sensory neurons (Lindsay, 1996a). BDNF may also act by an autocrine route in some of these sensory neurons (Davies and Wright, 1995).

The biological activity of BDNF is mediated by the tyrosine kinase receptors trkB and trkC. Of those two, trkB is the preferred receptor for BDNF (Glass et al., 1991; Ip et al., 1991; Lamballe et al., 1991a; b; Squinto et al., 1991). Trk receptors are autophosphorylated upon binding to their ligands. This leads to phosphorylation of downstream intracellular signaling proteins that induce cell growth and differentiation.

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In cultured neurons and hippocampal slices the transcription factor cyclic AMP response element-binding protein (CREB) regulates BDNF-induced gene expression. Exposure of neurons to BDNF stimulates CREB phosphorylation and activation via at least two signaling pathways: by a calcium/calmodulin-dependent kinase IV (CaMKIV)-regulated pathway that is activated by the release of intracellular calcium and by a Ras-dependent pathway. Activation of CREB, therefore, plays a central role in mediating BDNF responses in neurons (Finkbeiner et al., 1997). The mechanism of action for BDNF is similar to that of NGF, in activating calcium/calmodulin-dependent kinases, important regulatory pathways contributing to survival and regenerative growth.

BDNF prevents axotomized retinal ganglion cell death through MAPK and PI3K signaling pathways. This BDNF-mediated signaling pathway involves activation of both MAPK and Akt on the axotomized adult rat retina, and the collaboration of both MAPK and PI3K-Akt pathways seems to be necessary in neuroprotective signaling in axotomized retinal ganglion cells (RGCs) (Nakazawa et al., 2002). This provides additional evidence that BDNF and NGF share some common regulatory pathways. Indeed, in a peripheral nerve transection injury model, BDNF administration to the ends of the cut axons enhances functional recovery (Utley et al., 1996; Yin et al., 1998).

1.1.7.3 Neurotrophin-3 (NT-3).

The amino acid sequence of Neurotrophin-3 (NT-3) is 50% homologous to that of NGF and BDNF (Barde, 1990; Hohn et al., 1990), and is conserved across different species. NT-3 binds the trkC receptor and is abundant in both central nervous system, and peripheral nervous system sensory neurons (Katoh-Semba et al., 1996; Schecterson

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and Bothwell, 1992). Specifically, the DRG is a source of NT-3 that may be supplied to the spinal cord, and contributes to plasticity in the spinal cord after injury (Wang et al., 2008). NT-3 deficient mice show 65% loss of DRG neurons, suggesting an early role of NT-3 during neurogenesis (Airaksinen and Meyer, 1996; Maness et al., 1994). Thus it is of interest to determine whether NT-3 can promote peripheral sensory neuron survival and regeneration in the adult following injury (Yin et al., 1998).

1.1.7.4 Glial cell line derived neurotrophic factor (GDNF).

Glial cell line derived neurotrophic factor (GDNF) was first purified from midbrain dopaminergic neurons (Ebadi et al., 1997; Lin et al., 1993). GDNF is a potent survival factor for motoneurons that is present in peripheral nerve and muscle (Henderson et al., 1994), and for sympathetic, parasympathetic, proprioceptive, enteroceptive and small and large cutaneous sensory neurons (Buj-Bello et al., 1995). GDNF is found in a variety of peripheral organs more than in the neuronal tissues, indicating its function as a target-derived neurotrophic factor. GDNF mRNA is expressed in the sciatic nerve and DRG. Following sciatic nerve axotomy, GDNF mRNA levels increase dramatically in the sciatic nerve, implicating GDNF in the overall peripheral nerve resposnse to injury (Lapchak et al., 1996; Trupp et al., 1995). These reports suggest that GDNF is essential for the survival of multiple PNS and CNS neurons at different stages of their development (Ebadi et al., 1997).

GDNF signals through a multicomponent receptor complex consisting of RET receptor tyrosine kinase and a member of the GDNF family receptor alpha (GFRα). This complex eventually activates the RAS and PI3-K pathways. This leads to the activation

of CREB and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Hayashi et al., 2000; Poteryaev et al., 1999; Trupp et al., 1999). GDNF also activates the MAPK pathway (Chen et al., 2001). In early embryogenesis, GDNF-stimulated PI3K and Akt activities exert opposing effects on the ERK pathway, protecting neuroectodermal cells from apoptosis during their migration in the foregut (Mograbi et al., 2001). These findings show evidence that GDNF activates some pathways in common with other neurotrophins.

1.1.7.5 Ciliary neurotrophic factor (CNTF).

Ciliary neurotrophic factor (CNTF) was initially identified and characterized in the intraocular (ciliary) muscles of chick eyes as a target-derived neurotrophic molecule that supports the survival of parasympathetic chick ciliary neurons in culture (Adler et al., 1979; Barbin et al., 1984). The spectrum of CNTF biological activity infuences a much broader range of the components of the nervous system, since peripheral and central neurons also respond to CNTF (Ip et al., 1991). It has been shown that CNTF also supports the survival of sympathetic, sensory and spinal motoneurons (Thoenen, 1991).

CNTF is a member of the hematopoietic cytokine family and shares structural and functional properties with the members of this family such as LIF and IL6. It is composed of a four-helix bundle structure and shares the transmembrane signal transducing proteins, glycoprotein-130 (gp130) and leukemia inhibitory factor receptor (LIF-R). CNTF binding activates signal transduction cascades mediated by the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and Ras/MAPK signaling pathways (Inoue et al., 1996).

Evidence indicates that CNTF plays a key role in the injury response in the nervous system. Dramatic changes in the level of expression of CNTF occur following neural trauma in the PNS. Prior to nerve injury, high levels of CNTF mRNA and protein were localized within the Schwann cells of the sciatic nerve (Abe et al., 2001; Hu et al., 2005; Stockli et al., 1991). However, following peripheral nerve injury, the CNTF mRNA and protein levels decrease dramatically in the distal nerve (Rabinovsky et al., 1992), thus change in CNTF expression is one of the early markers of neural injury. CNTF binds to ciliary neurotrophic factor receptor a (CNTFRa) receptor to initiate its biological response in neuronal tissues, and shares receptor components with other hematopoietic cytokines (Ip and Yancopoulos, 1996). Interstingly, BDNF and CNTF may act cooperatively in modulating the development and functioning of synapses, indicating a different level of the complex nature of neurotrophin actions (Stoop and Poo, 1996b).

1.1.7.6 Leukemia inhibitory factor (LIF)

Leukemia inhibitory factor (LIF) is a cytokine structurally homologous to CNTF (Ip and Yancopoulos, 1996). Furthermore, the receptors for LIF and CNTF share two identical subunit components, which are believed to mediate their overlapping biological activity (Ip and Yancopoulos, 1996). However, LIF only requires the receptor components gp130 and LIFRβ for signal transduction (Davis et al., 1993). Because of the structural and biochemical similarities between the two factors, and because LIF induces biological activity on several neuronal classes (Ip and Yancopoulos, 1996), it is reasonable to ask whether LIF would also exert trophic activity on neuronal cells.

Interestingly and of direct relevance to this project is the fact that the signal of the

cytoplasmic regions of leukemia inhibitory factor receptor (LIFR) alpha-subunit and

gp130 involves signal STAT3 activation (Liu et al., 1999).

LIF expression is induced by injury and is required for the neuronal response to

injury (Sun et al., 1996; Sun and Zigmond, 1996a; b; Zigmond et al., 1996). It can act as

a survival factor for injured peripheral neurons. When applied to peripheral nerves in

vivo, the cytokine is retrogradely transported and rescues damaged sensory neurons

(Curtis et al., 1994).

1.1.7.7 Insulin-like growth factors-1 and 2 (IGF-1 and IGF-2).

Insulin-like growth factors (IGF-1 and IGF-2) are proteins that share a high

sequence similarity to insulin. They are recogized by their respective receptors IGFR1

and IGFR2. IGFs exhibit neurotrophic properties similar to NGF, including the capacity

to enhance neurite formation and extension (Ishii et al., 1985; Mill et al., 1985). IGFs

can act in combination with other growth factors such as fibroblast growth factors (FGFs)

to elicit their neurotrophic effects (Torres-Aleman et al., 1990a; b).

IGFs have been demonstrated to both promote and sustain the regenerative

process in a peripheral nerve injury model (Hansson, 1993; Ishii et al., 1994; Ishii et al.,

1993). Exogenous administration of IGF-2 increases the rate of peripheral nerve

regeneration, and endogenous IGFs in nerves are required to maintain a specific rate of

regeneration (Glazner et al., 1993). Target-derived and nerve-derived IGFs can regulate

peripheral nerve regeneration following injury (Ishii et al., 1994). In particular, sensory

neurons respond with enhanced regenerative growth to insulin and IGF-1 (Fernyhough et al., 1993).

IGF-1 has been reported to have an inductive effect on the expression of GAP-43 expression (Lutz et al., 1999). Of particular relevance to this project is the finding that in primary Schwann cell culture IGF-1 alone was ineffective, but in the presence of forskolin or dibutyryl cyclic AMP (dbcAMP), IGF-1 became a potent mitogen, stimulating Schwann cell proliferation (Schumacher et al., 1993). These results suggest a regeneration promoting effects of IGF-1 on peripherally transected sciatic nerve.

1.1.7.8 Fibroblast growth factor-2 (FGF-2).

Fibroblast growth factors are expressed in dorsal root ganglion neurons. Acidic fibroblast growth factor (aFGF) and its receptors (FGFR-1 and FGFR-2) have been reported to be expressed and maintained in postmitotic dorsal root ganglion neurons (Oellig et al., 1995).

Basic fibroblast growth factor (FGF-2) and its receptors have been described as important physiological modulators of neurotrophins and in peripheral nervous system regeneration (Grothe et al., 2006; Grothe et al., 1997; Grothe and Nikkhah, 2001; Grothe and Wewetzer, 1996). FGF-2 is one of more than 23 members of the FGF family (Ornitz and Itoh, 2001). FGFs can mediate their biological activities through two types of binding sites, the low-affinity binding sites represented by heparan sulfate proteoglycans (HSPGs) and the high-affinity tyrosine kinase transmembrane receptors (FGFRs) (McKeehan et al., 1998). FGF-2 protein exists in different isoforms representing different translation products from a single mRNA (Florkiewicz et al., 1991a;

Florkiewicz et al., 1991b; Florkiewicz and Sommer, 1989). Peripheral nerve lesion causes differential increase of the FGF-2 isoforms (Grothe et al., 1997; Meisinger and Grothe, 1997).

FGF-2 and its receptors are expressed in the DRG and the peripheral nerves and are upregulated in the DRG and in the proximal and distal nerve stumps following peripheral nerve injury. There are two major sources of FGF in the peripheral nerve, neurons in the ganglia, and at the lesion site of the nerve, where Schwann cells and invading macrophages represent the main sources of FGF-2 and its receptors FGFR1-3 (Grothe and Nikkhah, 2001).

FGF-2 is localized in a subpopulation of small and medium neurons in adult DRG from all axial levels. It is colocalized with the somatostatin/bombesin expressing subpopulation but not with substance P (Weise et al., 1992). FGF-2 signaling does not appear to regulate CGRP expression *in vivo* (Jungnickel et al., 2005).

When applied exogenously, FGF-2 rescues injured sensory neurons and facilitates neurite outgrowth of injured peripheral nerves. FGF-2 exerts a protective effect on the maintenance of transected sensory neurons. Specifically, FGF-2 protects DRG neurons from lesion-induced death, possibly via local accumulation of neurotrophic activities at the proximal nerve stump that have been elicited by FGF-2 (Otto et al., 1987). Moreover, FGF-2 promotes neurite extension and vascularization of the regenerating nerve fibers crossing the gap between the proximal and distal stumps of the transected sciatic nerve (Aebischer et al., 1989; Danielsen et al., 1988).

FGF-2 is also expressed endogenously within both the developing and adult DRG neurons. In the adult rat, FGF-2 is found in subpopulations of sensory neurons within the

DRG (Weise et al., 1992). In addition, FGF-2 and FGFR1 immunoreactivity are co-localized in adult rat DRG neurons (Grothe and Wewetzer, 1996; Meisinger et al., 1996a; Meisinger et al., 1996b). Thus FGF-2 and its receptors are constitutively expressed in developing and adult DRG neurons. FGF-2 is also expressed in Schwann cells and macrophages, thus it is suggested that FGF-2 can act in concert with other cytokines, factors, or neurotrophins (Grothe et al., 2006; Grothe and Nikkhah, 2001).

In frog retinal ganglion cells, FGF-2 upregulates the synthesis and alters the distribution of the axonal growth-associated protein GAP-43 expression after optic nerve injury (Soto et al., 2003), enhancing regenerative axon growth. It is not known whether a similar biological activity may also be involved in regenerative growth in DRG neurons.

Northern blot analysis of adult rat DRGs reveals the presence of FGF-2 transcript (Grothe and Meisinger, 1995). Ligation of the sciatic nerve does not lead to proximal or distal accumulation of FGF-2 immunoreactivity (Grothe and Wewetzer, 1996). Transection of the sciatic nerve elevates the expression of FGF-2 and FGFR-1 in DRG neurons in both postnatal and adult rats (Grothe and Wewetzer, 1996). This pattern of FGF expression is consistent with FGF involvement in the regulation of axon regrowth. Intraganglionic expression of FGF-2 may indicate the presence of an autocrine/paracrine loop (Murphy et al., 1994).

Mice lacking FGF-2 showed faster sensory recovery two weeks after peripheral nerve injury. The regenerating fibers of the mutant mice showed both significantly increased axon and myelin size, suggesting that loss of FGF-2 could expedite restoration of mechanosensory function by accelerating structural recovery, possibly by regulation of Schwann cell differentiation (Jungnickel et al., 2004; Jungnickel et al., 2009). This

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study, however, was performed on mice that were developmentally deficient in FGF-2. The scope of the implications of not having FGF-2 pathway present during normal neurogenesis of the sensory neurons could have influenced the outcome of this particular experiment; therefore, the results in the absence of FGF-2 should not be taken as the sole reason for having potential faster rate of regeneration.

On the other hand, the same research group reported that mice over-expressing FGF-2 had faster nerve regeneration after sciatic nerve injury. The number of regenerated axons was shown to be smaller in transgenic mice one week after crush injury, suggesting a role of the elevated expression of FGF-2 in early peripheral nerve regeneration by regulating Schwann cell proliferation, axonal regrowth, and remyelination (Jungnickel et al., 2006). Despite this reported increased rate of sciatic nerve regeneration, mice lacking FGF receptors FGFR-1 and FGFR-2 showed degeneration of sensory axons of Remak bundle and impaired thermosensory functions, resulting in c-fiber neuropathy (Furusho et al., 2009).

Different members of the FGF family are expressed following sciatic nerve injury. Using cDNA array, it was reported that FGF 2 and FGF 7 were significantly increased in the DRG after 28 days following nerve transection. Whereas about 60 % of the uninjured neurons expressed FGF 13, the percentage dropped to 18% following injury (Li et al., 2002). These data support the notion that FGF-2 plays a key role in the process of neuronal inury response mechanism.

In cultured DRG neurons, FGF-2 was found to repress GAP-43 expression in a dose dependent manner. This repression of GAP-43 by FGF-2 was reversed by

MEK/ERK inhibition, suggesting that the repressive action of FGF-2 occurs, at least in part, through the MEK pathway (Schreyer, 2004).

In contrast to this study, it was recently shown that Sprouty2, a negative feedback loop modulator that limits the intensity and duration of tyrosine kinase activation is expressed in adult sensory neurons (Hausott et al., 2009). Down-regulation of Sprouty2 enhances regenerative axon growth by adult sensory neurons, associated with enhanced FGF-2-induced activation of the ERK and Ras signalling pathway. In contrast, Sprouty2 overexpression inhibited axon growth (Hausott et al., 2009). It appears, therefore, that Sprouty2 may inhibit the influence of FGF-2 via Ras/Raf/ERK pathway. Because of the potential importance of FGF-2 in peripheral nerve regeneration and maintenance of sensory neurons, FGF-2 was selected in the current project as one of the main potential exogenous modulators of injury-induced biochemical markers.

1.2 Peripheral nerve injury-response.

As a consequence of peripheral nerve axotomy, between 10-40% DRG neurons die within a week of axotomy (Aldskogius and Risling, 1981; Arvidsson et al., 1986; Himes and Tessler, 1989; Liss et al., 1994; McKay Hart et al., 2002; Rich et al., 1989; Schmalbruch, 1987). Factors such as proximity of axotomy to the DRG (Ygge, 1989), the age of the animal (Bahadori et al., 2001; Kerezoudi et al., 1995), and the species used (Kline et al., 1964a; b) have been shown to influence the regenerative capacity and the outcome of neuronal death in response to injury. The closer the axotomy to the DRG ganglion perikarya, the more drastic is the rate of DRG neuronal loss (Ygge, 1989). The

process of cell death described here is likely to be apoptotic, due to the immediate loss of target derived neurotrophins and supporting factors.

The DRG neurons that survive following peripheral nerve transection undergo a morphological change termed chromatolysis, in which the injured neurons swell. Their nuclei move to an eccentric position, concomitant with increases in RNA synthesis. Morphological changes may be induced in injured DRG neurons as a result of restructuring of cytoskeletal elements such as neurofilaments (Oblinger et al., 1989).

Biochemical changes that ensue following injury to DRG neurons include, but are not limited to, changes in gene transcription, changes in translation, stability, or subcellular localization of translated proteins, and post-translational modification of proteins (Bhave and Gereau, 2004; Caroni, 1998). Most of these biochemical changes are restored to normal levels upon successful reinnervation of the target tissues. Other biochemical changes may arise at a distance from the neuronal cell body, namely at the injured axon tip. Local translation of axonal mRNA coding for proteins with nuclear localization signals (NLS), and of critical carrier proteins (including vimentin) that link diverse signaling molecules to the dynein retrograde motor at the injury site have been reported. These proteins are retrogradely transported to the cell body as part of the injury response (Hanz and Fainzilber, 2006; Hanz et al., 2003). Additionally, local translation of other cytoskeletal elements such as β-actin, α-tubulin, low molecular weight neurofilament, Tau, actin-depolymerizing factor (ADF), EphA2, and importins have also been reported at the site of injury (Willis et al., 2005).

DRG neuronal somata are electrically excitable, and some are able to generate action potentials repetitively on sustained depolarization. It has thus been hypothesized

that electrical properties of the soma may be required to insure reliable propagation of impulses past the DREZ and into the spinal cord (Devor, 1999). The disruption of this normalized electrical activity by axotomy is one of the earliest events in DRG neuronal response to injury (Liu et al., 2000). One of the major electro-physiological effects of peripheral nerve transection is to trigger an ectopic afferent action potential barrage carried by DRG neurons afferents into the spinal cord (Liu et al., 2000).

Axonal injury can alter the electrophysiological properties of DRG neurons (Abdulla and Smith, 2001a; b; Bishop, 1982; Ma et al., 2003). Peripheral nerve injury can change the receptor phenotype of both small and large neurons and may, as a result, have differential effects on the membrane electrical properties of these neuron types (Xu et al., 1997). Peripheral nerve injury can also lead to an abnormal afferent barrage generated in the cut end of the nerve (Wall et al., 1979). This abnormal discharge can amplify and or distort naturally generated signals (Wall and Gutnick, 1974a; b; Wall et al., 1974). The changes in electrical properties of the injured peripheral nerve have been linked to increased sensitivity and hyperalgesia associated with the injury (Wall, 1984). Following crush lesion, continuous conduction has been found in the regenerating processes of myelinated dorsal root fibers in the rat 12-20 days after injury (Feasby et al., 1981). The initial discharge of electrical activity may be one of the very earliest events that take place in response to injury, prior to any other visible morphological signs.

Ion channels present in the DRG neurons show changes as a result of neuronal injury. At least six different sodium channels are known to be expressed in DRG neurons, including several sensory-neuron-specific sodium channels that are not present in other parts of the nervous system (Waxman et al., 1999). Following injury to their

peripheral axons, DRG neurons down regulate some sodium channel genes and upregulate others. As a result, different groups of sodium channels are inserted into the DRG neuron cell membrane following injury (Goldin et al., 2000; Waxman et al., 2000). Axotomy reduces the action potential threshold by significantly increasing the expression of tetrodotoxin (TTX)-sensitive sodium currents (Zhang et al., 1997). Injured neurons also increase the expression of voltage-gated sodium channels $Na_v1.3$ (Berta et al., 2008). These changes are accompanied by alterations in physiological properties that contribute to hyperexcitability in these cells, and altered depolarization firing rates.

At least seven different families of voltage-gated potassium channels are normally involved in regulating and modifying the integration and transmission of electrical signals in the nervous system (Ishikawa et al., 1999). Cultured DRG neurons from axotomized adult rats show remarkable decreases in a number of potassium channels (Ishikawa et al., 1999). Similar decreases in potassium channels in the DRG neurons have been seen as a result of sciatic nerve chronic constriction injury (Kim et al., 2002), and axon axotomy (Park et al., 2003) . The decreases in some voltage gated potassium channels as a result of injury, along with simultaneous increases in some sodium channels, may lead to changes in electrical excitability of the DRG neurons (Ishikawa et al., 1999), and may, thus, be additional contributors to the overall injury response mechanism.

Calcium channels are present in DRG neurons (Fedulova et al., 1981; Morad et al., 1988). Calcineurin, a compound that regulates the activity of calcium channels, also exists in the DRG neurons (Lukyanetz, 1997). Increases in the pore forming part of calcium channels α2δ in DRG neurons of injured peripheral nerves have been reported

(Kim et al., 2001b; Luo et al., 2001; Newton et al., 2001). A concurrent increase in ectopic spontaneous discharges has been related to increased calcium channel activity (Xing and Hu, 1999). These drastic changes have been associated with injury-induced allodynia (Luo et al., 2001).

Application of brief or extended electrical stimulation (1 hour to 2 weeks of 20 Hz continuous electrical stimulation) to the proximal cut end of peripherally axotomized femoral nerve has been found to accelerate axonal regrowth (Al-Majed et al., 2000). Continuous 20 Hz electrical stimulation applied proximal to injury site for 1 h lead to a significant increase in DRG neurons regenerating into cutaneous and muscle branches, and increased the numbers of neurons that regenerated axons. This enhanced regenerative capacity is correlated with increased GAP-43 mRNA in the regenerating neurons, and with elevated expression of brain-derived neurotrophic factor (BDNF) (Geremia et al., 2007). The results of this experiment provided evidenc that linked electrical stimulation to increased expression of a neurotrophic factor, BDNF. This, in turn, leads to an increased expression of GAP-43 message. It would be of interest to study the potential correlation between this brief stimulation with other known neutrophic factors.

1.2.1 Response to peripheral axon injury.

Lesions or trauma of the peripheral axon branch induce a number of morphological, metabolic, and molecular alterations in the DRG neuronal cell body that may ultimately contribute to the initiation and maintenance of the axonal regeneration. There may be an additional role for non-neuronal cells to take an active part in the DRG

collective response to injury. For example, macrophages actively remove myelin debris, and Schwann cells proliferate and migrate. Non-neuronal cells may also be sources of biochemical factors that might influence axon regrowth.

The neuropeptide expression of DRG neurons appears to be sensitive to axon injury. Following peripheral nerve injury the population of DRG neurons shows a marked decrease in those that express SP (Barbut et al., 1981; Bisby and Keen, 1986; Jessell et al., 1979; Tessler et al., 1985), PPT (Kashiba et al., 1992; Noguchi et al., 1989), CGRP (Inaishi et al., 1992), and galanin (Kashiba et al., 1992). On the other hand, there is a robust increase in the number of DRG neurons that express other neuropeptides, such as VIP (Kashiba et al., 1992; Noguchi et al., 1989) and NPY (Frisen et al., 1992). Little is known about what role altered neuropeptide expression (and release) may play in axonal regeneration.

Peripheral axon injury also causes increased expression of intracellular proteins that are known to relay extracellular signals and control cytoskeletal dynamics such as cortical cytoskeleton-associated and calmodulin binding protein (CAP-23), neuron-specific growth associated protein/stathmen homolog (SCG10), and GAP-43 (Hoffman, 1989; Mason et al., 2002). Also, chaperonins such as HSP27 (Costigan et al., 1998), are robustly enhanced in peripherally axotomized sciatic nerve. These proteins are collectively called growth associated proteins, the expression of which is associated with the regenerative process.

Transcription factors such as c-Jun (Jenkins et al., 1993), STAT3 (Qiu et al., 2005), and ATF3 (Seijffers et al., 2006), also undergo changes following peripheral axon injury in DRG neurons. As a result of injury, MAPK signaling pathway is activated.

Consequently, phosphorylation and nuclear localization of a number of transcription factors including c-Jun, ATF3, and STAT3 may contribute to the alteration in gene expression of the injured neuron. Many of these transcription factors control the expression of neuropeptides, ion channel expression, and their own expression.

These collective phenotypic changes suggest that when DRG neurons suffer peripheral axon injury, there is a switch from the normal action potential transmitting phenotype to a phenotype more suitable for the initiation and support of axonal regeneration. The process of regeneration is dependent upon, and may be governed by intrinsic and extrinsic factors to neurons (Fawcett, 1992; Fu and Gordon, 1997), including trophic factors (Navarro et al., 2007).

Intrinsic factors refer to the biochemical factors that exist within the confinement of the neuron proper. In contrast, the term extrinsic factors refers to those factors that are present in the immediate surroundings of the neuron, and the neuron is subject to their potential biological actions.

1.2.2 Response to central axon injury.

The central axons of the DRG extend through the dorsal root, then into the spinal cord at the dorsal root entry zone. Central DRG axon branches synapse with dorsal horn interneurons to relay sensory information to the CNS. For many larger DRG neurons, collateral of the central axon branch may also ascend through the dorsal columns to ultimately synapse in the brainstem.

A lesion of the central axons of the DRG neurons, by itself, largely fails to induce the full range of morphological, physiological and biochemical changes that are normally

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seen following lesions to the peripheral axons. For example, no cell death is observed among L5 DRG neurons after dorsal transection (Himes and Tessler, 1989). Chromatolysis is also not seen in DRG neurons following central axon injury (Cragg, 1970). Injury of the central branch axon, by itself, is followed by poor regeneration of the central branch (Oblinger and Lasek, 1984).

Expression levels of neuropeptide markers such as VIP and PPT (Noguchi et al., 1989), and NPY do not change following dorsal root transection. Expression of transcription factors such as c-Jun (Broude et al., 1997; Jenkins et al., 1993; Kenney and Kocsis, 1997a), and ATF3 (Seijffers et al., 2006) also does not change dramatically as a result of dorsal transection. On the other hand, levels of neuropeptide CGRP (Inaishi et al., 1992) increase following dorsal root transection, whereas, neurofilament protein NF200 is one of the few proteins that show a marked reduction following a lesion to the central axon branch (Guseva and Chelyshev, 2006).

Molecules that interact with membrane signal-transduction mechanisms and control cytoskeletal dynamics such as CAP-23, SCG10, and GAP-43 remain unchanged after injury of the DRG central axon branch (Schreyer and Skene 1993) (Mason et al., 2002) even though they are robustly enhanced in peripherally axotomized sciatic nerve. Ion channels such as sodium channel type III (Black et al., 1999; Sleeper et al., 2000) and $\alpha2\delta$ 1 pore forming part of calcium channels (Li et al., 2004; Luo et al., 2001) remain unchanged as a result of dorsal transection, contrary to these channels' enhanced expression following peripheral axotomy. There are other proteins that do not change following dorsal root transection, but increase as a result of peripheral axotomy such as Neuropilin-1 (NP-1) (Gavazzi et al., 2000).

1.2.3 Distal nerve stump injury-response (Wallerian Degeneration):

Following peripheral branch nerve injury, axons distal to the lesion are disconnected from the cell body and undergo a process termed 'Wallerian degeneration' (Koeppen, 2004; Pearce, 2000; Waller, 1850) reviewed in (Fawcett and Keynes, 1990; Hall, 2005; Navarro et al., 2007; Zochodne, 2008). Damage to the axonal membrane, or axolemma, induces calcium-mediated proteolytic activity that will break down the axon, causing myelin to break down, and recruits resident and circulating macrophages to the damaged site (Akassoglou et al., 2000; Coleman and Perry, 2002; George and Griffin, 1994; Kiryu-Seo et al., 2000). This infiltration of macrophages into the area of injury accomplishes two important processes: first, it allows for interactions between macrophages and Schwann cells; secondly, it clears the myelin and axonal debris by phagocytosis (Martini et al., 2008).

Eliminating myelin debris is an important step to successful regeneration, because myelin associated proteins are known to impede and inhibit successful regeneration. The inhibitors include myelin-associated glycoprotein (MAG) (Chen et al., 2006), oligodendrocyte-myelin glycoprotein (OMgp) (Xie and Zheng, 2008) and Nogo (Schwab, 2004), all three of which appear to act via the Nogo receptor (NgR) despite the fact that they have little structural similarity (Mandemakers and Barres, 2005). It is believed that the microenvironment of the intact peripheral nerve does not support axons that had been cut and undergoing regrowth (Hall, 1993). It is only upon injury and initiation of the regenerative response that Schwann cell proliferation and axonal sprouting and elongation are permitted to take place (Alvarez et al., 2000; Tapia et al., 1995). These

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processes are normally inhibited in intact nerves by signals that originate from Schwann cells (Hall, 2005).

The basement membrane that surrounds the axon and Schwann cell remains intact and serves as a pathway or a tract for Schwann cells to line up, elongate and form bands of Büngner or Schwann tubes (Stoll and Muller, 1999). Schwann cells then begin to synthesize growth and neurotrophic factors that act as tropic (guidance cues), and trophic (maintenance) factors for the newly forming axonal sprouts from the proximal end of the injured nerve (Campana, 2007; Cheng et al., 1996; Pellitteri et al., 2006). After providing the proper extracellular environment that supports axonal growth, Schwann cells adopt a different phenotype and begin to remyelinate the newly regenerated axons (Stoll and Muller, 1999).

After nerve injury and successful repair, the newly regenerated peripheral nerve is thinner with shorter internodes than it had prior to injury (Hall, 2005; Navarro et al., 2007). The axons have smaller diameters, and suboptimal conduction velocities and excitability (Fields and Ellisman, 1986a; b). Regenerated Schwann cell myelin is also thinner.

1.2.4 Proximal nerve stump injury-response.

Peripheral nerve injury leaves the surviving proximal nerve axon stumps potentially available to respond to exogenous substances available in the immediate extracellular microenvironment (Yoo et al., 2003). Some of these substances may initiate, or act as, "positive" signals that are retrogradely conveyed back to the DRG neuron cell bodies to initiate a regenerative response. Immediately following axotomy, the membranes at the severed proximal end of the axons begin to seal off (Kristensson

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and Olsson, 1981). Before membrane sealing is complete, calcium ions may enter damaged axonal elements and activate calcium-dependent proteases (LoPachin and Lehning, 1997).

Simultaneously, axotomy deprives the DRG neuron cell bodies of any target-derived molecules that are normally retrogradely transported from peripheral target tissues. Deprivation of a normally tonically available signal is often described as a "negative" signal. Examples of such retrogradely-acting tonic signaling molecules include FGF-1 that counteracts the injury induced expression of NPY (Ji et al., 1996). These proteins and their role in the injury response will be described in more detail below. The presence of new, retrogradely transported molecules (positive signals) from the microenvironment of the injury site, or the absence of normally available target-derived molecules (negative signals) could each presumably play a role in initiating a metabolic response to injury in DRG neurons.

DRG neurons that survive peripheral axotomy will initiate the injury response, leading to regenerative growth, ultimately culminating in functional reinnervation. Within the first few hours following axotomy, a small area of the axon's proximal segment adjacent to the injury undergoes Wallerian degeneration back to the first node of Ranvier (Terenghi, 1999). The axon may initially form several regenerative sprouts (Desouches et al., 2005; Wong and Mattox, 1991). These sprouts start to migrate distally, likely supported by Schwann cells, and in active search for a suitable growth supporting pathway (Bixby et al., 1988). The regenerating axonal sprouts respond to guidance (tropic) and survival (trophic) factors supplied by dedifferentiating Schwann cells and peripheral targets. Despite these early signs of regenerative response, it may be

several days before any axonal outgrowth extends beyond the proximal nerve stump (Al-Majed et al., 2000).

If the gap between the distal and proximal nerve stumps is not more than a few millimeters, axon growth into the distal stump may be successful. The regenerating axon can then take advantge of the local permissive environment of the Wallerian degenerating distal nerve stump and axons successfully navigate toward their original targets. However, if the gap between the proximal and the distal stumps is wide, growing axons may not reach the distal stump, and no functional recovery takes place.

1.2.5 Conditioning lesion.

A conditioning axon lesion is defined as an earlier, suitably timed injury that may alter regenerative success in response to a later 'test' injury of the same axons (Forman et al., 1980). Indeed, an earlier conditioning lesion in the same peripheral nerve has been found to enhance the regenerative response to a subsequent, more proximal test lesion of the same nerve (McQuarrie, 1978; McQuarrie et al., 1977). The improvement in regeneration caused by a prior conditioning lesion appears to involve faster initiation of regeneration, faster growth during regeneration, and a larger number of regenerating axons.

The conditioning lesion effect is dependent upon the type of the initial conditional lesion itself. The more severe the initial lesion, the longer the effect lasts (Bondoux-Jahan and Sebille, 1986). The increase in the number of axons that ultimately regenerate as a result of the conditioning lesion effect also depends on the time interval between the initial and test lesions (Arntz et al., 1989; Jenq et al., 1988). In some cases, more than

one conditioning lesion at the same site, separated by a week, resulted in an increased rate of regeneration (Bisby, 1985; Bisby and Pollock, 1983). In addition, a conditioning lesion in a rat sciatic nerve not only accelerated regeneration of the same nerve when subsequently tested, but also lead to enhanced regeneration of previously unconditioned nerves on the contralateral side. This was accompanied by enhanced expression of cytokines in the contralateral DRG neurons (Ryoke et al., 2000). It is not clear how a conditioning lesion on one side might influence regenerative ability in contralateral neurons that did not receive the conditioning lesion. The influence could presumably be carried either through systemic circulation or through neuronal connectivity across the midline.

The unique axonal morphology of the DRG neuron has allowed the test of whether conditioning lesions of one axon branch (i.e. the DRG peripheral axon branch) can influence the regenerative ability of a second, non-conditioned axon branch from the same neuron (i.e. the central axon branch). The central axon branches of DRG neurons regenerate very poorly if subjected to an unpaired 'test' lesion, but regenerate much more readily if their corresponding peripheral axon branches are also given a 'conditioning' lesion either beforehand, or simultaneously (Richardson and Issa, 1984; Richardson and Verge, 1986; 1987) . Regeneration of the central axons can also be enhanced by peripheral exposure to inflammatory challenge (Lu and Richardson, 1991). If an injury occurs in the central branch after peripheral branch lesion, the central branch can regenerate beyond the injury site and into a (Wallerian degenerating) peripheral nerve graft, or even into the inhibitory environment of the spinal cord itself (Neumann and Woolf, 1999; Richardson and Issa, 1984). Peripheral conditioning injury of the sciatic

nerve resulted in an increase in the number of myelinated axons at the corresponding

dorsal root entry zones and on the surface of the spinal cord (Chong et al., 1999).

The effects of a conditioning lesion may result from early initiation of the cell

body response to injury, including altered gene expression and increased protein

synthesis to meet the demand of regenerating axons. Changes in non-neuronal cells

(Schwann cells and satellite cells) may also be involved (Sjoberg and Kanje, 1990;

Torigoe et al., 1999). Neurons cultured from DRGs that have undergone a sciatic nerve

conditioning injury one week earlier show enhanced growth of neurites *in vitro*. The

effect seems to involve earlier initiation of neurites, more rapid growth, and altered

sensitivity to tropic factors (Smith and Skene, 1997). The experiments demonstrating

that a conditioning lesion of DRG peripheral axons can contribute to increasing the

growth capacity of injured DRG central axons implies that the effect works through a

signaling mechanism in the cell bodies of the injured neurons.

1.2.6 Evidence for a negative signal inducing the injury response.

Microtubules are the cytoskeletal substrate for fast anterograde (away from cell

body) axonal transport driven by kinesin proteins and for retrograde (toward cell body)

axonal transport driven by dynein proteins (Cyr and Brady, 1992; Hirokawa, 1997). The

existence of a retrograde axonal transport mechanism and its importance in sensory

neuron survival is well documented (Dumas et al., 1979; Fink et al., 1985; Max et al.,

1978; Schwab and Thoenen, 1977; Streit, 1980; Streit et al., 1980).

Distinct subpopulations of DRG neurons are selective in terms of their retrograde

transport of trophic factors (DiStefano et al., 1992). For example, small diameter,

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nociceptive sensory neurons, respond mainly to and transport NGF (Lindsay, 1996a). Additionally, small to medium diameter neurons preferentially transport BDNF (Zhou and Rush, 1996), while large, proprioceptive neurons tend to be responsive to and transport NT-3 (DiStefano et al., 1992). Although GDNF is transported by all sensory neurons, the small RET-positive, IB4-positive, non-TrkA-expressing neurons show a preferential ability to transport GDNF (Leitner et al., 1999; Matheson et al., 1997). A similar subpopulation of small, CGRP positive, TrkA expressing sensory neurons favorably transport LIF (Thompson et al., 1997).

Pharmacological blockade of retrograde axoplasmic transport using microtubule disrupting agents, such as vinblastine or colchicine, initiates a number of neuronal cell body reactions similar to the response seen following peripheral axotomy (Aldskogius and Svensson, 1988; Woolf et al., 1990). If dynein-based retrograde transport is specifically inhibited, trophic stimulation of axon terminals will not support neuronal survival (Heerssen et al., 2004). Disrupting anterograde transport, on the other hand, does not initiate a peripheral nerve injury response (Csillik et al., 1982).

It has been hypothesized that there are repressive factors originating from the peripheral target tissues, and through retrograde transport, these repressive factors maintain low expression of growth-associated proteins and other injury-induced features (Andersen et al., 2000; Liabotis and Schreyer, 1995; Schreyer and Skene, 1993). Support for this retrograde repressor hypothesis comes from a number of experimental findings. The magnitude of GAP-43 expression following peripheral axotomy does not depend on the distance of the site of lesion from the DRG perikarya. This is consistent with a mechanism involving loss of a tonic retrograde repressive signal derived from the

periphery (Liabotis and Schreyer, 1995). The observations from a number of studies suggest that unknown substrates from the periphery, or peripheral target tissue, might regulate GAP-43 expression. In corticospinal neurons, GAP-43 expression is elevated during the perinatal period. This increase gradually declines as target tissues are innervated in normal development. When colchicine, a compound that disrupts retrograde neuronal transport, is applied to those target approaching axons, GAP-43 expression remains elevated (Karimi-Abdolrezaee and Schreyer, 2002; Karimi-Abdolrezaee et al., 2002; Margolis et al., 1991).

Peripheral axotomy of the sciatic nerve upregulates GAP-43 expression in the DRG neurons, whereas, central axotomy does not (Schreyer and Skene, 1993). A similar pattern of reaction to peripheral, but not central axotomy is seen with c-Jun (Kenney and Kocsis, 1998), STAT3 (Schwaiger et al., 2000), NPY, and VIP (Sterne et al., 1998). Such results suggest that retrograde repressive signals (or negative regulators) are derived only from peripheral target tissue because central axotomy apparently does not interrupt them.

GAP-43 expression in developing cultured DRG neurons appears to be decreased when the DRG neurons are cultured in the presence of peripheral target tissues (Baizer and Fishman, 1987). However, the biochemical nature of such "repressive" signals is yet to be determined. Neurotrophins can retrogradely influence expression of neuropeptides such as SP (Mulderry, 1994), and NPY (Sterne et al., 1998), but do not appear to directly repress GAP-43 expression in cultured DRG neurons (Schreyer et al., 1997). Experiments using skeletal muscle extracts (Kimura and Schreyer, unpublished) indicate that a target-derived GAP-43 repressive signal can be in soluble or membrane-bound form.

Considerable evidence exists in the literature implicating FGFs as potential modulators of injury-induced expression of neuropeptides and growth associated proteins. FGF-1 has been shown to counteract injury-induced expression of galanin and NPY (Ji et al., 1996). In CNS, FGF-2 stimulates the proliferation of GAP-43 positive O-2A precursor cells (common precursor for oligodendrocytes and type-2 astrocytes) as well as increase the level of GAP-43 mRNA in these cells (Deloulme et al., 1993). Additionally, FGF-2 has also been shown to stimulate GAP-43 phosphorylation at serine 41 (ser41), thereby modifying its intracellular localization in cultured hippocampal neurons (Tejero-Diez et al., 2000). In injured frog retinal ganglion cells, FGF-2 modulates expression and distribution of GAP-43 after optic nerve injury (Soto et al., 2003).

Recently, bone marrow stromal cells are have been shown to differentiate into various neuronal cells both *in vivo* and *in vitro* (Egusa et al., 2005). An important and relevant finding shows that FGF-2 effectively induces neuronal differentiation from stromal cells, as evidenced by elevated levels of GAP-43. This effect of FGF-2 requires FGFR-1, MAPK/ERK, and an activator protein 1 (AP-1) recognition site (Yang et al., 2008). The AP-1 site is also part of the GAP-43 gene.

1.2.7 Evidence for a positive signal inducing the injury response.

Positive retrograde injury signals have also been postulated to exist at the injury site. Proteins may be activated at the injury site by undergoing post-translational modification, and then traveling retrogradely to the cell body. The post-translational modification, activated by injury, could include phosphorylation, or proteolysis (Perlson

et al., 2004). One example is the phosphorylation of MAP kinases Erk1 and Erk2 at the injury site, and their subsequent retrograde transport to the cell body (Agthong et al., 2006; Perlson et al., 2005).

Some transcription factors, such as STAT3, ATF3, and c-Jun have also been identified in peripheral axons and may participate in positive retrograde signaling (Agthong et al., 2006; Lee et al., 2004; Lindwall and Kanje, 2005). Recent works suggest that this positive retrograde transport requires the presence of carrier proteins, including importins that interact with the retrograde transport motor dynein (Hanz et al., 2003).

Formation and maintenance of the regrowing axon seems to involve protein synthesis within the distal axon (Willis et al., 2005). It is therefore plausible that newly synthesized proteins could be retrogradely transported from the site of axotomy and act as a positive signal to initiate the injury response. As part of the neuronal response to injury, adult sensory neurons that are conditioned by axonal crush can rapidly extend processes *in vitro* by regulating the translation of existing mRNAs that encode ribosomal proteins essential in neurite regeneration and growth (Twiss et al., 2000). Blocking protein synthesis in these regenerating axons causes a rapid retraction of their growth cones (Twiss and van Minnen, 2006; Zheng et al., 2001). Axons of adult mammalian sensory neurons can synthesize proteins independent of transcriptional control, leading to synaptic plasticity and/or pathfinding and injury responses (Wang et al., 2007).

1.2.8 Proteins of the injury response.

Peripheral nerve regenerative propensity is reflected by the expression and upregulation of a number of genes referred to variously as 'growth-associated proteins' (GAPs) (Benowitz and Routtenberg, 1997; Benowitz and Schmidt, 1987; Skene, 1984; 1989; 1990; Skene and Willard, 1981) or as 'regeneration associated genes' (RAGs) (Al-Majed et al., 2004; McPhail et al., 2004; Schmitt et al., 2003). These genes and their encoded protein products include transcription factors, growth stimulating substances, intracellular signaling enzymes, cytoskeletal proteins and cytoskeleton interacting proteins (McLean et al., 1995; Schreyer and Skene, 1993; Skene, 1984). For many of these proteins, there is evidence suggesting that their presence leads to successful nerve regeneration.

1.2.8.1 Growth-associated protein-43 (GAP-43).

One of the first well characterized examples of a protein that is upregulated in association with developmental and regenerative axon growth is GAP-43, also known as B-50, F-1, pp-46, and neuromodulin (Benowitz and Routtenberg, 1997; Meiri et al., 1986; Skene, 1989). GAP-43 is a 24 kDa neuronal-specific phosphoprotein that is expressed in virtually all developing neurons at high levels as they initiate axon growth. Although the actual size of rat GAP-43 protein is 24 kDa, it migrates with an apparent size of 40-60 kDa on sodium dodecyl sulfate (SDS) polyacrylamide gels due to its unusual amino acid composition (rich in acidic amino acids and alanine, but with few hydrophobic residues) and aberrant SDS binding properties (Skene, 1989). As development proceeds and axons reach their target tissues, the level of GAP-43

expression declines (Chong et al., 1992), but does not disappear altogether in some neurons.

In adult DRG neurons and in other neurons of the PNS, GAP-43 is markedly upregulated following (peripheral) axotomy (Chong et al., 1994; Mason et al., 2002; Schreyer and Skene, 1993). Once synthesis is increased, GAP-43 is transported to both the peripheral and the central DRG axon branches (Schreyer and Skene, 1991; Woolf et al., 1990).

GAP-43 appears to be involved in transducing intra- and extracellular signals to regulate cytoskeletal organization in the axon (Benowitz and Routtenberg, 1997; Frey et al., 2000; Laux et al., 2000; Meiri et al., 1996). The amino acid sequence of GAP-43, highly conserved among mammals, has a unique calmodulin binding domain (Alexander et al., 1988; Skene, 1989). GAP-43 has a high affinity to bind calmodulin in the absence of free Ca^{+2} (Masure et al., 1986). It is proposed that GAP-43 binds and localizes calmodulin at specific sites within the cell and that free calmodulin is released locally in response to phosphorylation of GAP-43 by protein kinase C and/or to increases in intracellular free Ca^{+2} (Alexander et al., 1987; Alexander et al., 1988). Phosphrylated GAP-43 may be a substrate for calcineurin and both calcineurin and protein kinase C may regulate the levels of free calmodulin available in neurons (Liu and Storm, 1989). Biochemical properties that are central to the biological effects of the GAP-43 are its membrane- and calmodulin-binding properties, which could allow it to sequester a large fraction of calmodulin to the submembranous regions, and to release free calmodulin in response to protein kinase C activation (Skene, 1990). In addition, such calmodulin binding properties could be useful in modulating the responses of membrane and

cytoskeletal assembly events to calcium signals in the growth cones of regenerating

axons (Skene, 1990). The calmodulin pathway also appears to link elevated intracellular

calcium to gene induction (Morgan and Curran, 1988).

GAP-43 interacts with the GTP binding protein Go, a member of the G protein

family that links receptors and second messengers (Strittmatter and Fishman, 1991;

Strittmatter et al., 1991). Because of its ability to associate with the neuronal membrane,

to interact with calmodulin, and to associate with cytoskeletal elements, GAP-43 is

important in coordinating activity between the neuronal membrane and the arrangements

of cytoskeletal elements, as well as coordinating the effects of both extracellular signals

and intracellular growth proteins.

The GAP-43 gene contains three exons. The first exon codes for the first 10

amino acids of the protein that contain two reversible palmitolylation sites.

Palmitoylation allows for strong association with membranes, and is essential for

targeting the protein to the growing axonal terminal (Benowitz and Routtenberg, 1997;

Skene and Virag, 1989). The second exon contains most of the coding region, including

serine-41, the site phosphorylated by protein kinase C (Benowitz and Routtenberg, 1997;

Dent and Meiri, 1992). Phosphorylation at serine-41 inhibits calmodulin binding to

GAP-43 (Liu and Storm, 1990). The third exon codes for the carboxy terminus and

contains regions with structural similarity to intermediate filaments giving it a unique

ability to interact with actin and actin associated proteins (Meiri and Gordon-Weeks,

1990).

GAP-43 has been associated with axon growth cone pathfinding (Strittmatter et

al., 1995), consistent with a possible role in axon guidance. High concentrations of GAP-

43 in a growth cone alter the way an axon terminal responds to environmental stimuli acting through calcium and calmodulin (Lankford et al., 1990; Skene, 1990). Thus, GAP-43 was suggested to modulate complex cellular properties such as growth cone guidance and motility, synaptic plasticity and neurotransmitter release (Coggins and Zwiers, 1991).

Null mutation of the GAP-43 gene disrupts axonal pathfinding and is generally lethal shortly after birth (Benowitz and Routtenberg, 1997). During development, GAP-43-deficient retinal axons remain stalled in the optic chiasm, unable to navigate past this midline decision point. However, cultured neurons of GAP-43-deficient mice extend neurites and growth cones in a very similar fashion to wild type. Thus, it is suggested that, while GAP-43 protein may not be essential for axonal growth, is required to guide the growing axon at certain decision points, such as the optic chiasm (Strittmatter et al., 1995). This is compatible with the hypothesis that GAP-43 serves to transduce extracellular pathfinding signals at the growth cone (Strittmatter et al., 1995).

We know little about how axon injury leads to increased expression of GAP-43. Clues as to the nature of the mechanism(s) that control GAP-43 expression have come from analysis of its gene structure. The GAP-43 gene, located on chromosome 3 in humans and chromosome 16 in mice, spans at least 50 kb (Benowitz and Routtenberg, 1997; Denny, 2006; Grabczyk et al., 1990). The 5'-flanking sequence lacks CAAT or TATA elements. This sequence however does have the ability to initiate RNA transcription from several sites, depending on the neuronal type. The 1.5 kb mRNA is spliced from three widely separated exons: the first includes a 5' untranslated region (5'UTR) of variable length and the first ten codons; the second contains most of the

coding region, while the third includes the last few codons and a long 3'UTR (Grabczyk et al., 1990). The rat GAP-43 promoter region has seven E-boxes, but only one of them (E1) was found to control GAP-43 promoter activity (Chiaramello et al., 1996). Basic helix-loop-helix (bHLH) transcription factors may regulate the expression of the GAP-43 gene (Chiaramello et al., 1996). The E1 box can be a positive or a negative regulator of the GAP-43 promoter, depending on which bHLH transcription factor binds to it (Denny, 2006).

The GAP-43 gene promoter contains AP-1 site that contributes to gene activity in neurons. The fact that GAP-43 expression is minimally detected in mature neurons after growth is complete, suggests that repressive elements play an important role in allowing AP-1 signaling pathways to control the activity of the GAP-43 gene in neurons (Weber and Skene, 1998). In injured CNS neurons, c-Jun, a transcription factor that binds AP-1 sites, has been implicated in the upregulation of GAP-43 expression (Haas et al., 2000).

1.2.8.2 Tα1 tubulin.

Tubulin is a globular, cytoskeletal protein that exists as a dimer. The tubulin dimer has one α and one β subunit. Tubulin dimmers are the building blocks of microtubules that support cellular structural integrity and play a central role in the trafficking and movement of intracellular components. The regulation of Tα1 tubulin expression after injury differs according to the neuronal cell type and the distance of axotomy from the cell body (Bisby and Tetzlaff, 1992). In the PNS an upregulation of Tα1 tubulin mRNA (Miller et al., 1989) and protein (Mohiuddin and Tomlinson, 1997) is observed in all neurons after peripheral nerve injury concomitant with elevated GAP-43

expression (Al-Majed et al., 2004; Geremia et al., 2007). This suggests that expression of

Tα1 tubulin and GAP-43 may be regulated together as part of the overall injury response.

In addition to Tα1 tubulin, other members of the tubulin proteins are upregulated in

axotomized DRG neurons. These include βII, βIII tubulins. Other tubulin family

members such as βIV, however, do not show any changes in injured neurons (Hoffman

and Cleveland, 1988; Miller et al., 1989; Moskowitz et al., 1993; Wong and Oblinger,

1990).

Tα1 tubulin and GAP-43 expression are coregulated in response to neurotrophins.

A study using BDNF and NT-4/5 showed that both neurotrophins stimulate GAP-43 and

Tα1 tubulin mRNA expression, and promote axonal regeneration (Kobayashi et al.,

1997). Moreover, expression of these growth associated proteins increases as a result of

brief electrical stimulation in the regenerating femoral nerve (Al-Majed et al., 2004). In

the CNS, CCAAT enhancer binding protein-beta (C/EBPbeta), a transcription factor

implicated in cellular injury and regeneration has been shown to be essential for the

neuronal injury response. Of particular relevance to the current project, C/EBPbeta acts

to transcriptionally activate both GAP-43 and Tα1 tubulin gene expression (Nadeau et al.,

2005). Beta3-tubulin (βIII tubulin) gene expression is induced by vinorelbine through an

AP-1 site (Saussede-Aim et al., 2009), a site that is also shared with GAP-43 promoter.

1.2.8.3 Heat shock protein 27 (Hsp-27).

Heat shock proteins (HSPs) are molecular chaperones that function in proper

folding and trafficking of proteins (Kampinga, 2006). They can also play an important

role in mediating the assembly of proteins into oligomeric structures. Primarily, they act

to prevent the formation of incorrect structures which may result from the transient exposure of charged or hydrophobic surfaces normally involved in interactions between or within polypeptide chains (Ellis et al., 1989). Hsp-27 is a member of this family of molecular chaperone proteins and is induced in cells that have been exposed, not only to sub lethal heat shock treatment, but also to oxidative stress or various other environmental stresses (Arrigo, 2007). Hsp-27 possesses anti-apoptotic properties and is upregulated following peripheral nerve injury in a very similar fashion to GAP-43.

In the PNS, Hsp-27 is expressed at low levels in medium sized DRG neurons and upregulated following chronic constriction injury (Kim et al., 2001a). There is up to a nine fold increase following peripheral nerve transection (Costigan et al., 1998). Hsp-27 expression is induced by transcription factors ATF3 and c-Jun (Nakagomi et al., 2003). Because of this correlation with GAP-43 expression, its potential protective role in neuronal cell survival (Klettner, 2004; Latchman, 2005) and its contribution to rearrangement of cytoskeletal elements (Gerthoffer and Gunst, 2001), Hsp-27 is classified among the growth associated proteins that are upregulated in the neuronal response to injury.

1.2.8.4 Neuropeptides.

NPY is a 36 amino acid peptide widely distributed within neurons of the CNS and PNS (Gray and Morley, 1986). NPY is upregulated in medium to large DRG neurons following peripheral axotomy (Hokfelt et al., 1994; Landry et al., 2000; Verge et al., 1995; Wakisaka et al., 1991; Zhang et al., 1993). The role of the upregulated NPY in DRG neurons as a result of injury is not fully understood, although antinociceptive

effects have been attributed to NPY (Xu et al., 1994). A strong correlation between

GAP-43 expression and that of NPY in response to NT-3 (Ohara et al., 1995) suggests

that NPY upregulation may also be a part of the DRG neuronal response program to

peripheral injury.

SP and CGRP are transmitters for a proportion of DRG neurons and are

implicated in the nociceptive transmission of the primary sensory system. Substance P

protects neurons from apoptosis via PKC-Ca^{2+}-MAPK/ERK pathways (Lallemend et al.,

2003).

The newly identified small proline-rich repeat protein 1A (SPRR1A) is

upregulated by the main subpopulations of DRG neurons following an injury to their

peripheral, but not central, axon branches (Bonilla et al., 2002; Starkey et al., 2009). This

follows the pattern of GAP-43 and many other elements of the injury response.

1.2.9 Transcription factors.

There are several transcription factors whose synthesis and activation are

modified as part of the injury response (Raivich and Makwana, 2007). Examples of such

factors are ATF3, cJun, and STAT3. They may therefore be involved as higher-order

regulators of other aspects of the neuronal injury response. These three transcription

factors will be the major focus of the experimental work described in this thesis.

1.2.9.1 Activating transcription factor 3 (ATF3).

ATF3 is a member of activating transcription factor/cyclic AMP response element

binding protein (ATF/CREB) family of transcription factors that share a common motif

of bZip DNA binding domain which binds to the consensus site 'TGACGTCA' (Hai and Hartman, 2001; Hai et al., 1999). ATF was first described as a protein that activates transcription of multiple E1A-inducible adenovirus early promoters (Lee et al., 1987). cAMP response element binding protein (CREB) was described as protein product that binds to cAMP responsive element (CRE) (Montminy, 1997). ATF/CREB transcription factors can heterodimerize with the transcription factor AP-1, utilizing a common dimerization motif. This suggests that there may be cross-talk between the two types of transcription factors (Karin et al., 1997). Genomic AP-1 consensus sequences have also been found to interact with c-Jun and other transcription factors.

ATF3 was first isolated from a library derived from HeLa cells (Hai et al., 1989). It is an immediate-early gene induced by a number of stimuli such as carbon tetrachloride, ischemia, radiation, and anticancer drugs such as tetradecanoylphorbol acetate (TPA). It is also rapidly and highly expressed in regenerating liver (Hsu et al., 1991) and has thus been called liver regenerating factor-1 (LRF-1). Other names that refer to ATF3 are LRG-21, CRG-5, and TI-241 (Hai and Hartman, 2001). The *ATF3* gene is activated in a variety of organs as a result of chemical or surgical insults. For example, the *ATF3* gene is activated in ischemic heart (Yin et al., 1997), in hepatectomized or carbon tetrachloride treated liver (Chen et al., 1996), as well as in epileptic brain (Chen et al., 1996), and in transected peripheral nerves (Tsujino et al., 2000). ATF3 is an unusual transcription factor in that it can both repress and activate transcription. The ATF3 homodimer is a transcriptional repressor. However, heterodimeric complexes of ATF3 with c-Jun function as transcriptional activators (Hai and Hartman, 2001).

ATF3 expression and nuclear localization increase robustly in DRG neurons following peripheral nerve injury (Tsujino et al., 2000), indicating that the induction of ATF3 is part of the overall neuronal response to injury. It is also upregulated following chorda tympani nerve injury, concomitant with increases in GAP-43 (Tsuzuki et al., 2002). In the DRG neurons, ATF3 mRNA is detected by 12 hours, peaks at 1 day, and remains high 2 weeks following axotomy (Tsujino et al., 2000). Thereafter, ATF3 gradually decreases but is expressed at a low level beyond 10 weeks after axotomy.

The *ATF3* gene is considered to be one of the immediate early genes whose activation regulates other, downstream genes, initiating a network of transcriptional regulation. Thus, regulation of *ATF3* gene expression has been an area of recent active research. Treatment of cultured HeLa cells with anisomycin leads to activation of the p38 pathway by mitogen-activated protein kinase kinase 6 (MAPK 6), a kinase upstream of p38. This activation is sufficient to induce the expression of the *ATF3* gene. This induction is possibly carried out through phosphorylation of CREB by p38, and does not seem to involve extracellular regulated kinase (ERK) nor c-Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathways (Lu et al., 2007). However, in a peripheral nerve injury model the ATF3 appears to be induced through retrograde signaling involving JNK. Retrograde axonal transport of JNK signaling components contribute to the injury induced activation of the *ATF3* gene (Lindwall and Kanje, 2005). Because of the variation in range of factors (stressful and nonstressful) and conditions that control ATF3 expression, *ATF3* has been described as an adaptive response gene that initiates cellular processes that adapt to various cellular stress signals (Lu et al., 2007).

Transfection of cells with siRNAs that block the expression of SOX11, a gene that encodes a member of the SOX family of transcription factors caused a transcriptional and translational level reduction in ATF3 expression *in vitro* and *in vivo* (Jankowski et al., 2009). Growth arrest and DNA damaging/ C/EBP-homologous protein 10 (gadd153/Chop10), another bZip protein, heterodimerizes with ATF3 and inhibits its expression (Chen et al., 1996). Inversely, ATF3 represses the expression of gadd153/Chop10 (Wolfgang et al., 1997), making gadd153/Chop10 a potential target gene of the repressor activity of ATF3. Interestingly, ATF3 can repress its own promoter, providing a potential mechanism of homeostatic autoregulation (Wolfgang et al., 2000). Thus, ATF3 induction in DRG neurons may be a part of a protective response to injury, in addition to any direct role in promoting axon growth.

1.2.9.2 c-Jun.

c-Jun was first identified and isolated from transformed cultured chick embryo fibroblasts (Bos et al., 1986). c-Jun dimerizes with other transcription factors (such as Fos) through the leucine zipper domains, and the Jun/Fos heterodimers bind to DNA and regulate transcription of numerous other genes (Ransone and Verma, 1989; Vogt and Bos, 1989; Vogt and Morgan, 1990). Jun protein is a major component of the transcription factor complex AP-1, which regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis (Hartl et al., 2003). c-Jun is also induced in cancer cells (Whitmarsh and Davis, 2007). Phosphorylation of c-Jun by JNK (jun N-terminal kinase) leads to c-Jun nuclear translocation, whereby it can affect transcription. c-Jun has a number of binding domains such as DNA binding domain, a leucine zipper region, a transcription-activating or transactivating module, and binding

domains for the mitogen-activated protein kinases. c-Jun forms homodimers and heterodimers with Fos and other transcription factors such as ATF3. c-Jun homo-and heterodimers bind the AP-1 promotor complex, thus initiating transcriptional regulation of genes that are involved in cell growth and differentiation.

c-Jun is upregulated in rat lumbar DRG neurons following sciatic nerve axotomy (Herdegen et al., 1992; Jenkins and Hunt, 1991; Kenney and Kocsis, 1997b). The timing of c-Jun induction is dependent upon how far the lesion is from the lumbar ganglion (Kenney and Kocsis, 1997b). The trigger signal for c-Jun upregulation following axotomy has been extensively examined and appears to be a negative signal, such as interruption of the retrograde flow of unidentified molecule(s). This is supported by observations that blockade of retrograde transport alone, without concurrent nerve injury, leads to c-Jun elevation in DRG neurons (Leah, Herdegen et al. 1991; Jenkins, McMahon et al. 1993).

Although c-Jun is upregulated substantially following a peripheral nerve transection, it is not upregulated following a central root transection (Broude et al., 1997). This substantiates the idea that target-derived factor(s) from peripheral target tissue contribute to the suppression of transcription factors that are a part of the injury response, in a pattern similar to GAP-43. It is certainly of interest to investigate the possible molecular regulatory pathways that may connect c-Jun expression to that of GAP-43.

Immunohistochemistry of ATF3 protein in the axotomized DRG does not reflect complete colocalization with c-Jun. More DRG neurons were labeled for ATF3 than for those labeled for c-Jun (Tsujino et al., 2000). This indicates that the factor(s) that may regulate the expression of ATF3 may not be the same as those that regulate c-Jun,

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although in PC12 and Neuro-2a neuron-like cell lines, co-transfection of c-*jun* and *ATF3* genes doubled the number of cells producing neurites, compared with expression of c-Jun alone (Pearson et al., 2003).

1.2.9.3 STAT3.

Signal transducers and activators of transcription (STATs) are a family of transcription factors that mediate a wide variety of biological functions in the PNS and CNS. STATs are phosphorylated by janus kinases (JAKs) in response to cytokine or growth factor activation of a cell surface receptor. Following phosphorylation by receptor tyrosine kinases, STATs then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. Activation of STAT3 protein through phosphorylation of the Tyr705 residue initiates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis.

STAT3 promotes neuronal survival by inducing neuroprotective genes (Dziennis and Alkayed, 2008). In the nervous system, STAT3 is activated in acute spinal cord injury (Tsai, Yang et al. 2007), and in peripheral nerve inflammation (Tamura et al., 2005). Sciatic nerve axotomy results in phosphorylation and activation of the STAT3 transcription factor in DRG neurons. Perineural infusion of the JAK2 inhibitor AG490 at the proximal nerve stump blocks STAT3 phosphorylation following sciatic nerve transection and results in reduced GAP-43 upregulation. It significantly attenuates central branch regeneration in the spinal cord after a preconditioning sciatic nerve axotomy (Qiu, Cafferty et al. 2005). STAT3 activation is thus necessary for increased

DRG neuronal growth and improved axonal regeneration within the spinal cord after a conditioning injury (Qiu, Cafferty et al. 2005).

STAT3 by itself can be a potential retrograde signaling transcription factor. Peripheral nerve lesion leads to a rapid activation of STAT3 in axons at the lesion site. This activation extends from the lesion site to the DRG neurons and motor neurons in the spinal cord. This indicates the possibility that axonal STAT3, activated at the injury site, may act as a retrograde signaling transcription factor, thus promoting the survival and regeneration of both sensory and motor neurons (Lee, Neitzel et al. 2004).

1.3 Modulation of axonal regeneration.

1.3.1 Exogenous growth factor/neurotrophin supplementation.

A number of methods to deliver growth factors, neurotrophins, and other factors of therapeutic interest *in vivo* have been reported and reviewed (McDonald and Zochodne, 2003). The methods include direct near-nerve local injection (Finkelstein et al., 1996; Ro et al., 1998), delivery by osmotic pump with catheter to the repair site (Ishii et al., 1993; Leong et al., 1999; Mohiuddin et al., 1999; Newman et al., 1996; Utley et al., 1996), infection with neurotrophic factor-transducing recombinant adenoviruses (Gravel et al., 1997; Isenmann et al., 1998), and subcutaneuous and intraperitoneal injections and intrathecal delivery.

Molecular delivery of exogenous growth factors or neurotrophins has also been attempted *in vitro* through introduction of an adenoviral vector to cultured neurons. A single infection with an adenoviral vector coding for NT-3 maintains active production of

NT-3 for at least 20 days in culture, as evidenced by continued neurite extension assay (Dijkhuizen et al., 1997).

Manipulation of the extacellualr matrix material proteins, mixed with neurotrophins in the vicinity of injury, has also been attempted. For example, grafting NT-3 impregnated fibronectin mats into the site of injured sciatic nerve attenuated the upregulation of NPY expression, a neuropeptide protein that is associated with injury (Sterne et al., 1998).

Exogenous delivery of BDNF antibody or antisense oligodeoxynucleotide into injured DRGs reduced the sprouting of sympathetic axons witin both ipsi- and contra-lateral DRGs. Delivery of exogenous BDNF into an intact L5 DRG resulted in a 4.2 fold increase this sympathetic sprouting (Deng et al., 2000). These findings indicate that BDNF that is endogenously synthesized and secreted from the DRG proper is involved in the sympathetic axonal sprouting that follows the peripheral nerve injury. In another study, exogenously and peripherally delivered BDNF to an already condition lesioned sciatic nerve enhanced regeneration of ascending sensory neurons in the spinal cord (Song et al., 2008). Endogenous infusion of BDNF antibodies intrathecally via a mini-osmotic pump for 3 days at the level of the fifth lumbar dorsal root ganglion, immediately following unilateral spinal nerve injury decreased expression of the injury/regeneration-associated genes GAP-43 and Tα1 tubulin in the injured sensory neurons. Similar results were observed following intrathecal delivery of or siRNA targeting BDNF 3 days prior to injury (Geremia et al., 2009). These results substantiate the importance of endogenous BDNF in the initial induction of the cell body response in injured sensory neurons.

Intrathecal delivery of NGF but not GDNF induced extensive sprouting of sympathetic neurons within lumbar DRGs (Jones et al., 1999). A similar method of NGF delivery dramatically upregulated pituitary adenylate cyclase-activating polypeptide (PACAP) expression in intact and injured L4-L6 DRG neurons (Jongsma Wallin et al., 2001). Moreover, NGF infusion increased BDNF expression in both intact and injured trkA-positive neurons, accompanied by reduced trkB expression (Karchewski et al., 2002), suggesting that NGF could regulate BDNF expression. Exogenous infusion of NT-3 and NGF also differentially modulated PACAP expression in DRG neurons (Jongsma Wallin et al., 2001), suggesting a dynamic role in injury response and inflammation.

Intrathecal infusion of NT-3 on intact, uninjured adult rat dorsal root ganglion neurons was investigated. NT-3 did not appear to alter trkC expression, but reduced trkA, high-affinity NGF binding sites and SP levels (Gratto and Verge, 2003).

Osmotic minipump infusion of GDNF to injured DRG prevented a number of injury induced manifestations. These included the loss of binding of IB4, downregulation of the purinergic receptor P2X(3), upregulation of galanin and NPY immunoreactivity in large diameter DRG cells, and expression of the transcription factor ATF3. The findings confirm that exogenous GDNF has a broad neuroprotective role in injured primary afferents (Wang et al., 2003).

Materials that can be exogenously applied include a variety of other growth factors. For example, exogenous injection of transforming growth factor-beta 1 (TGFβ1) to the proximal crushed end of the sciatic nerve increased the expression of FGF-2 in the anterior horn motoneurons of a regenerating spinal cord. Distal to the crush injury,

TGFβ1 increased both the number and diameter of regenerating myelinated axons. These results indicate that TGFβ1 promotes peripheral nerve regeneration, and up-regulates the FGF-2 expression in the anterior horn motoneurons of spinal cord during the peripheral nerve regenerative process (Pei et al., 2005).

Other proteins not characterized as growth factors have also been introduced to injured nerves. An osmotic pump delivering oxidized galectin-1, a carbohydrate binding protein with an affinity for ß-galactosides, peripherally to the site of sciatic nerve injury restored and significantly improved functional recovery of the injured nerve. There was also an increase to the number and size of regenerating myelinated fibers (Kadoya et al., 2005). Another example of a protein that does not belong to growth factors is the tissue plasminogen activator (tPA), an essential component of the proteolytic cascade that lyses blood clots. Following sciatic nerve injury in the mouse, concomitant application of exogenous tPA promoted axonal regeneration, remyelination, and functional recovery. A significant increase in the total number of axons and myelinated axons was observed, as manifested by enhanced expression of neurofilament preotein. In addition, tPA markedly reduced the deposition of fibrin and fibrinogen after nerve injury, increased the number of macrophages and induced matrix metaloprotease-9 expression at the injury site. Moreover, exogenous tPA application reduced collagen scar formation and accelerated clearance of myelin and lipid debris after treatment. Mice lacking tPA, or plasminogen genes showed delayed functional recovery after sciatic nerve crush (Siconolfi and Seeds, 2001). These findings support the notion that tPA promotes axonal regeneration and remyelination, making it a potential candidate for the treatment of peripheral nerve injury (Zou et al., 2006).

1.3.2 cAMP.

Cyclic 3',5'-adenosine monophosphate (cAMP) is a second messenger for various extracellular signals. When external stimuli, such as hormones and activating or growth factors bind certain receptors on the effector cell membrane, receptor occupation triggers dissociation and activation of heterotrimeric G proteins and the membrane-bound adenylate cyclase is activated. This enzyme catalyses the intracellular production of cAMP. The substrate for the catalytic action of adenylate cyclase is the complex Mg-ATP. cAMP exerts its regulatory action via the activation of protein kinase A (PKA) and can phosphorylate a number of key proteins. The substrate proteins for PKA can be enzymes, structural proteins (cytoskeletal elements, ribosomes, and synaptic membranes), nuclear proteins (histones), and growth associated proteins. The attachment of a phosphate to either a serine (in most cases) or a by PKA to a substrate protein may result in activation or inhibition of catalytic or binding properties. Phosphorylation of proteins is reversed by the activity of a special class of enzymes, the phosphoprotein phosphatases.

Degradation of intracellular cAMP is controlled by phosphodiesterases which convert the active cAMP into the inactive 5'- AMP. The intracellular levels of cAMP, and thus the kinases that cAMP regulates, are continuously governed by the balance of activities of adenylate cyclase and phosphodiesterase (Wiegant, 1978). A number of diverse families of adenylate cyclases and phosphodiesterases have now been identified, allowing for complex and diverse mechanisms to modulate the levels of intracellular cAMP (McKnight, 1991).

Cyclic AMP can exert its effects on gene expression through PKA mediated phosphorylation of transcription factors tht bind to enhancers located in the regions of DNA upstream of the transcription start sites to the genes. Examples of the two best characterized enhancer elements are the CRE (5'-TGACGTCA-3') and the AP-2 site (5'-CCCCAGGC-3'). The activity of the CRE is regulated by CRE binding proteins (CREBs). These proteins contain leucine zipper regions that allow for either homo- or hetero dimerization (Walton and Rehfuss, 1990). CREB-containing dimers, once phosphorylated, bind to the CRE and stimulate transcription of cAMP-responsive genes (Montminy et al., 1990b). Phosphorylation of CREB is reversible and is regulated by cytoplasmic PKA (Montminy et al., 1990a). Another nuclear factor that regulates CRE is, cAMP-responsive element modulator (CREM) (Delmas and Sassone-Corsi, 1994; Habener et al., 1995).

In the brain, damaged hippocampal dentate granule cells secrete neuroprotective molecules that activate CREB by phosphorylation of Ser133 and through the Akt signaling pathway (Walton and Dragunow, 2000). It would be important to investigate the role of CREB and the downstream survival genes that are activated by CREB in mediating the neuroprotective actions.

In the nervous system, cyclic nucleotides can regulate growth cone movement and directionality. Chemorepulsion induced in cultured *Xenopus* spinal neurons can be reversed by activation of the guanosine 3',5'-monophosphate (cGMP) or adenosine 3',5'-monophosphate (cAMP) signaling pathways. These findings suggest that it may be possible to counteract the inhibition of nerve regeneration by repulsive factors in the CNS (Song et al., 1998). The cellular machinery that regulates actin- and tubulin in the growth

cone, integrates stimulatory and inhibitory signals from the local environment and translates them into axon growth or growth cone collapse (Snider et al., 2002).

Neurotransmitters, neurotrophic factors, prostaglandins, and other extracellular signaling molecules can activate adenylate cyclase in neurons leading to increased production of cAMP. Evidence from this lab and others shows that cAMP signalling promotes neuronal survival and axonal elongation in neuronal cells. Dibutyryl cAMP (dbcAMP), a membrane permeable cAMP analogue, and forskolin, an adenylyl cyclase activator, stimulate neurite outgrowth in cultured DRG neurons (Anderson et al., 2000). These findings indicate that increased levels of cAMP in neurons may have an important role in axonal elongation.

Treatment with dbcAMP *in vivo* or addition of forskolin *in vitro* markedly increases axon growth, supporting the notion that cAMP triggers the intrinsic growth program of mature DRG neurons (Qiu et al., 2002b). However, in other neuronal cultures, cAMP addition alone does not support growth, nor does PKA inhibition prevent conditioning lesion-induced axon growth on a favorable substrate (Qiu et al., 2002b). This work suggests that elevation of cAMP levels alone may not be sufficient to mediate the enhanced growth state activated by peripheral axotomy, but may regulate the response to inhibitory cues (Snider et al., 2002).

Studies on the effect of introducing cAMP to injured sciatic nerve have been carried out. In one such study, clamping the sciatic nerve for 2 seconds, and injecting dbcAMP (50 mg/kg) intramuscularly for 7 days enhanced the return of sensorimotor functions, as demonstrated by foot-flick response (Pichichero et al., 1973). However, another study showed that a daily intramuscular injection of dbcAMP had no effect on

crushed sciatic nerve (McQuarrie et al., 1977). Although the latter study reported no effect of dbcAMP, the duration of the clamping of the sciatic nerve was longer than in the former study. It is documented that increasing cAMP levels in neuronal cell bodies elicits a partial regenerative response, altering expression of tubulin isotypes but not expression of other growth-associated genes or the rate of axonal transport (Han, Shukla et al. 2004). This approach suggests that control of the regenerative response may be complex and that multiple regulatory mechanisms may be involved (Liu and Brady, 2004).

In this lab, peliminary studies have shown that FGF-2 partially represses expression of GAP-43 in cultured adult DRG neurons. This effect occurs, at least in part, through a MEK pathway. Interestingly, combined application of both dbcAMP and FGF-2 completely represses GAP-43 expression in these cultures, indicating a combinatorial role for FGF-2 with cAMP in maintaining the mature, uninjured state (Schreyer, 2004). Additional experiments indicated that selective inhibition of FGF-2 increases GAP-43 levels of expression.

Microinjection of dbcAMP into lumbar DRG markedly increases the regeneration of central axon branches injured within the spinal cord. Regenerating central axons reach well into the spinal cord lesion, often passing the original injury site. Thus, cAMP signaling appears to mimick the effect of a conditioning peripheral nerve lesion through increasing the intrinsic growth capacity of injured sensory axons by overcoming the central nervous system myelin inhibitory effects (Han et al., 2004; Neumann et al., 2002). Intraganglionic injection of dbcAMP also increases the expression of growth-associated tubulin isotypes. However, it does not increase the average axonal transport rates of

tubulin delivered to the tips of growing axons. Injection of dbcAMP therefore induces

some, but not all of the changes that may be necessary to increase intrinsic axon growth

capacity (Han et al., 2004).

1.3.3 Mechanisms of Signaling

The well-understood consequence of adenyl cyclase activation is that cAMP

signaling leads to the activation of protein kinase A (PKA), phosphorylation of both the

mitogen-associated protein kinase (MAPK) and cAMP-response element-binding protein

(CREB), and subsequent transcription of growth-associated genes. Convergent signalling

through Ca^{2+} elevation can also lead to MAPK activation via the Ras pathway (Waltereit

and Weller, 2003).

The guanine nucleotide exchange protein activated by cAMP (Epac), which

belongs to the cAMP-binding protein family, represents an alternative pathway through

which adenyl cyclase can have diverse effects on cellular functions including

hormone/transmitter secretion, cell adhesion, and intracellular Ca^{2+} mobilization. Epac

mediates the PKA-independent effects of cAMP on cellular activities. Thus, cAMP

regulates cellular activities by coordinating both PKA-dependent and PKA-independent

pathways, possibly within different domains of the cell (Seino and Shibasaki, 2005). A

finding that supports the possible influence of cAMP through a PKA independent

pathway is that in cultured DRG neurons, signaling from cAMP to PKCε, (epsilon

isoform of protein kinase C) is not mediated by PKA, but by Epac. Epac, in turn, is

upstream of PLC and PLD, both of which are necessary for translocation and activation

of PKCε. In DRG neurons, this signaling pathway was specific to IB4-positive

nociceptors. Thus, cAMP signaling through Epac may involve both the MAPK pathway and PKC in parallel (Hucho et al., 2005).

Little is known about the signals that control how adult neurons switch from a normally transmitting, differentiated state to a regeneration state, although BDNF appears to be a critical part of this process (Geremia et al., 2009). There is no clear indication as to whether expression of the various components of the injury response are directed by a common regulatory mechanism or whether each GAP gene, or protein is controlled by individual inducing or silencing factors that act in parallel.

It is well established that deprivation of target-derived trophic factors (Lee et al., 1998; Raivich et al., 1991) and the arrival of activating signals (chemokines and cytokines) from the injured axon tip and from the surrounding non-neuronal cells, can initiate the regenerative signaling cascades. In the few days after injury, macrophages and reactive Schwann cells infiltrate the degenerating nerve and release cytokines and neurotrophic factors, the effects of which may reach the injured neuronal cell body through retrograde axonal transport to switch on, promote, and sustain the regenerative program of the neuron.

A variety of mechanisms in response to signals are responsible for the initiation and maintenance of the regenerative neuronal response. Some signals that are common with cAMP signaling pathway have been reported. For example, the high frequency burst of action potentials generated at the lesion site results in the rapid influx of Ca^{+2} ions immediately following injury. This can activate several protein kinase pathways, including CMAK2, protein kinases PKA, PKC, and MAPK (Ghosh and Greenberg, 1995). The activation of PKC and calmodulin dependent kinases can influence GAP-43

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expression and other growth associated proteins. Additionally, the intense burst of excitation causes transmitters, such as glutamate, neuropeptides and BDNF to be released at the spinal cord. These can also activate PKA, PKC and MAPK postsynaptically (Navarro et al., 2007).

Other signaling pathways that may be common with the cAMP signaling pathway in response to nerve injury are activated in DRG neuronal cell bodies. Among these pathways are kinases such as the MAPKs Erk1 and Erk2, JNK and p38 kinase (Navarro et al., 2007). Following axotomy, Erk activation has been observed in axotomized sciatic nerve and DRG (Agthong et al., 2006; Katsura et al., 2007; Obata et al., 2007; Obata et al., 2004a; b; Obata et al., 2003). This activation of Erk is influenced by neurotrophic factors such as NGF and GDNF (Wiklund et al., 2002). JNK is rapidly activated after nerve injury and remains elevated for weeks until either neuronal death or axonal regeneration occurs (Kenney and Kocsis, 1998; Waetzig et al., 2006). Similarly, p38 is transiently activated following peripheral axotomy in small diameter DRG neurons (Ji et al., 2002; Murashov et al., 2001) as well as in small-to-medium DRG neurons after chronic constriction injury (Obata, Yamanaka et al. 2004).

Activation of these kinases as a result of axotomy influences downstream events that include upregulation or activation of a number of transcription factors. Activated JNK increases the expression and phosphorylation of c-Jun, JunD and Fos, and their translocation into the nucleus. This leads to the formation of AP-1 complexes that activate many downstream genes (Kenney and Kocsis, 1998; Raivich and Behrens, 2006; Raivich and Makwana, 2007). Activation of AP-1 sites can influence the expression of GAP-43 and Tα1 tubulins and perhaps other growth associated proteins. The presence of

basal levels of transcription factors characterized as immediate early genes in DRG

neurons has been reported (Sheng et al., 1995), but many of these are increased following

axotomy or lesion of the sciatic nerve (Herdegen et al., 1992; Kajander et al., 1996;

Kenney and Kocsis, 1998; Wang et al., 2002).

In the CNS, c-Jun deficient neurons do not express CD44, galanin, and α7β1

integrin, genes that normally participate in the injury response (Raivich et al., 2004).

Several protein kinase pathways activate transcription factor CREB in the early stages

after injury, some through the Erk pathway. ATF3 expression is upregulated in all DRG

neurons after peripheral, but not central axotomy (Tsujino, Kondo et al. 2000). ATF3

expression in subpopulations of DRG cells can be modulated by exogenous trophic

factors, such as NGF and GDNF (Averill et al., 2004). Activated STAT3 is required in

the regeneration process of DRG neurons and the central axonal growth in the spinal cord

after a conditioning injury (Qiu, Cafferty et al. 2005). The PI3K-Akt pathway seems to

also be involved in DRG neuronal response to injury (Leinninger et al., 2004).

CHAPTER 2
2.0 RATIONALE OF THE CURRENT PROJECT

We have insufficient knowledge about the mechanisms by which positive or negative signals associated with peripheral axon injury may lead to the injury response and enhancement of intrinsic regenerative ability in DRG neurons. Evidence from this lab and others show that the activation of cAMP-signalling promotes neuronal survival and axonal elongation in cultured DRG neurons and stimulates peripheral nerve regeneration *in vivo*. These findings indicate that increased levels of cAMP in neurons have an important role in axonal elongation. Paradoxically, this lab has found that cAMP signaling suppresses expression of the prominent growth-associated protein GAP-43 in cultured adult DRG neurons (Andersen et al., 2000).

FGF-2 acts specifically on central and peripheral neurons during development and regeneration (Unsicker, 1993). FGF-2 is expressed by the Schwann cells of the peripheral nerves and by the satellite cells of the DRG, and is important in wound repair and regenerative events in the injured peripheral nerves (Duobles et al., 2008).

In this lab, recent unpublished studies have shown that exogenous FGF-2 also suppresses GAP-43 expression in cultured adult DRG neurons. FGF-2 was shown to repress GAP-43 at least in part through a MEK pathway. Combined application of both cAMP and FGF-2 completely represses GAP-43, indicating a complimentary role for FGF-2 with cAMP in maintaining the repressed state of GAP-43. Additional experiments indicated that selective inhibition of FGF-2 signaling increases GAP-43 level of expression in the brain.

The survey of the literature and the preliminary results from tissue culture experiments leads to a reasonable assumption that cAMP and/or FGF-2 may act as

retrograde signaling molecules to maintain the basal metabolic state in uninjured neurons, (for example suppressing GAP-43 expression), and whose absence contributes to the generation of the injury response.

The current project endeavors to answer two key questions: 1) Is the expression of transcription factors ATF3, c-Jun, and STAT3 by DRG neurons similar to that of GAP-43? 2) If so, could these transcription factors be regulated by signaling mechanisms that aslo regulate GAP-43?

CHAPTER 3
3.0 HYPOTHESES

1) FGF-2 regulates expression of GAP-43 in DRG neurons *in vivo*, through modulation

of transcription factors ATF3, c-Jun and/or STAT3.

2) cAMP regulates expression of GAP-43 in DRG neurons *in vivo*, through modulation of

transcription factors ATF3, c-Jun and/or STAT3.

CHAPTER 4
4.0 SPECIFIC AIMS

1. Establish through Western blot analysis, immunohistochemistry and image analysis whether expression patterns of ATF3 and GAP-43 are similar in their response to peripheral sciatic nerve transection or dorsal root transection of DRG neurons.

2. Establish through Western blot analysis, immunohistochemistry and image analysis whether expression patterns of c-Jun and GAP-43 are similar in their response to peripheral sciatic nerve transection or dorsal root transection of DRG neurons.

3. Establish through Western blot analysis, immunohistochemistry and image analysis whether expression patterns of STAT3 and GAP-43 are similar in their response to peripheral sciatic nerve transection or dorsal root transection of DRG neurons.

4. Develop, design and test an improved *in vivo* method using mini osmotic pumps to study the possible modulatory role of FGF-2 and cAMP on GAP-43 expression, when applied to the site of injured sciatic nerve.

5. Develop, design and test an improved *in vivo* method using mini osmotic pumps to study the possible modulatory role of FGF-2 and cAMP on expression of ATF3, c-Jun and STAT3 transcription factor, when applied to the site of injured sciatic nerve.

CHAPTER 5
5.0 SIGNIFICANCE

The elucidation of the nature of signal(s) that lead to the reexpression of the GAPs in response to injury could help to understand the requirements for peripheral neuronal growth and might suggest ways to improve regeneration in the CNS. From a cell biological point of view, it will be important to relate renewed expression of GAPss following injury to the expression and activation of transcription factors. These studies of FGF-2 and cAMP application could help identify extrinsic influences (positive or negative) which are crucial to the modulation of the injury response in a model system in which axon regeneration readily occurs. Moreover, detailed knowledge of the role of transcription factors in mediating the response to injury could be utilized to design new therapeutic strategies for PNS and CNS trauma to complement the current clinically available techniques.

CHAPTER 6
6.0 MATERIALS AND METHODS

6.1 Animals.

All animals used in this project were cared for and used in accordance with the guidelines of the Canadian Council on Animal Care, and the regulations of the University of Saskatchewan Committee on Animal Care and Supply.

Female Sprague Dawley rats (Charles River, Wilmington, MA, USA) weighing between 250 - 300 g were maintained in our animal facilities with 12 hour light/dark cycle at 25ºC, and fed rat chow *ad libitum.*

6.2 Peripheral sciatic nerve transection.

Animals were anesthetized using halothane (Halocarbon Laboratories, River Edge, NJ, USA) or isoflurane (Baxter Corporation, Toronto ON, Canada) delivered by inhalation using an anesthetic vaporizer (Medishield Products Ltd. Rexdale, ON, Canada). The vaporizer was connected to an oxygen cylinder (100% O_2). The oxygen and the anesthetic at a concentration of 1.5-2% were delivered in a closed circuit at a flow rate of 1 L/min to a nose cone attached to the rat's snout. Deep anesthesia was confirmed by pinching the hind paw with forceps and noting the absence of a withdrawal reflex. Anesthesia administration and the entire surgical procedure were done under aseptic conditions and. Body temperature was maintained using an electrically heated pad. Care was taken to continuously monitor the breathing signs of the rat, as well as the color of the paws during the entire surgery. The skin over the left (ipsilateral) thigh region was shaved, cleansed with 70% alcohol, and a 0.5 cm incision was made in the

skin using size 21 stainless steel Paragon sterile surgical blades at the mid-thigh level.
The biceps femoris muscle was then teased with small curved tip scissors to allow access
to the common sciatic nerve, which is located immediately beneath this muscle. The
nerve was then carefully transected using 6 mm blade spring scissors from separating the
proximal from the distal stumps. The damaged muscle was sutured using 6.0 ophthalmic
silk sutures. The external incision in the skin was then sutured using 4.0 silk sutures
(Ethicon Inc., Johnson and Johnson, NJ, USA). The right sciatic nerve (contralateral)
was left intact to serve as an internal control. At the end of surgery, the nose cone was
removed from the rat and recovery was monitored. The rat was then transferred back to
the cage, and placed in the recovery room for 1 (or 2) wk.

Transcardial perfusion was done after an intraperitoneal injection of sodium
pentobarbital (Somnitol; 30 mg/kg or Euthanyl (65 mg/kg)) to achieve terminal
anesthesia. Deep anesthesia was confirmed and a ventral midline incision was made
from mid-thoracic to the most caudal part of the abdominal region. The diaphragm was
immediately cut transversely and the thoracic cage was reflected to expose the heart.
Immediately the right ventricle was nicked and a perfusion needle was inserted through
the left ventricle, reaching the transparent aorta. The needle was stabilized using a small
pair of suturing forceps, and the animal was flushed with ice cold phosphate bufferd
saline (PBS) at pH 7.4, at a flow rate of 50 ml/min. After a total volume of 200 ml of
PBS was used, an equal volume of 4% ice cold paraformaldehayde in 0.1M phosphate
buffer was delivered. Both ipsilateral and contralateral fourth lumbar (L4) and fifth
lumbar (L5) DRG (N = 4 animals) were then carefully removed, post-fixed in 4%
paraformaldehyde for 2 hours, and cryoprotected with 20 % sucrose (in PBS) overnight at

4°C. The tissues were then coated with Tissue-Tek OCT compound in disposable vinyl cryomolds and kept frozen at -80^{0}C until cryosectioning and immunohistochemical analysis was performed (detailed below in section 6.6).

6.3 Dorsal root transection.

The animals were deeply anesthetized using isoflurane as described above to shave the entire back, and the skin was sterilized using 70 % alcohol. A dorsal midline incision was made from twelfth thoracic vertebra (T12) to the sixth lumbar vertebra (L6). The paraspinal muscles were separated from the spinal processes on the both sides of the vertebral column spanning the L3-L5 region. The transverse processes of L3 and L5 were exposed by scraping off attached ligaments using rongeurs, and the spinous processes of L3, L4, and L5 were all removed. Hemilaminectomy was performed on the left side at L3 and L4 to expose the L4 and L5 dorsal roots. The dura was carefully opened using the tip of a 27.5 gauge needle to expose the dorsal spinal roots within the vertebral canal. The occasional bleeding that took place during surgery was blotted using small pieces of sterilized filter paper or Gelfoam to clot and minimize the bleeding. Both the left L4 and left L5 dorsal roots were identified under a dissecting microscope and cut using 6 mm blade spring scissors. The separated paraspinal muscles were sutured together using 6.0 silk ophthalmic sutures. A piece of Gelfoam comparable to the size of the incision was placed in areas where muscles had been removed. The external skin incision was sutured using 4.0 silk sutures.   The right dorsal roots of both L4 and L5 were left as internal controls. Postoperative pain was controlled by 0.05 mg/kg subcutaneous injections of buprenorphine hydrochloride (Buprenex) every eight hours during the first three days of

the postoperative period. Seven days after surgery, rats were perfused with 4%

praformaldehyde in 0.1M phosphate buffer (as described above) and both ipsilateral and

contralateral DRG (N = 4 animals) were collected and processed for cryosectioning as

described above.

6.4 Mini-osmotic pump preparation.

Altzet® mini-osmotic pumps Model 2001 (Durect Corporation, Cupertino, CA,

USA) were used in order to ensure the delivery of controlled and constant amount of

FGF-2, and/or dbcAMP to the cut end of the proximal nerve stump for seven consecutive

days (Figure 6-1). Each mini-osmotic pump is designed by the manufacturer to hold up

to 200 µl of material in the reservoir and can be surgically implanted subcutaneously with

ease anywhere in the body. As a result of osmotic pressure from bodily fluids, the pumps

deliver at a flow rate of 1 µl/hr. This pumping rate is ideal for our experimental

procedures, since we leave the animals for seven days postoperatively.

For our experiment, control pumps were filled with vehicle (1 mg/ml rat serum

albumin plus 10 µl/ml penicillin-streptomycin mixture in 0.1M PBS). The experimental

pumps are filled with either FGF-2 in vehicle at a final concentration of 50 ng/ml, or

dbcAMP in vehicle at a final concentration of 0.5 mM.

6.4.1 Mini-osmotic pump and chamber surgical implantation.

Under aseptic conditions, surgical implantation of the mini-osmotic pumps was carried

out after animals were deeply anesthetized using isoflurane, as described above. An

incision (2 cm) was made on the dorsal aspect of the rat, between the thoracic and lumbar

region. The skin was then separated using the blunt tip of large scissors in order to create

the necessary space for the pump implantation. The pump was implanted on the
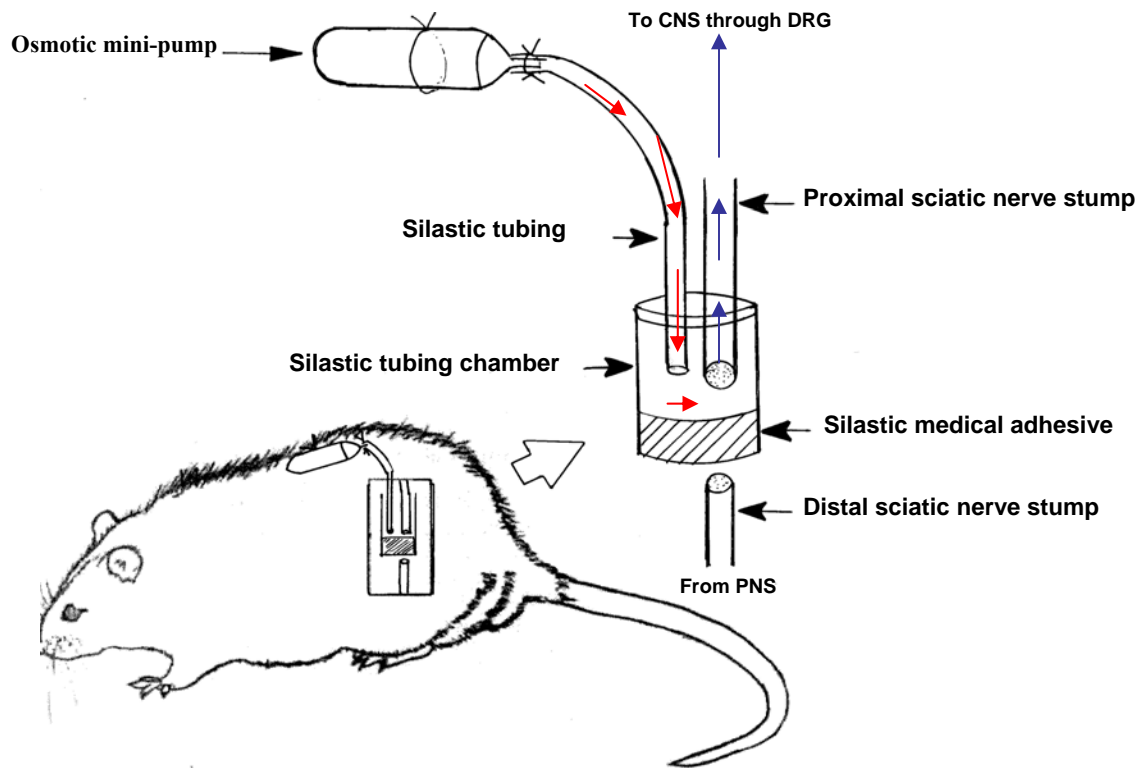
Figure 6-1. Implantable mini-osmotic pump nerve chamber model.   Surgical implantation of osmotic mini-pumps for fluid delivery to the cut sciatic nerve. The connection between the pump and the site of injury is done using silastic tubing. At the site of injury an appropriate size chamber is made using silastic tubing of a larger size. This chamber ensures that the material that is coming from the pump is confined to the proximal end of the nerve, and minimizes the effects of any reterograde signal that might emminate from the immediate surrounding environment.

6.4.1 Mini-osmotic pump and chamber surgical implantation.

Under aseptic conditions, surgical implantation of the mini-osmotic pumps was

carried out after animals were deeply anesthetized using isoflurane, as described above.

An incision (2 cm) was made on the dorsal aspect of the rat, between the thoracic and

lumbar region. The skin was then separated using the blunt tip of large scissors in order to create the necessary space for the pump implantation. The pump was implanted on the dorsal thoraco-lumbar region, and secured by 6.0 ophthalmic sutures to ensure stability. The pump was connected by 5 cm long small diameter silastic tubing the dimensions of which are: 0.025 in. ID (0.64 mm) x 0.047 in. OD (1.19 mm). This length was chosen to ensure that the contents of the mini-osmotic pump reached specifically the proximal cut end of the sciatic nerve in the mid-thigh region. Another incision was made at the mid-thigh level and a peripheral sciatic nerve transection was carried out just as described above. A large diameter silastic tubing (0.5 cm long) the dimensions of which are: 0.14 in. ID (2.64 mm) x 0.192 in. OD (4.88 mm) was sealed from one end by silastic medical adhesive silicone. The open end served as a chamber in which the small diameter silastic tubing connected to the pump was inserted. The proximal cut end of the nerve was also inserted in the same chamber. The chamber was then carefully placed in the mid-thigh region, where it was secured by additional 6.0 sutures to the muscles in the area. This set up was ideal since it took advantage of the anatomical confinements and utilized them to secure foreign objects for a period of time. The animals tolerated the chamber and the implanted mini-osmotic pump well. The incision wound from the implantation of the mini-osmotic pump was adequately healed one day following surgery. At the end of the experimental period (7 d), animals were either perfused as described above for DRG tissue immunohistochemical processing (Vehicle: N = 4 animals, FGF-2: N = 4 animals, dbcAMP: N = 4 animals), or killed with carbon dioxide asphyxiation for Western blot analysis (Vehicle: N = 4 animals, FGF-2: N = 4 animals, dbcAMP: N = 4 animals).

6.5 Western blot analysis.

L4 and L5 DRG tissues ipsilateral and contralateral to the sciatic nerve injury were immediately removed from each of the four animals in each experimental group and pooled in ice cold PBS solution. Attached nerve and roots were carefully removed under the dissecting microscope, and the epineurium cut to expose the neurons. The tissues were then placed in lysis buffer (20 mM HEPES (pH 7.5), 50 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, Sigma anti-protease cocktail, and 1% NP-40). Tissues were then homogenized at high speed using Tissue Tearer (Biospec Products, Inc, OK, USA) to break down connective tissue components. The homogenate was further subjected to pulse sonication (three times for 5 sec with 1 min interval on ice). The sonicated homogenate was centrifuged at 14,000 rpm at 4ºC for 30 minutes. The supernatant was then collected and its protein content was assayed using the Bradford method (Bradford, 1976), or Lowry's method (Lowry et al., 1951).

Electrophoresis was done using a BioRad Mini-Protean Tetra Cell system. Proteins (20 µg per lane) were loaded and separated using polyacrylamide gel electrophoresis (2 h at room temperature, 120V). 10 % acrylamide gels were used for higher molecular weight detection and 12% acrylamide gels were used for smaller molecular weight detection. The proteins were transferred and blotted on Pall BioTrace NT nitrocellulose membranes, (Pensacola, FL USA) using standard transfer buffer (12.08 g Tris base, 56.6 g glycine, 800 ml methanol, added to water in a total volume of 4 liters). Transfer time was 2 hours at 4ºC. Membranes were initially soaked in PBS for 10 minutes prior to blocking. Non-specific binding was blocked overnight on a shaker

(Fisher Scientific clinical rotator) using blocking solution containing 5% (w/v) non-fat milk (Biorad, Hercules, CA, USA) in PBS + 0.1% Tween 20.

The following primary antibodies were diluted in blocking solution: mouse anti-GAP-43 monoclonal antibody 9-1E12 (Schreyer and Skene, 1991) (1:5000), rabbit anti-c-Jun (Santa Cruz, 1:300), rabbit anti-ATF3 (Santa Cruz, 1:200), rabbit anti-STAT3 (Santa Cruz, 1:300), rabbit anti-pSTAT3 (Cell Signaling, 1:500), and mouse anti-c-Jun (Cell Signaling, 1:200). All incubations of membranes in primary antibodies in blocking solution were done overnight. After several washes with PBS, two types of detection methods were used: chemiluminescent and fluorescent. For enhanced chemiluminescence (ECL) detection, membranes were incubated in mouse or rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The chemiluminescence was detected using enhanced ECL luminol substrate of the peroxidase captured on Kodak film. For fluorescent detection, membranes were incubated in donkey anti-mouse conjugated to IRDye 800 (green), or with goat anti-rabbit conjugated to IRDye 680 (red) fluorescent secondary antibodies (Licor Biosciences). Fluorescence detection was carried out using an Odyssey Infrared Imaging System (Licor Biosciences, NE, USA). This system is uniquely equipped with two infrared channels (800 nm and 680 nm) for direct fluorescence detection on membranes.

6.6 Immunohistochemistry (IHC).

A separate set of experiments under identical conditions set forth in the Western blot analysis were performed and employed for IHC analysis. For peripheral transection

immunohistochemistry, DRG from ipsilateral (cut) and contralateral (uncut) L4 and L5

DRG from the four animals were embedded in the same mold and placed on the same

slide to ensure that all sections underwent similar processing conditions for primary and

secondary antibodies, and for further fluorescence image analyses. L4 and L5 DRG

collected from dorsal transection experiments underwent similar tissue processing

procedures (Figure 6-2).   Spararte slides were prepared for each antibody (i.e. ATF3, c-

Jun, STAT3, and pSTAT3), from the same group of experiments. Since these antibodies

were raised in rabbit, and since GAP-43 was raised in mouse, we double labeled all the

slides with GAP-43. This procedure was repeated for reproducibility.
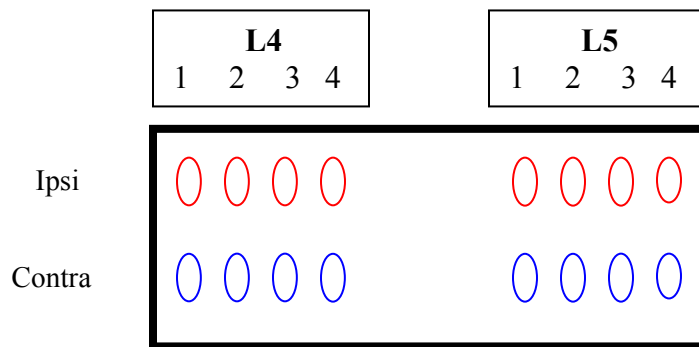
**Figure 6-2. Arrangement of DRG sections from peripheral nerve or dorsal root
transection experiments.** Contralateral (blue ovals) and ipsilateral (red ovals) DRG
tissues from animals that have andergone peripheral sciatic nerve transection, or dorsal
root transection. Tissues were arranged so that tissues from four similar animals are
placed on the same slide for immunohistochmical analysis. 1-4 denotes the animal
number.

For mini-pump experiments, ipsilateral and contralateral L4 or L5 DRG sections

from four animals from each of the three experimental treatment groups were arranged on

gelatin coated slides and processed on the same slides under identical experimental

conditions (Figure 6-3). This arrangement avoids bias resulting from variance in

immunofluorescence intensities from slide to slide. Representative slides that contained

roughly similar number of neurons in each DRG section were chosen for further

quantitative analysis. L4 and L5 DRG tissues were segregated onto different slides.
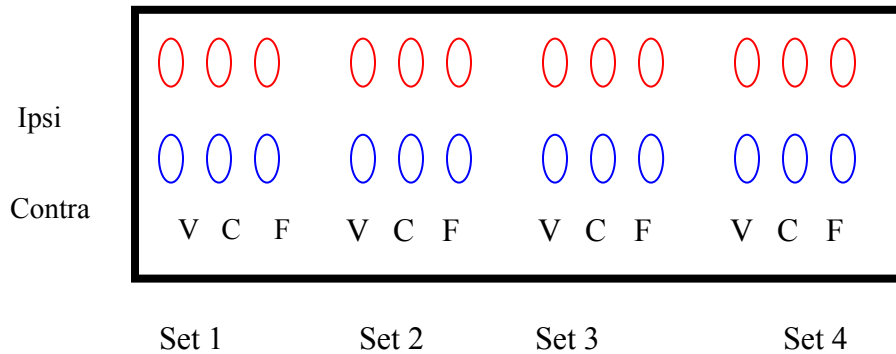
Figure 6-3. Arrangement of DRG sections from mini-pump experiments.
Contralateral (blue ovals) and ipsilateral (red ovals) L4 DRG tissues from animals
surgically implanted with mini-pumps containing: vehicle (V), dbcAMP (C), or FGF-2
(F) were arranged so that each set of experiments are placed on the same slide for
immunohistochmical analysis.

Briefly, DRG tissues were cryo-sectioned at 6 μm and placed on gelatin coated

slides. Sections were blocked (2% horse serum, 2% goat serum, 1% BSA, 0.1% Triton

X-100 in PBS) for 1hr at room temperature. Sections were double labeled and incubated

overnight at 4°C with the primary antibodies mouse anti-GAP-43 (1:5000) and rabbit

anti-ATF3 (Santa Cruz, 1:1000) in blocking solution. Sections were washed (3 x 5

minutes) with PBS and then incubated with fluorescently labeled secondary antibodies

that included Cy2-and Cy3-conjugates of goat-anti-rabbit IgG (Jackson ImmunoResearch

Laboratories, West Grove, PA) both at a dilution of 1:300 in blocking solution for 1 hr at

room temperature. Sections were washed (3 x 5 min) with PBS. For nuclear labeling, all

slides were incubated for 5 min in 4',6-diamidino-2-phenylindole (DAPI) at a

concentration of 1:20,000. Sections were coverslipped with Citifluor and viewed using a

Zeiss Axioskop fluorescence microscope (Zeiss Canada).

6.7 Immunohistochemical image analysis.

To analyze the images of slides prepared for immunohistochemistry, we used a Zeiss Axioskop microscope (Zeiss, Germany) fitted with epifluorescence optics. This system uses an X-cite fluorescence source (Photonic Solutions, Inc. Canada) to ensure even illumination and images were captured using a Qimaging, Retga Ex*i* camera. Images were digitized and transferred to Northern Eclipse imaging software (Empix Imaging Inc., Mississauga, ON, Canada), and the fluorescent labeling intensities of individual neurons and were quantified using a specifically designed program for our laboratory. The software program was designed to measure the intensity of the fluorescent signal in the cytoplasm (or nucleus), as well as the mean diameter of that neuron.

Four animals were used in each group. In each contralateral and ipsilateral DRG, at least 200 neurons that were DAPI positive (i.e. nucleus was visible) were manually traced and included in the quantification process. Each individual neuron was quantified for the GAP-43 and ATF3 labeling intensities, as well as the size of the individual neuron. The same technique was used for the ipsilateral DRGs. A similar quantification method was used for dorsal root transection experiments.

For double labeling with two different antibodies, the software was designed to measure the intensity of the two different antibodies in the exact same neuronal cytoplasm (or nucleus) giving us the advantage of quantifying and correlating the expression of antibodies in individual neurons. The labeling intensity data were transferred to Excel program (Microsoft) spreadsheets, for further statistical and graphical analyses.

6.8 Statistical analysis of immunohistochemical data.

The relative changes in immunofluorescence labeling intensities from one group to another were recorded for sections mounted on the same slide to avoid bias resulting from slide to slide variations. Data from at least 800 DRG neurons from 4 different rats were included to calculate the mean labeling intensity in each experimental group (both contralateral and ipsilateral). For the peripheral transection experiments, the mean labeling intensity of at least 800 DAPI positive neurons from the contralateral DRG of four different animals were normalized to 1. The mean labeling intensity of about a similar number of ipsilateral neurons from the same animals were included and compared to the normalized contralateral.

In double labeling experiments (example: GAP-43 and ATF3) slides were prepared as in Figure 6-2. The fluorescence measurements of cytoplasmic and nuclear intensities were taken using a specific filter that detected the secondary antibody conjugated to GAP-43 first. The filter was changed so that it could detect the secondary antibody conjugated to ATF3. Thus, this process generated, for each neuron, two sets of data one for each of the primary antibodies tested. This process repeated to collect at data from at least 200 neurons from each animal. This entire process was repeated to include data from the rest of the 3 animals. The resulting data would have a total of 4 contalateral set of measurements and 4 corresponding ipsilateral set of measurements. The contralateral data for all of the 4 different animals were normalized to 1. The ipsilateral cytoplasmic labeling intensity data were compared to the normalized contralateral cytoplasmic; and the ipsilateral nuclear labeling intensity data were compared to the normalized contralateral nuclear labeling intensity data.

To ascertain whether changes between ipsilateral and contralateral labeling intensities were significant, Kruskal-Wallis nonparametric ANOVA test was performed. This particular test was employed because our data collected did not assume a normal Gaussian distribution, and to ensure that we collected data from a heterogeneous neuronal size range. This analysis was done using Prism software (Prism v.4 GraphPad Software Inc., San Diego, CA) to compare the cytoplasmic and nuclear labeling intensities of each transcription factor in individual sensory neurons in each of the three treatment groups (vehicle, dbcAMP, and FGF-2). In each experimental mini-pump group, we measured the mean labeling intensities of each transcription factor of contra-and ipsilateral neurons in four different animals, and the selection of neurons was based on DAPI staining. This particular ANOVA test was followed by Dunn's multiple comparison tests to determine significant differences between specific groups of data. Statistical significance was accepted at $P < 0.05$ level.

Scatter plots were generated whereby the neuronal diameter data for each animal was plotted against the mean fluorescent labeling intensity for each of the antibodies tested. This type of graph provides qualitative assessment of the overall neuronal response to experimental conditions. Moreover, any variation between the responses of size subpopulations of neurons to a particular experimental condition can be easily seen, and subjected to further quantitative statistical analyses.

CHAPTER 7
7.0 RESULTS

7.1 Normal injury response to peripheral sciatic nerve transection

7.1.1 GAP-43 and ATF3 expression seven days after injury.

GAP-43 is normally expressed at low levels in uninjured neurons and is confined primarily to the small diameter neurons (Figure 7-1 A). Seven days following peripheral sciatic nerve transection, GAP-43 expression is elevated in the majority of neurons, including large diameter neurons (Figure 7-1 B). The nuclei of injured DRG neurons that are GAP-43 positive often exhibit an eccentric localization, displaying a typical chromatolytic morphology. In order to minimize sampling bias based on the size of the neuronal perikarya, only neurons that display a clear DAPI nuclear stain were included in the statistical calculations represented in the bar graphs below.

Western blot analysis of DRG neurons with anti- GAP-43 shows a specific band at MW 43 kDa corresponding to GAP-43, which is increased in the ipsilateral DRG (Figure 7-1 C).

Immunohistochemical results show that one week following sciatic nerve transection (N=4), GAP-43 and ATF3 are both upregulated in L4 DRG ipsilateral to the injury (Figure 7-2 A and C), and in L5 DRG (Figure 7-3A and C), when compared to the corresponding contralateral controls. Upregulated ATF3 is principally localized in the nuclei of injured neurons; whereas, GAP-43 is localized principally in the cytoplasm of injured neurons.

Western blot analysis of DRG neurons using affinity purified polyclonal antibody raised against a peptide mapping the C-terminus of human ATF3 shows a band at about

MW 26 kDa (Figure 7-3 E). This band was prominent in samples from ipsilateral injured

DRG, but undetectable in samples from contralateral vontrol DRG.
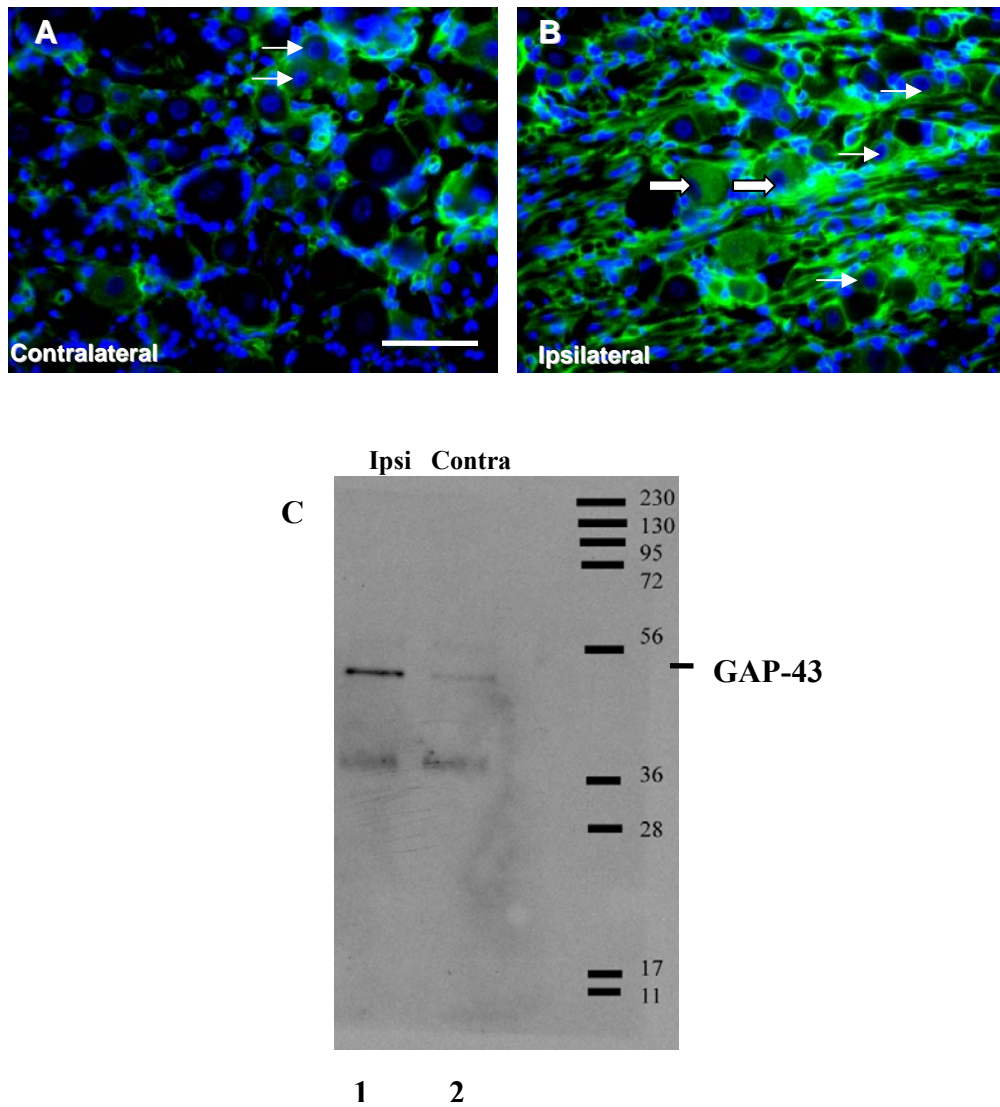
Figure 7-1. GAP-43 expression increases in DRG 7 d following sciatic nerve transection. Representative micrographs display the effect of injury on the expression of GAP-43 in the DRG neurons. (A) In uninjured DRG (contralateral), GAP-43 (green) is expressed at low levels in the neurons, especially in the small diameter neurons (small arrows). (B) In injured DRG (ipsilateral) GAP-43 expression is more pronounced and includes large diameter neurons (block arrows), which also display visible chromatolytic morphology. Cell nuclei are stained with DAPI (blue). (C) Western blot analysis for GAP-43 in L4 and L5 DRG neurons confirms the immunohistochemical data. Lane 1, ipsilateral; lane 2 contralateral to the injury. Scale bar represents 100 μm.
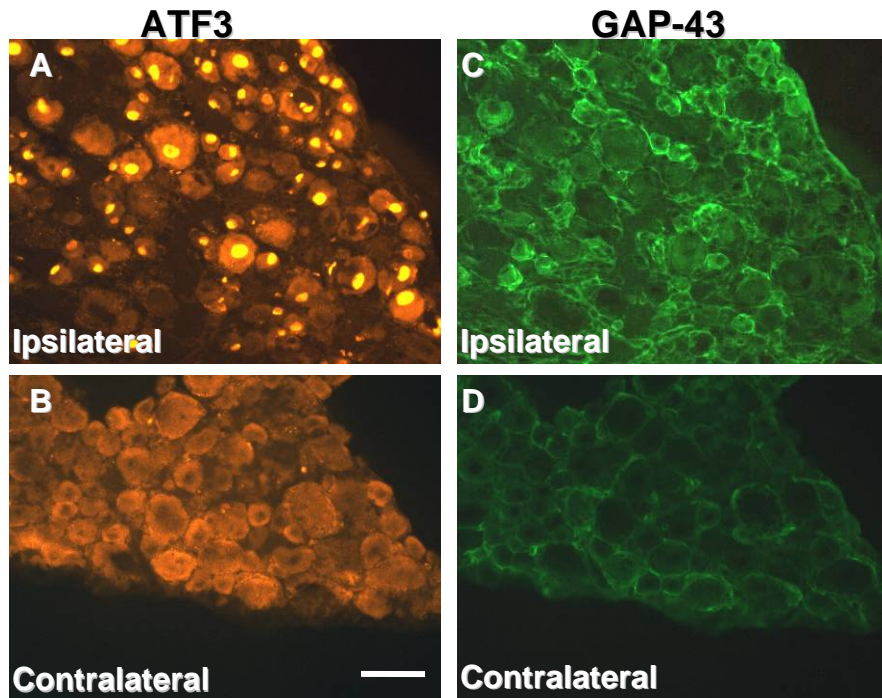
Figure 7-2. ATF3 and GAP-43 expression increases in L4 DRG 7 d following sciatic nerve transection. Micrographs of identical sections of L4 DRG neurons double labeled with ATF3 (red, A and B) and GAP-43 (green, C and D). (A) In injured DRG (ipsilateral), ATF3 (red) expression is enhanced compared to contralateral DRG (B). GAP-43 (green) expression is increased in ipsilateral (C) compared to the contralateral (D). Scale bar represents 100 µm.
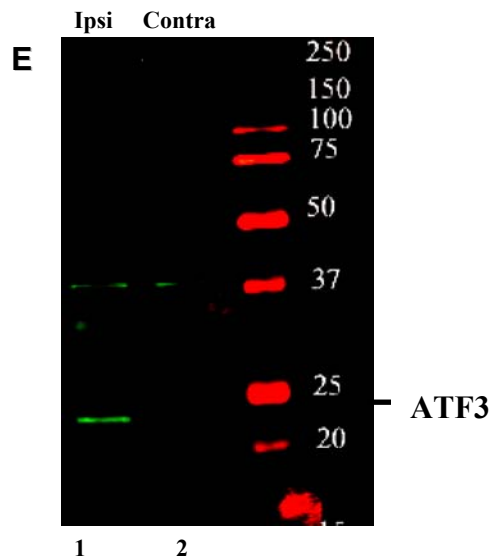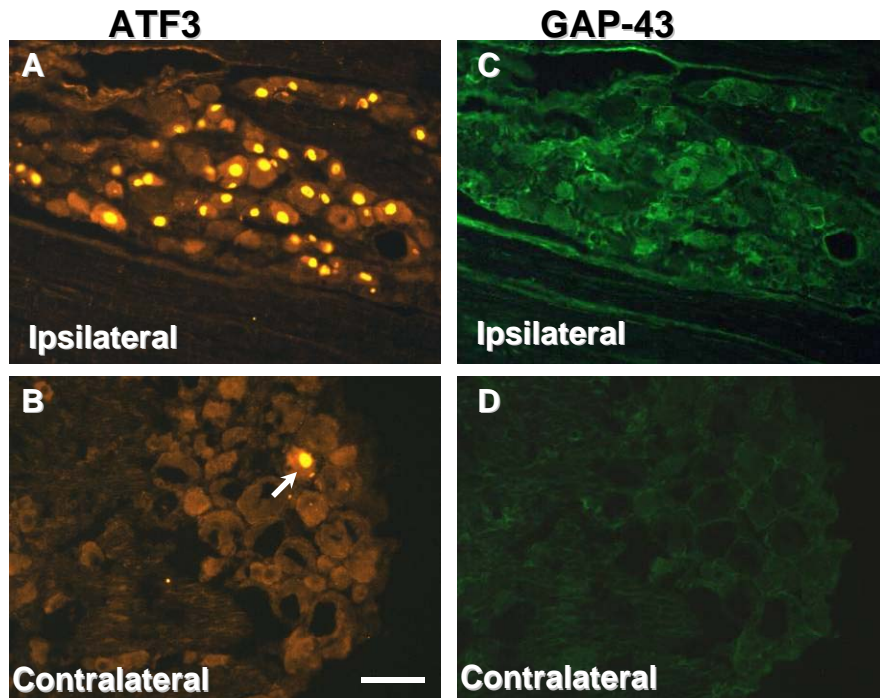
Figure 7-3. ATF3 and GAP-43 expression increases in L5 DRG 7 d following sciatic nerve transection. Micrographs of identical sections of L5 DRG neurons double labeled with ATF3 (red, A and B) and GAP-43 (green, C and D). (A) In injured DRG (ipsilateral), ATF3 (red) expression is enhanced compared to contralateral DRG (B). GAP-43 (green) expression is increased in ipsilateral (C) compared to the contralateral (D). Arrow in (B) points to occasional ATF3 positive neurons. (E) Western blot analysis for ATF3 in L4 and L5 DRG neurons confirms the immunohistochemical data. Lane 1, ipsilateral; lane 2 contralateral to the injury. Scale bar in micrographs represents 100 μm.

The nuclear and cytoplasmic labeling intensity of ATF3 and GAP-43 were measured in both contralateral (uninjured) and ipsilateral (cut) DRG neurons double labeled with both antibodies. In all cases, the mean contralateral value was normalized to 1 and the average values for ATF3 and or GAP-43 in ipsilateral injured DRG neurons were compared to the normalized contralateral value. A combined number of about 800 neurons from four different rats were used in each experimental group. Analysis of the data using two-way ANOVA followed by Kruskal Wallis test was performed comparing the combined number of neurons. Nuclear ATF3 values in the transected nerve (2.58 $\pm$ 0.02) were significantly higher than measured in the contralateral, uninjured nerve ($P <$ 0.001). The cytoplasmic ATF3 values also showed a significantly higher level than the contralateral side (1.17 $\pm$ 0.02) ($P < 0.001$). GAP-43 expression was significantly higher value in the injured nerve than in the contralateral nerve (1.39 $\pm$ 0.02) ($P < 0.001$) (Figure 7-4). Thus, quantification of the immunohistochemical data from DRG neurons show that sciatic nerve transection at mid-thigh level significantly increases the expression of the transcription factor ATF3 and that of GAP-43.

Northern Eclipse imaging analysis was used to quantify the labeling intensity of ATF3 and GAP-43 as a function of neuronal diameter. The image analysis takes into account the average grey area of the fluorescence emitted by the conjugated secondary antibody to the primary antibody. Manual tracing of individual neurons and of their respective nuclei generate numbers reflective of both neuronal diameter and labeling intensity values which can be entered in Excel and or Prism, where they can be analyzed. This type of data presentation shows changes in the pattern of labeling intensity values across the variable sizes of the subpopulation of DRG neurons.

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ATF3 and GAP-43 (peripheral nerve transection)

Figure 7-4. Quantification of ATF3 and GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following sciatic nerve transection. The mean immunoreactivity (IR) $\pm$ SEM of normalized data to control (blue bars) values. DRG neurons ipsilateral (red bars) to transection show a significant (*** $P < 0.001$) increase in the expression of ATF3 and GAP-43. Each bar represents the mean value of 800 or more neurons from 4 different rats.

No statistical analyses were done on this and on the following scatter plots presented throughout the remainder of this thesis. We used at least four different rats. All four different animals show a similar pattern when we plotted the GAP-43 and ATF3 labeling intensity values against the neuronal diameter. Representative scatter plot values of nuclear (Figure 7-5 A), cytoplasmic ATF3 (Figure 7-5 B) as well as the cytoplasmic GAP-43 (Figure 7-5 C) are shown. There was a noticeable increase in the nuclear ATF3 labeling intensity as a result of injury across the the size range of DRG neurons. However, the increase in the cytoplasmic ATF3 levels was uneven, with the larger

neurons expressing markedly higher levels of intensity. GAP-43 was increased across

the injured neuronal subpopulation.

7.1.1.1 GAP-43 and ATF3 expression 14 days after injury.

To determine if the injury effects last longer than 1 week, we studied the

expression of both GAP-43 and ATF3 at 14 days following peripheral sciatic nerve

transection. The expression of both GAP-43 and that of ATF3 remained high (Figure 7-

6). However, at 14 days, ATF3 expression was more pronounced in the injured nuclei of

small to medium diameter neurons than compared with the nuclei of the large diameter

neurons.
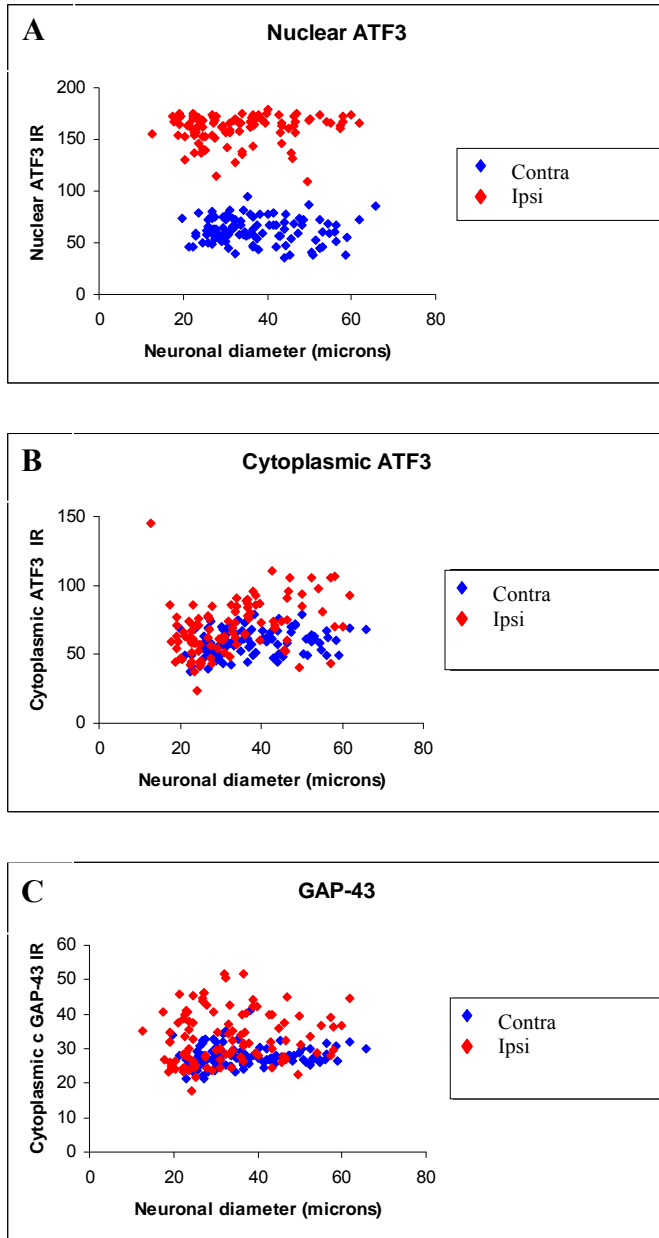
Figure 7-5. Sciatic nerve transection at mid-thigh level increases the expression of nuclear and cytoplasmic ATF3, and GAP-43 7 days after injury. Representative scatter plot of immunoreactivity (IR) of nuclear ATF3 (A), cytoplasmic ATF3 (B), and GAP-43 (C) in L5 DRG neurons comparing the contralateral (blue diamonds) with ipsilateral (red diamonds) data. L4 showed similar results (data not shown).

Figure 7-6. ATF3 and GAP-43 expression increases in L4 DRG 14 d following sciatic nerve transection. Micrographs of identical sections of L4 DRG neurons double labeled with ATF3 (red, A and B) and GAP-43 (green, C and D). (A) In injured DRG (ipsilateral), ATF3 (red) expression is enhanced compared to contralateral DRG (B). GAP-43 (green) expression is increased in ipsilateral (C) compared to the contralateral (D). Scale bar represents 100 µm.

Analysis of the data at 14 d after sciatic nerve transection data by two-way ANOVA followed by Kruskal Wallis test revealed nuclear ATF3 values in the transected nerve (4.20$\pm$ 0.05), were significantly higher than the contralateral, uninjured nerve ($P <$ 0.001). The cytoplasmic ATF3 values also showed a significantly higher level than the contralateral side (1.50 $\pm$ 0.02) ($P < 0.001$). GAP-43 expression was also significantly higher in the injured DRG neurons than in the contralateral side (1.80 $\pm$ 0.04) ($P < 0.001$) (Figure 7-7). Although the elevated nuclear and cytoplasmic ATF3 expression and GAP-43 expression were similar to the pattern observed with the 7 d injured DRG neurons, the amount of increase was higher in the 14 d injured DRG neurons than the 7 d injured DRG

neurons for nuclear ATF3 (4 fold vs 3 fold), cytoplasmic ATF3 (1.5 fold vs.1.2fold), and of GAP-43 (1.8 fold vs.1.4 fold).
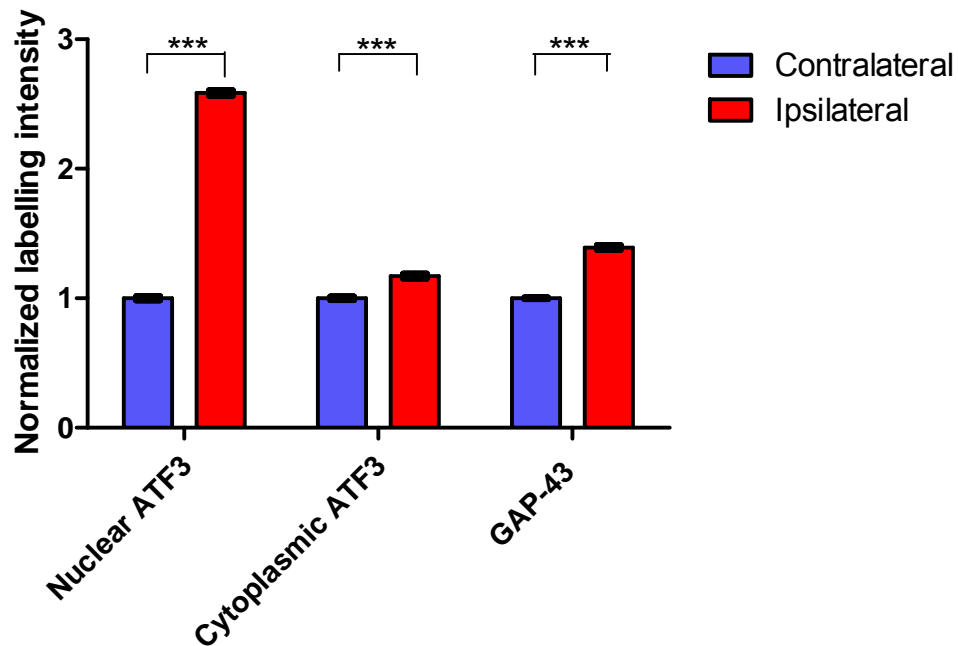
ATF3 and GAP-43 14d (peripheral nerve transection)
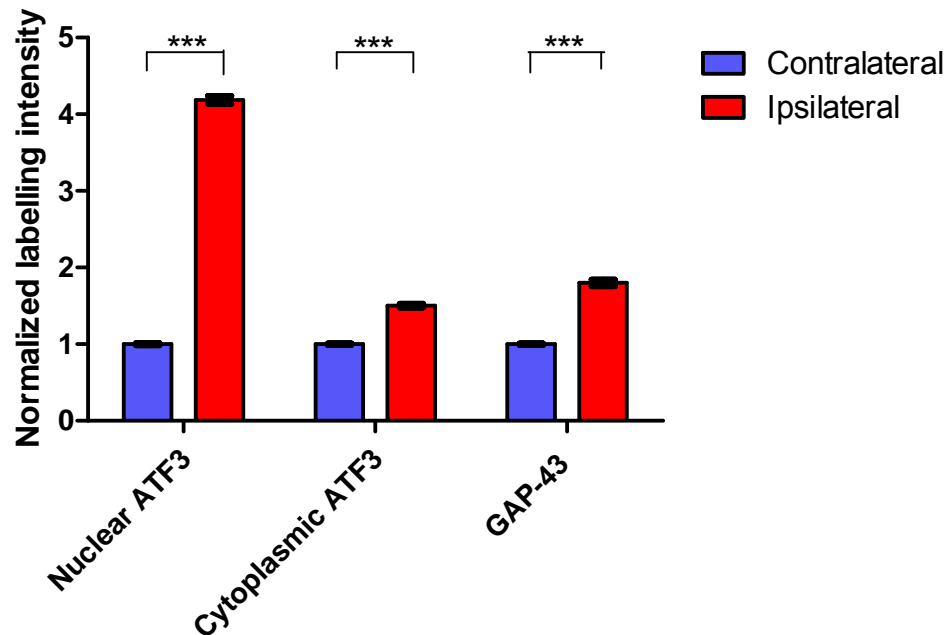
Figure 7-7. Quantification of ATF3 and GAP-43 immunohistochemical staining intensity in L4 DRG 14 d following nerve transection. The mean immunoreactivity (IR) $\pm$ SEM of normalized data to control (blue bars) values. DRG neurons ipsilateral (red bars) to transection show a significant (*** $P < 0.001$) increase in the expression of ATF3 and GAP-43. Each bar represents the mean value of 800 or more neurons from 4 different rats.

Representative scatter plots of nuclear (Figure 7-8 A), cytoplasmic ATF3 (Figure 7-8 B) as well as the cytoplasmic GAP-43 (Figure 7-8 C) were examined. There was a noticeable increase in the nuclear across all neuronal diameters, and cytoplasmic ATF3 labelling intensity as a result of injury especially in the small to medium neurons (20 – 40 μm in diameter). GAP-43 expression was increased as well across the entire size range of injured neurons.

When comparing the results obtained from the 14 d injury to those obtained from 7 d injury, there was a noticeable increase in the expressions of nuclear ATF3 and GAP-43. There was an increase in the neuronal response to peripheral injury, especially during the second week of injury.

7.1.2 GAP-43 and c-Jun expression seven days after injury.

Immunohistochemical results showed that when the sciatic nerve was transected, GAP-43 and c-Jun were both upregulated in DRG neurons (Figure 7-9 A and C). c-Jun was expressed at basal low levels in some of the nuclei of uninjured neurons (Figure 7-9 B). When DRG neurons were injured, c-Jun was more enhanced and upregulated in the nuclei of all neuronal subpopulation of cells, whereas, GAP-43 was upregulated in the cytoplasm. While all GAP-43 positive injured neurons were also c-Jun positive, not all c-Jun positive injured neurons were GAP-43 positive (Figure 7-10, white arrows).

7.1.3 GAP-43 and STAT3 expression seven days after injury.

Immunohistochemical results showed that when the sciatic nerve was severed at mid-thigh level, GAP-43 expression was upregulated in DRG neurons ipsilateral to the transection. Total STAT3 expression did not change as a result of injury, beyond the basal contralateral levels (Figure 7-13). However, a more careful look at the nuclei of injured neurons, a slight increase could be seen from the micrographs (Figure 7-13).

Figure 7-8. Sciatic nerve transection at mid-thigh level significantly increases the expression of nuclear and cytoplasmic ATF3, and GAP-43 14 days after injury. Representative scatter plot of immunoreactivity (IR) of nuclear ATF3 (A), cytoplasmic ATF3 (B), and GAP-43 (C) in L4 DRG neurons comparing the contralateral (blue diamonds) with the ipsilateral (red diamonds) data. L5 showed similar results (data not shown).
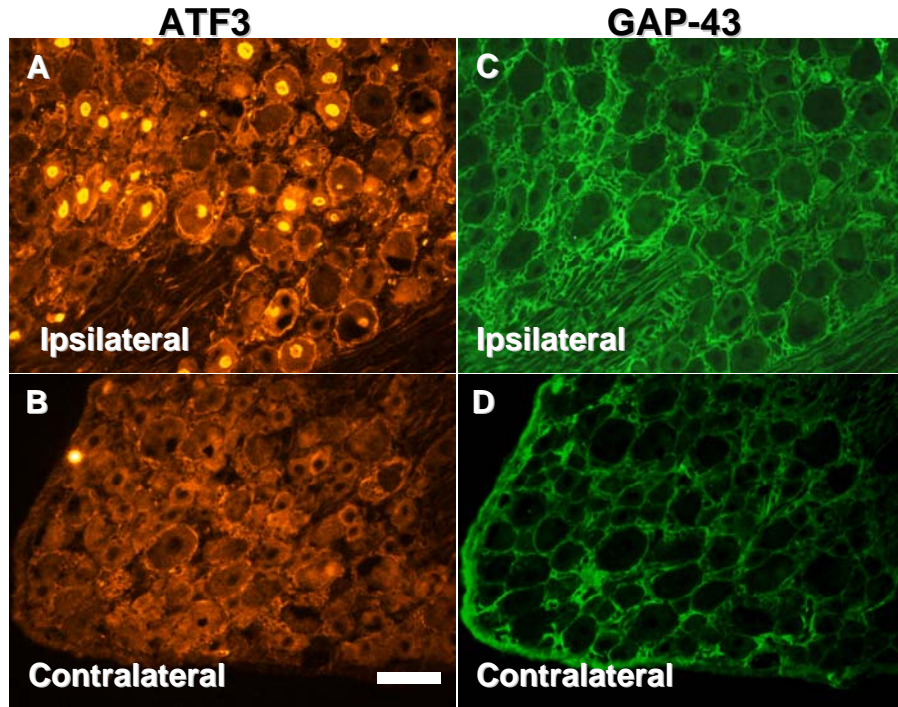
Figure 7-9. c-Jun and GAP-43 expression increases in L5 DRG 7 d following sciatic nerve transection. Micrographs of identical sections of L4 DRG neurons double labeled with c-Jun (red, A and B) and GAP-43 (green, C and D). (A) In injured DRG (ipsilateral), c-Jun (red) expression is enhanced compared to contralateral DRG (B), which expresses low (basal) levels of c-Jun as seen in the nuclei. GAP-43 (green) expression is increased in ipsilateral (C) compared to the contralateral (D). White arrows point to injured neurons that express elevated levels of both c-Jun and GAP-43; Yellow arrows point to injured neurons that express elevated c-Jun, but not GAP-43. Scale bar represents 100 µm.
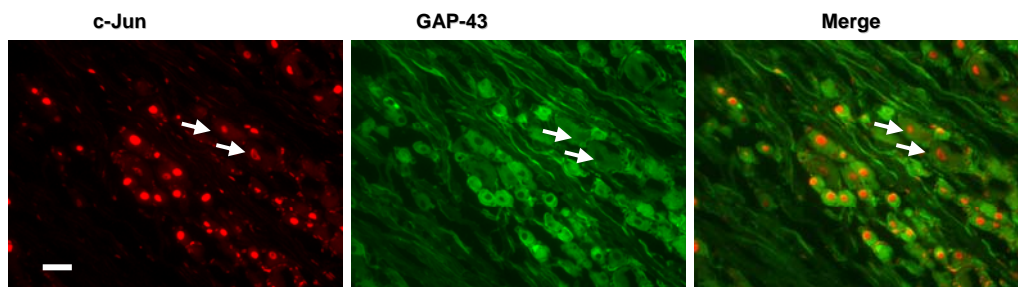
Figure 7-10. Colocalization of GAP-43 and c-Jun in injured neurons.
c-Jun (red) expression is, for the most part, colocalized with GAP-43 (green). A small percentage of neurons exist that express c-Jun, but not GAP-43 (arrows). Scale bar represents 100 µm.

Analysis of the data by two-way ANOVA followed by Kruskal Wallis test showed that nuclear c-Jun values in the injured neurons (1.58 ± 0.06), were significantly higher than in the contralateral, uninjured neurons ($P < 0.001$). The cytoplasmic c-Jun values of the injured DRG showed no significant difference ($P > 0.05$) from the contralateral side (0.97 ± 0.05 vs 1.00 ± 0.04). GAP-43 was significantly higher in the injured DRG neurons compared to the contralateral neurons (1.33 ± 0.06), ($P < 0.01$) (Figure 7-11).

Representative scatter plot of nuclear (Figure 7-12 A) and cytoplasmic c-Jun (Figure 7-12 B) as well as cytoplasmic GAP-43 (Figure 7-12 C) are shown. There was a noticeable increase in the nuclear c-Jun labeling intensity as a result of injury across the neuronal subpopulations, a trend that has also been observed in the injured cytoplasmic GAP-43 levels for all sizes of neurons, whereas there was no difference in the cytoplasmic expression of c-Jun for all neurons between injured vs. contralateral side.

c-Jun and GAP-43 (peripheral nerve transection)
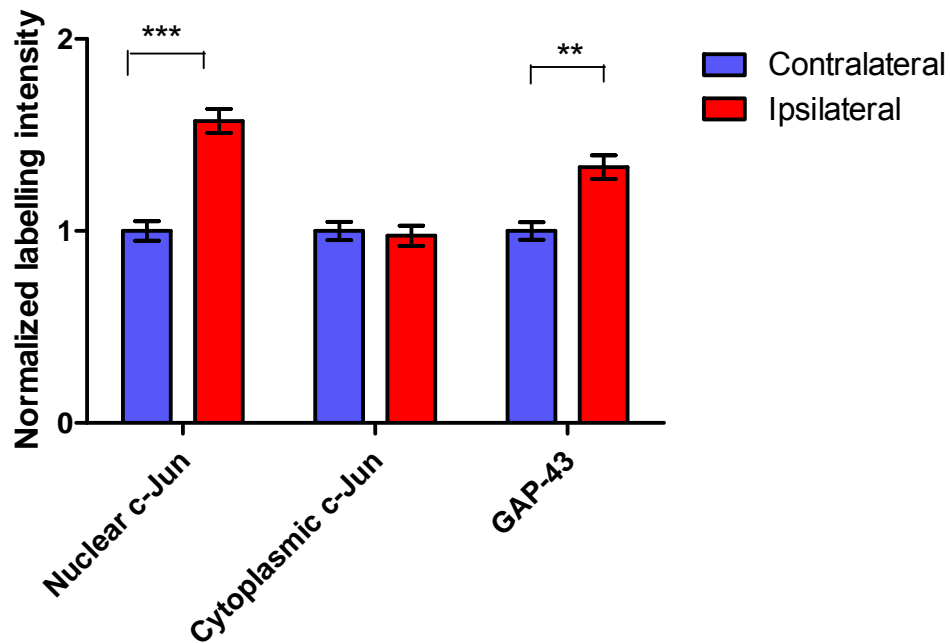
Figure 7-11. Quantification of c-Jun and GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following sciatic nerve transection. The mean immunoreactivity $\pm$ SEM of normalized data to control (blue bars) values. Nuclei of DRG neurons ipsilateral (red bars) to the transection show a significant (*** $P < 0.001$) increase in the expression of nuclear c-Jun, and GAP-43 (** $P < 0.01$). No difference is observed in the cytoplasmic c-Jun values. Each bar represents the mean value of 800 or more neurons from 4 different rats.
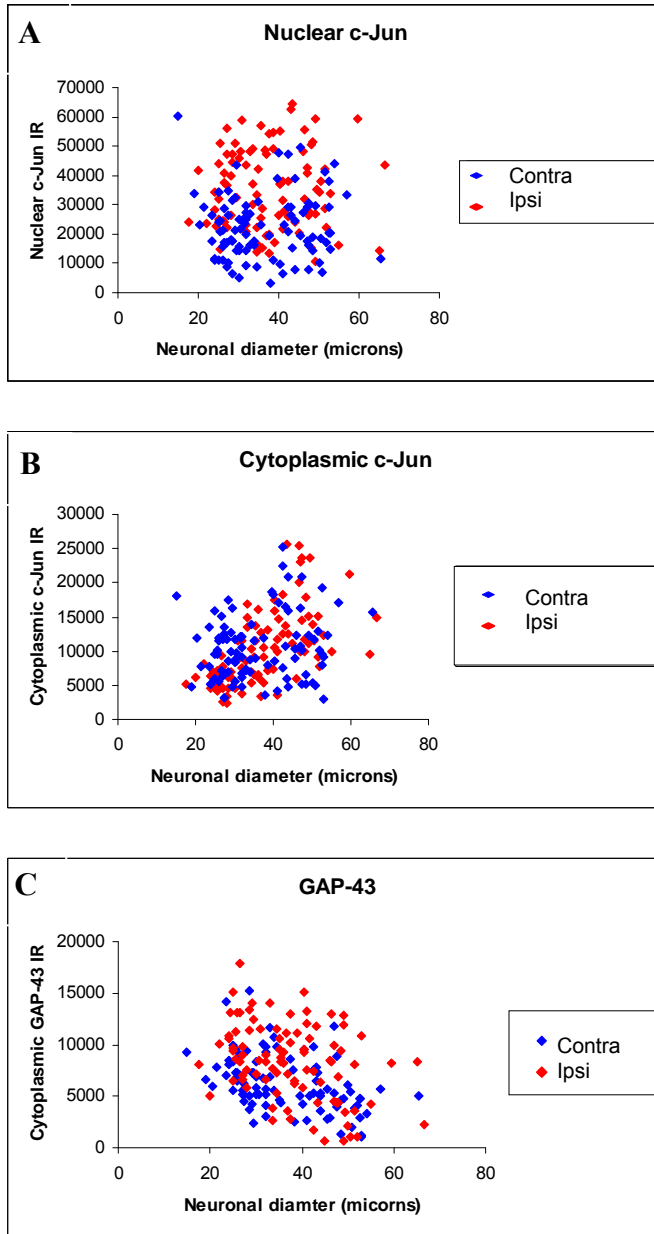
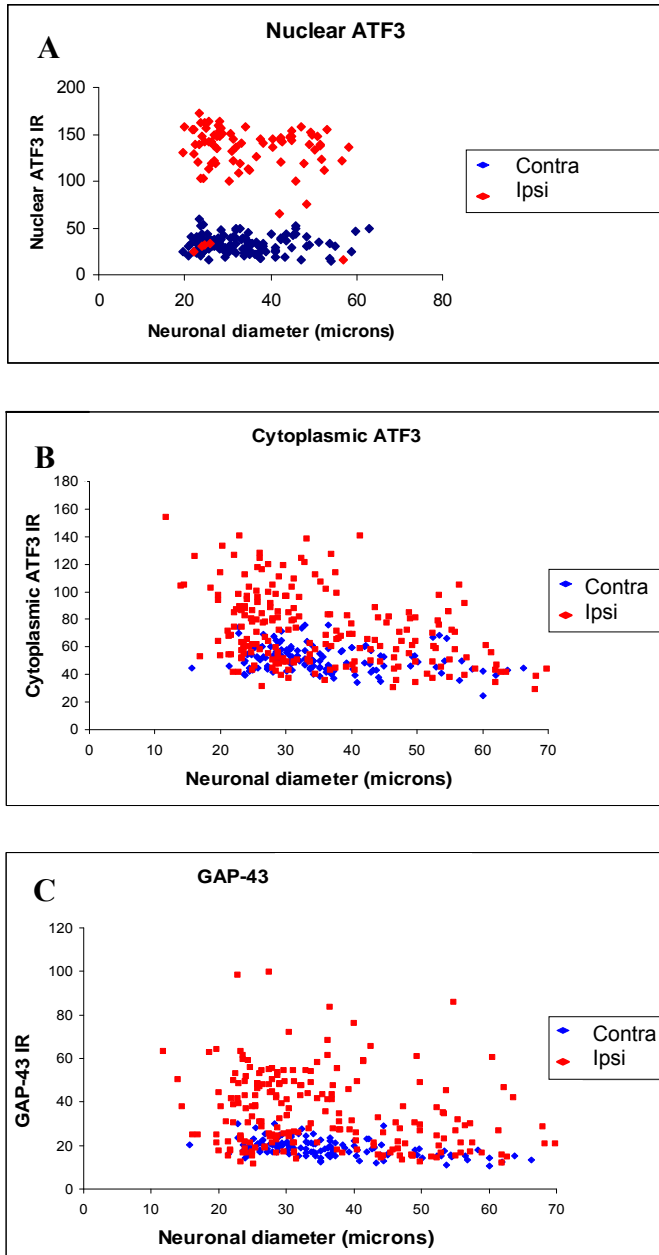**Figure 7-12. Sciatic nerve transection at mid-thigh level notably increases the expression of nuclear c-Jun, and GAP-43 7 days after injury.** Representative scatter plot of immunoreactivity (IR) of nuclear c-Jun (A), cytoplasmic c-Jun (B), and GAP-43 (C) in L5 DRG neurons comparing the contralateral (blue diamonds) with the ipsilateral (red diamonds) data. L4 showed similar results (data not shown).
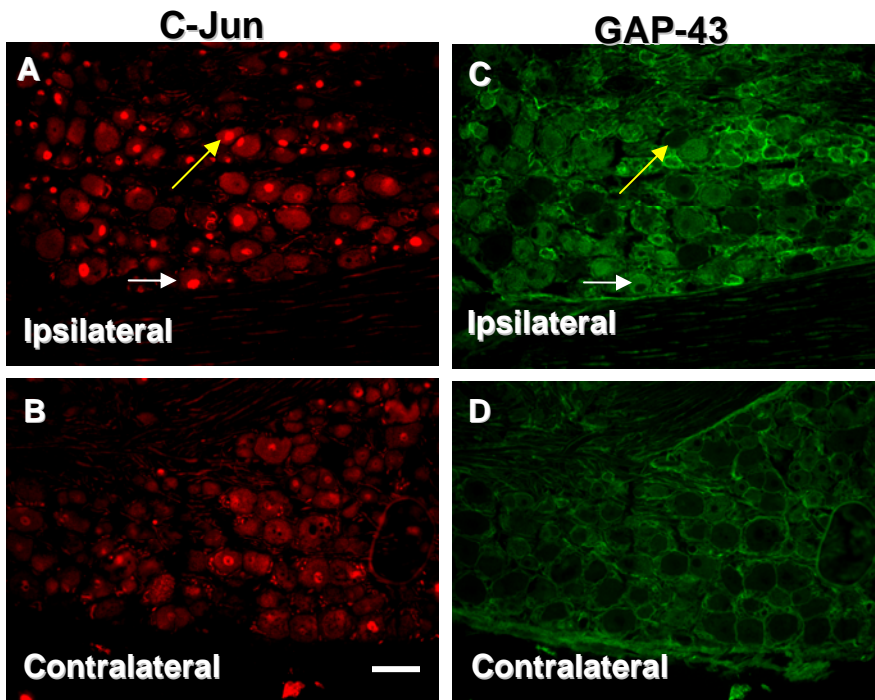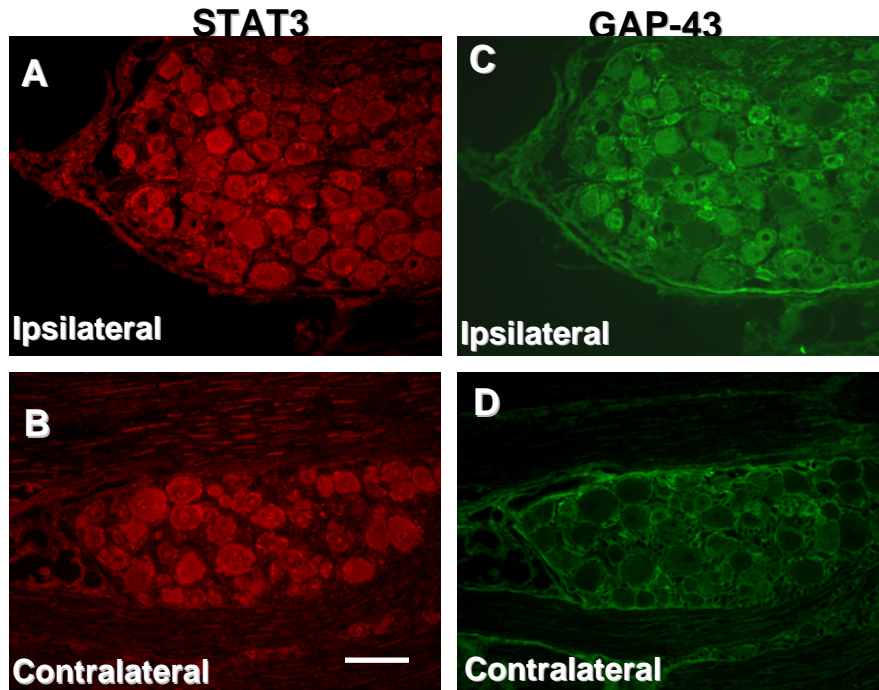
Figure 7-13. Total STAT3 expression changes slightly but GAP-43 expression increases in L5 DRG 7 d following sciatic nerve transection. Micrographs of identical sections of L5 DRG neurons double labeled with total STAT3 (red, A and B) and GAP-43 (green, C and D). (A) In injured DRG (ipsilateral), STAT3 (red) expression increase slightly compared to contralateral DRG (B), especially when the injured nuclei are closely observed. GAP-43 (green) expression is increased in ipsilateral (C) compared to the contralateral (D). Scale bar represents 100 μm.

Analysis of the data by two-way ANOVA followed by Kruskal Wallis test showed that there was no significant difference between injured and uninjured nuclear or cytoplasmic total STAT3 ($P < 0.05$) following peripheral nerve transection. GAP-43 was significantly higher in the injured DRG neurons compared to the contralateral neurons (1.70 ± 0.01), ($P < 0.001$) (Figure 7-14).

STAT3 and GAP-43 (peripheral nerve transection)

Figure 7-14. Quantification of total STAT3 and GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following sciatic nerve transection. The mean immunoreactivity ± SEM of normalized data to control (blue bars) values. Both cytoplasmic and nuclear STAT3 of DRG neurons did not change significantly as a result of peripheral transection, while GAP-43 expression dramatically increased (*** $P <$ 0.001). Each bar represents the mean value of 800 or more neurons from 4 different rats.

Representative scatter plot of nuclear STAT3 (Figure 7-15 A), cytoplasmic STAT3 (Figure 7-15 B) as well as the cytoplasmic GAP-43 (Figure 7-15 C) are shown. There was no apparent increase in total STAT3 labeling intensity whether observed in the nucleus or the cytoplasm as a result of injury. GAP-43 (Figure 7-15 C) levels, however, showed a typical pattern of increased expression as a result of injury, across the neuronal subpoulation.
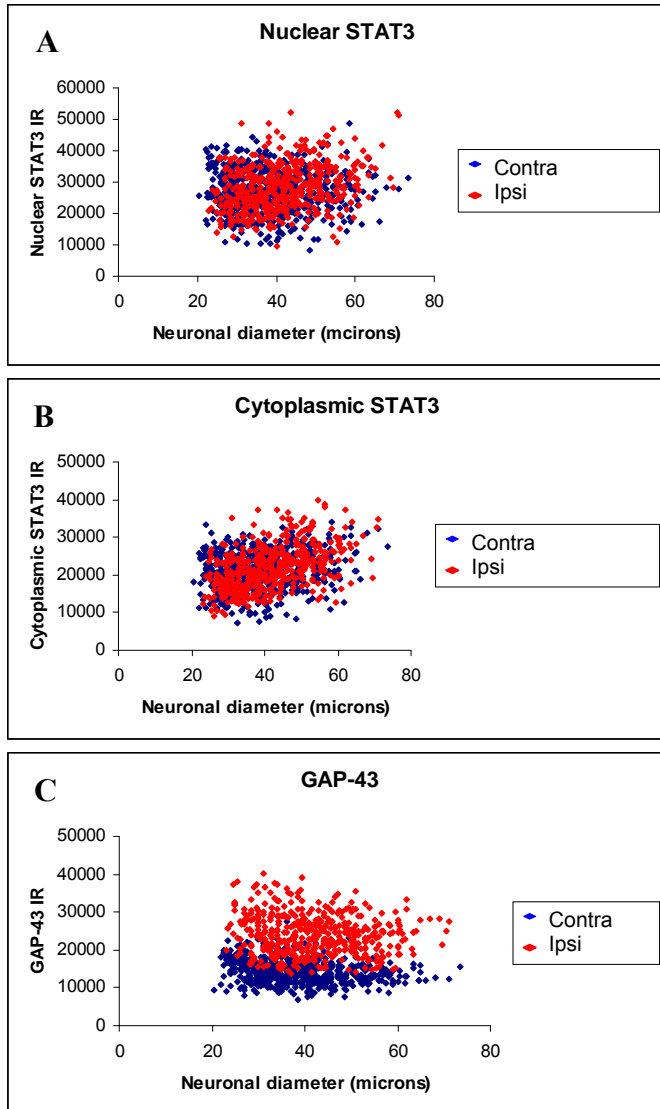
Figure 7-15. Sciatic nerve transection at mid-thigh level does not dramatically increases the expression of total STAT3. Representative scatter plot IR of nuclear (A) and cytoplasmic (B) STAT3 and GAP-43 (C) in L5 DRG neurons comparing the contralateral (blue diamonds) with the ipsilateral (red diamonds) data. No noticeable increase is seen in the expression of STAT3, whether in the nuclei or in the cytoplasm of injured neurons.

7.1.4 GAP-43 and pSTAT3 expression seven days after inury.

Immunohistochemical results showed that when the sciatic nerve was transected,

phosphorylated STAT3 in the nucleus and GAP-43 expression in the cytoplasm were

upregulated in injured DRG neurons (Figure 7-16 A and B). While the majority of GAP-

43 positive neurons were also pSTAT3 positive, not all pSTAT3 positive neurons were

GAP-43 positive (arrows in Figure 7-16).
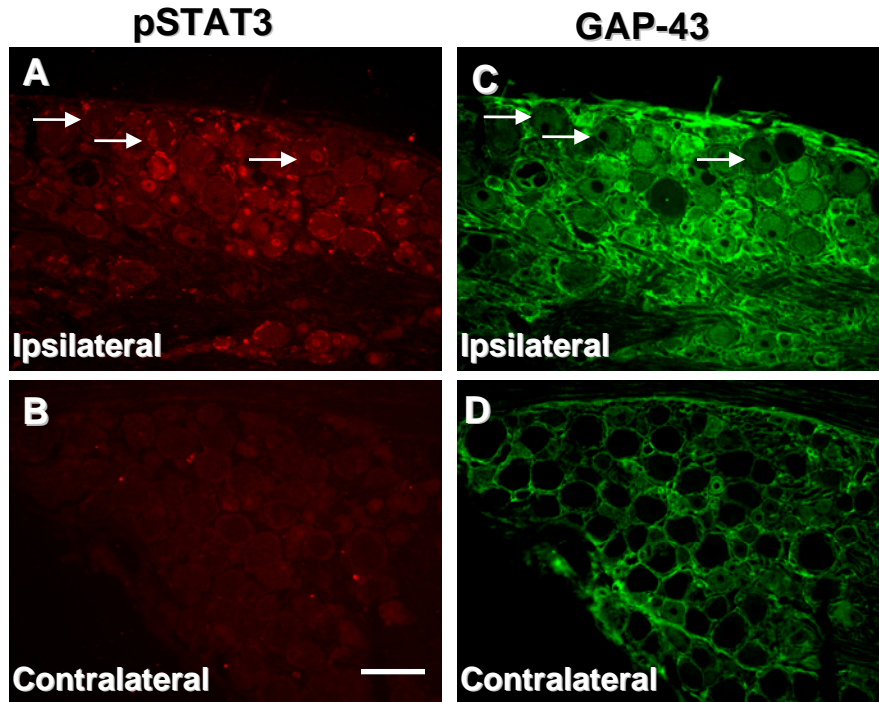
Figure 7-16. Sciatic nerve transection at mid-thigh level significantly increases the expression of GAP-43 and nuclear phosphorylated pSTAT3 in injured DRG neurons. Micrographs of identical sections of L4 DRG neurons double labeled with pSTAT3 (red, A and B) and GAP-43 (green, C and D). (A) In injured DRG (ipsilateral), pSTAT3 (red) expression is enhanced compared to contralateral DRG (B).   GAP-43 (green) expression is increased in ipsilateral (C) compared to the contralateral (D). Arrows point to injured neurons that are pSTAT3 positive, but GAP-43 negative. Scale bar represents 100 μm.

Analysis of the data by two-way ANOVA followed by Kruskal Wallis test

showed a significant increase in the nuclear pSTAT3 in injured DRG neurons ($1.47 \pm$

0.04264), ($P < 0.001$). Cytoplasmic pSTAT3 expression showed a significant but slight

decrease (0.89 ± 0.02619) ($P < 0.05$) in the injured neurons. GAP-43 expression was

significantly higher in the injured DRG neurons compared to the contralateral neurons

(1.20 ± 0.03), (*P* < 0.01) (Figure 7-17).
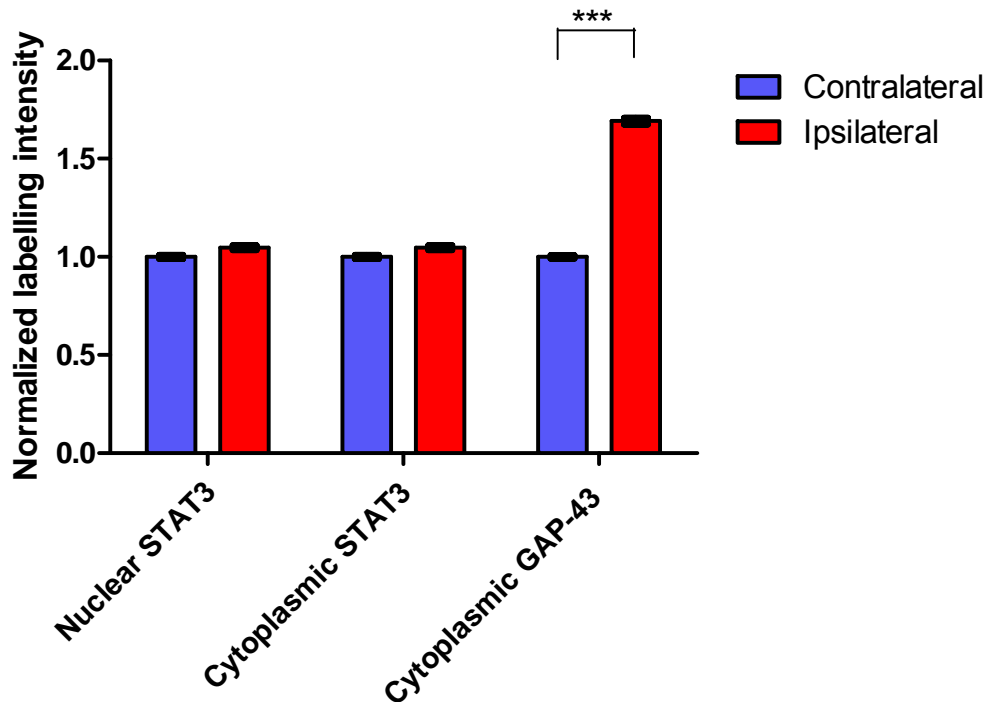
pSTAT3 and GAP-43 (peripheral nerve transection)

Figure 7-17. Quantification of pSTAT3 and GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following sciatic nerve transection. The mean immuno-reactivity ± SEM of normalized data to control (blue bars) values. Both GAP-43 (***P* < 0.001) and nuclear pSTAT3 (***P* < 0.001) of DRG are elevated as a result of transection. Cytoplasmic pSTAT3 is also reducedas a result of peripheral nerve injury. Each bar represents the mean value of 800 or more neurons from 4 different rats.

Representative Scatter plot of nuclear (Figure 7-18 A), cytoplasmic pSTAT3

(Figure 7-18 B) as well as the cytoplasmic GAP-43 (Figure 7-18 C) are shown. A

noticeable increase could be seen in the nuclear pSTAT3 expression as a result of injury

across the neuronal size range. GAP-43 expression levels were also increased spanning

the entire neuronal population.
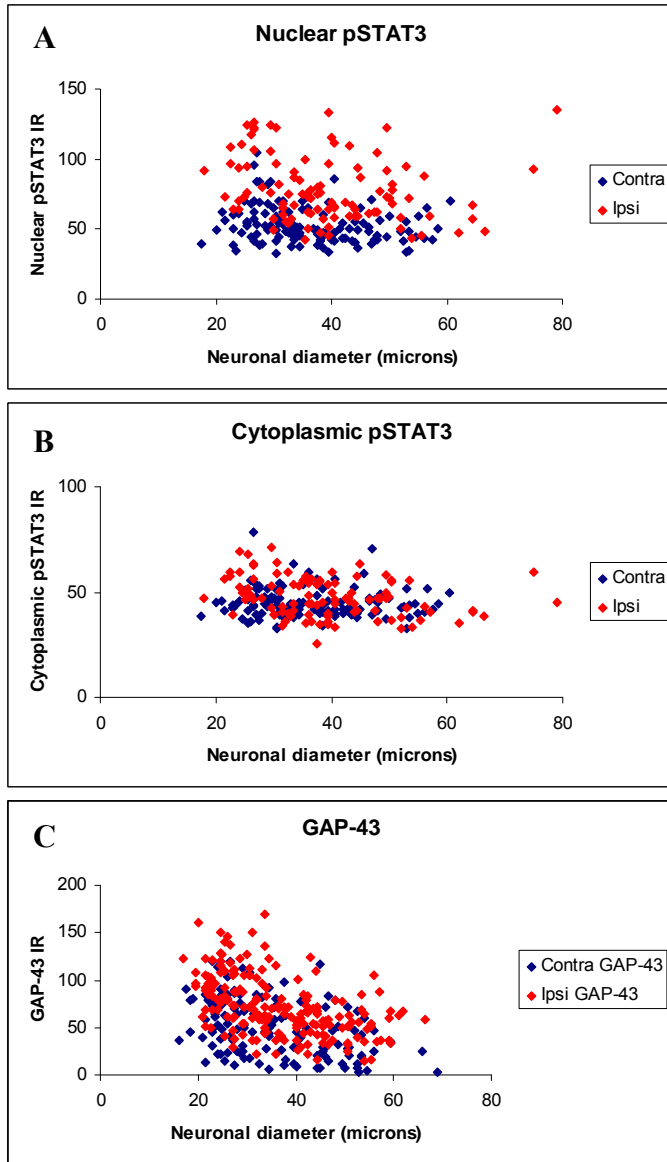
Figure 7-18. Sciatic nerve transection at mid-thigh level increases the expression of nuclear pSTAT3. Representative scatter plot of nuclear pSTAT3 (A) and cytoplasmic pSTAT3 (B), as well as cytoplasmic GAP-43 (C) in L5 DRG neurons, comparing contralateral (blue diamonds) with ipsilateral (red diamonds) analysis. Expression of nuclear pSTAT3 is increased as a result of peripheral nerve transection. L4 showed similar results (data not shown).

7.2 Injury response to dorsal root transection.

7.2.1 GAP-43 and ATF3 expression seven days after injury.

Immunohistochemical results show that when dorsal root transection was performed, the levels of GAP-43 and ATF3 do not change beyond basal levels in the DRG (Figure 7-19). No evidence for chromatolysis can be observed.   This absence of ATF 3 immunoreactivity is very important since it contrasts with what we observed following peripheral nerve transection. Moreover, it confirms earlier studies that suggest a retrograde repressive signal(s) is derived from peripheral target tissue. These repressive signal(s) normally communicate through peripheral, but not central branches. Some peri-neuronal cells can be immunoreactive to ATF3.
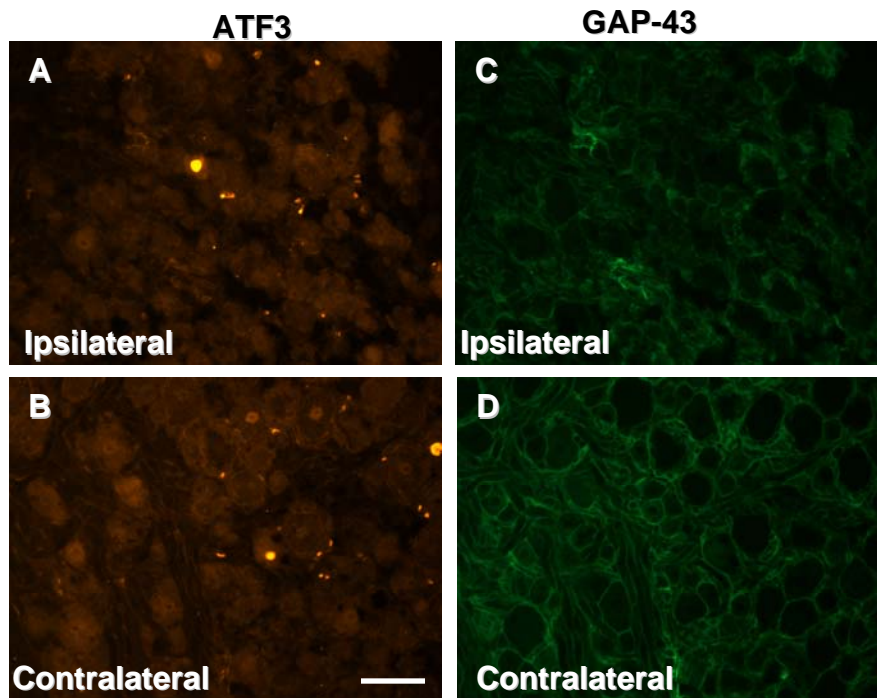
Figure 7-19. Dorsal root transection does not change the expression of GAP-43 or ATF3 7 days after injury. Micrographs of identical sections of L4 DRG neurons double labeled with ATF3 (red, A and B) and GAP-43 (green, C and D. (A) In injured DRG (ipsilateral), ATF3 (red) expression does not change compared to contralateral DRG (B). GAP-43 (green) expression similarily does not increase in ipsilateral (C) compared to the contralateral (D). Scale bar represents 100 μm.

Analysis of the data by two-way ANOVA followed by Kruskal Wallis showed no significant changes in the nuclear ATF3 in centrally injured DRG neurons (1.03 ± 0.050) ($P > 0.05$). Similarly, cytoplasmic ATF3 (1.01 ± 0.02) ($P > 0.05$), and GAP-43 (0.93 ± 0.02) ($P > 0.05$) did not change in centrally injured neurons (Figure 7-20).

ATF3 and GAP-43 (dorsal root transection)

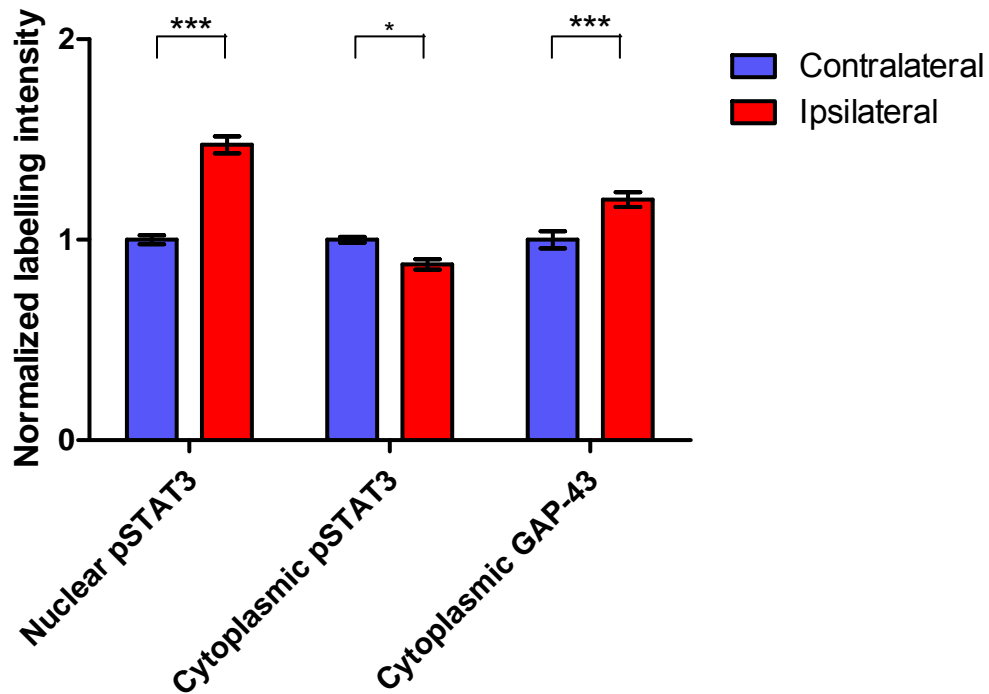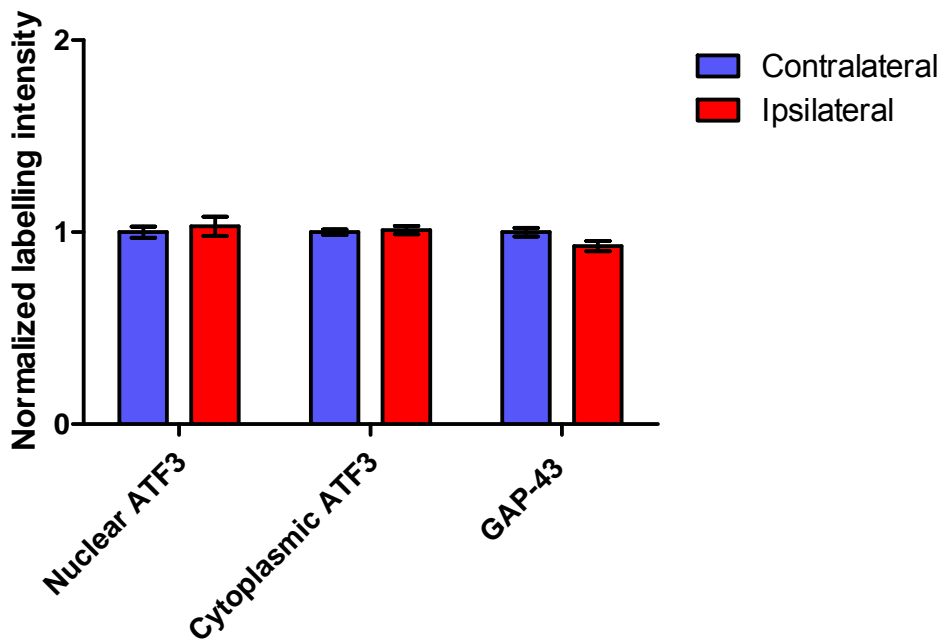**Figure 7-20. Quantification of ATF3 and GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following dorsal root transection.** The mean immunoreactivity $\pm$ SEM of normalized data to control (blue bars) values. GAP-43 ($P > 0.05$), nuclear and cytoplasmic ATF3 ($P > 0.05$) do not change. Each bar represents the mean value of 800 or more neurons from 4 different rats.

A representative scatter plot of measurements of the immunoreactivity of nuclear and cytoplasmic ATF3, and that of GAP-43 revealed no dramatic changes resulting from dorsal root transection (Figure 7-21 A and B, and C). These results are in agreement with immunohistochemical data as analyzed statistically. It is noteworthy that the results obtained from dorsal root transection experiments are in sharp contrast to those obtained from our peripheral sciatic nerve transection experiments (compare photomicrographs in Figures 7-2 and 7-3 to those in Figure 7-19), in which both ATF3 and GAP-43 increased significantly in the DRG.
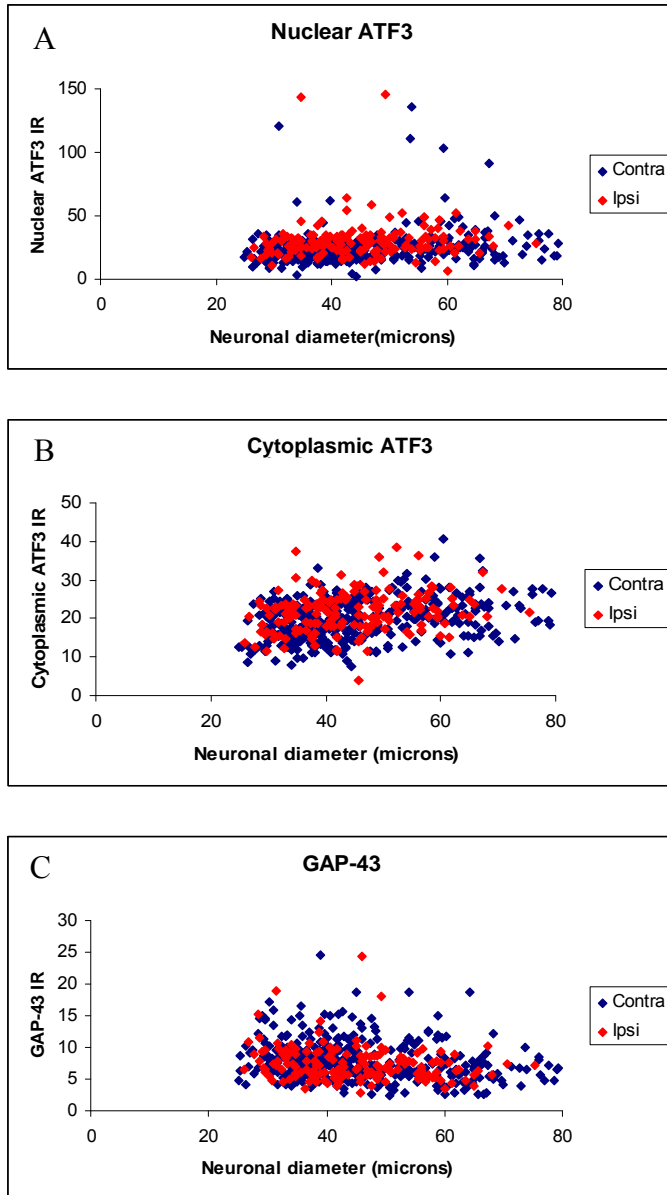
Figure 7-21. Dorsal transection does not induce changes in the expression of nuclear or cytoplasmic ATF3. Representative scatter plot of immunoreactivity of nuclear (A) and cytoplasmic (B) ATF3, and (C) GAP-43 in L5 DRG neurons comparing the contralateral (blue diamonds) with the ipsilateral data (red diamonds).

7.2.2 GAP-43 and c-Jun expression seven days after injury.

Although the perineuronal GAP-43 immunolabelling appears to be more intense in the ipsilateral DRG neurons, no increase was noted within the DRG neurons.

Immunolabelling indicative of c-Jun expression did not change beyond basal levels as a result of dorsal root transection (Figure 7-22). This finding is very important since it contrasts with the pattern of c-Jun expression following peripheral sciatic nerve transection, where a marked increase in nuclear c-Jun and GAP-43 expression was noted. Moreover, the lack of increased expression of c-Jun resembles the absence of increased exression of ATF3 and GAP-43 as a result of dorsal root transection.
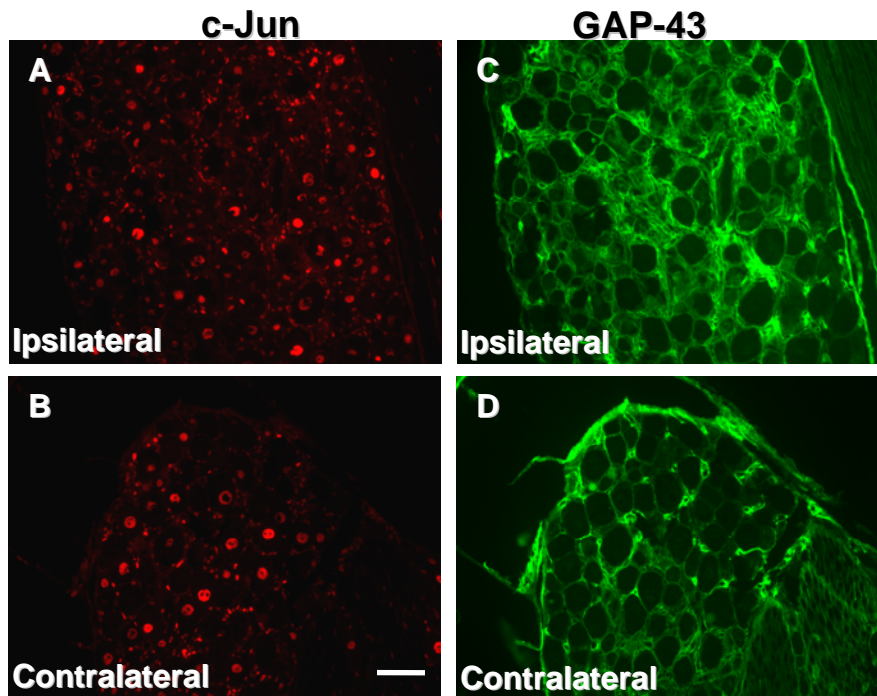
Figure 7-22. Dorsal root transection does not change the expression of GAP-43 or c-Jun 7 days after injury. Micrographs of identical sections of L5 DRG neurons double labeled with c-Jun (red, A and B) and GAP-43 (green, C and D. (A) In injured DRG (ipsilateral), c-Jun (red) expression does not change compared to contralateral DRG (B). GAP-43 (green) expression similarily does not increase in ipsilateral (C) compared to the contralateral (D). Scale bar represents 100 μm.

Analysis of the data by two-way ANOVA followed by two-way ANOVA followed by Kruskal Wallis showed no significant changes in the nuclear or the cytoplasmic c-Jun in centrally injured DRG neurons (1.04 ± 0.02) and (1.01 ± 0.02),

respectively, (*P* >0.05). Similarly, GAP-43 (0.95 $\pm$ 0.01) did not change in centrally

injured neurons (*P* >0.05) (Figure 7-23).

c-Jun and GAP-43 (dorsal root transection)

Figure 7-23. Quantification of c-Jun and GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following dorsal root transection. The mean immunoreactivity $\pm$ SEM of normalized data to control (blue bars) values. GAP-43 (*P* >0.05)**,** nuclear and cytoplasmic c-Jun (*P* >0.05) do not change. Each bar represents the mean value of 800 or more neurons from 4 different rats.

A careful examination of the representative scatter plots revealed no dramatic

changes in c-Jun or GAP-43 immunoreactivity as a result of dosal root transection across

the size range of DRG neurons, In agreement with immunohistochemical and statistical

data (Figure 7-24 A and B, and C). The results here, especially of nuclear c-Jun, are in

contrast to the results obtained form peripheral sciatic nerve transection, where a higher

nuclear expression was noted.

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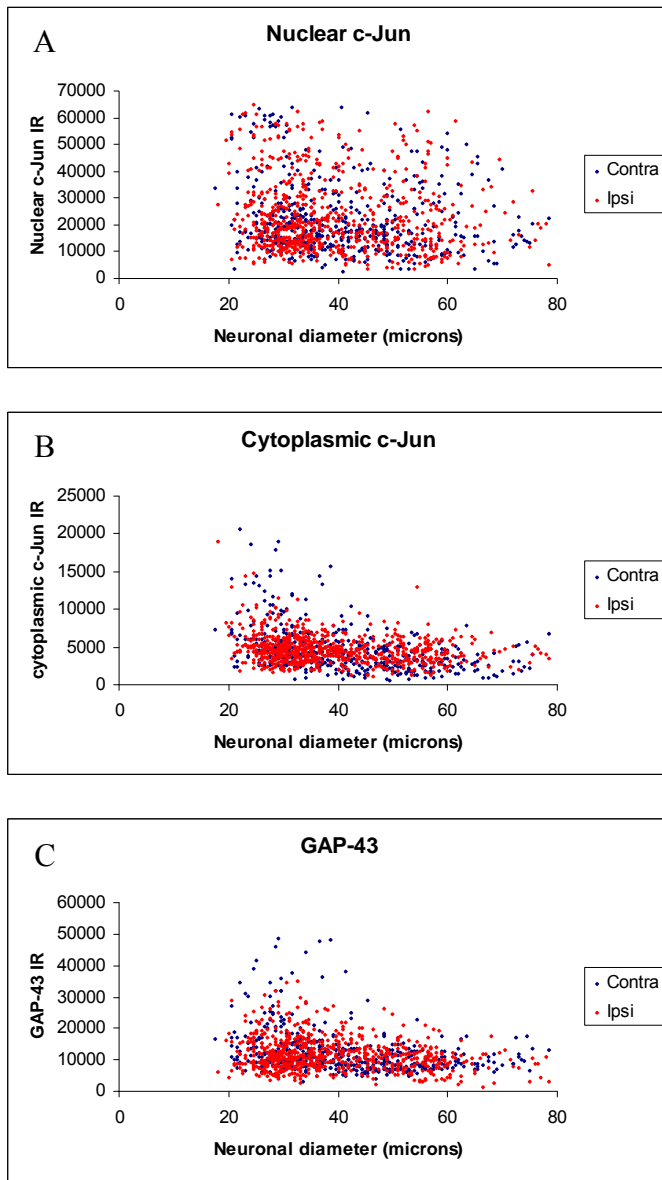
Figure 7-24. Dorsal transection does not induce changes in the expression of nuclear or cytoplasmic c-Jun. Representative scatter plot of immunoreactivity of nuclear (A) and cytoplasmic (B) c-Jun, and (C) GAP-43 in L5 DRG neurons comparing contralateral (blue diamonds) with ipsilateral (red diamonds).

7.2.3 GAP-43 and STAT3 expression seven days after injury.

In contrast to ATF3 and c-Jun, STAT3 is unique because it is the only transcription factor we tested that was upregulated following dorsal root transection. Not only was the increase noted in the cytoplasmic domain, but also in the nuclei of injured DRG. Small to medium neurons display higher labeling intensity. No change in GAP-43 is observed. Immunohisto-chemical micrographs clearly demonstrate this increase (Figure 7-25). Total STAT3 results here are also in contrast to the results obtained from peripheral nerve transection, where no significant change was observed.
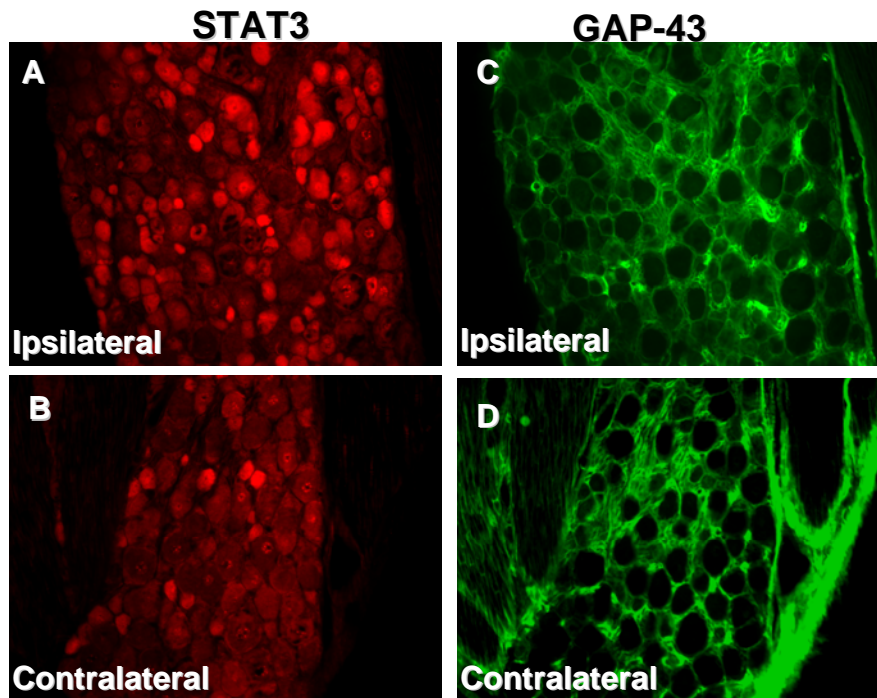
Figure 7-25. Dorsal root transection increases the levels of cytoplasmic STAT3 7 days after injury. Micrographs of identical sections of L5 DRG neurons double labeled with total STAT3 (red, A and B) and GAP-43 (green, C and D. (A) In injured DRG (ipsilateral), cytoplasmic and nuclear total STAT3 (red) expression increases compared to contralateral DRG (B). GAP-43 (green) expression does not increase in ipsilateral (C) compared to the contralateral (D). Scale bar represents 100 μm.

Analysis of the data by two-way ANOVA followed by Kruskal Wallis test confirmed the cytoplasmic (1.319 ± 0.18, $P < 0.001$) and nuclear (1.46 ± 0.02, $P < 0.001$) increases of total STAT3 in the neurons ipsilateral to the dorsal root transection (Figure 7-26).

STAT3 and GAP-43 (dorsal root transection)
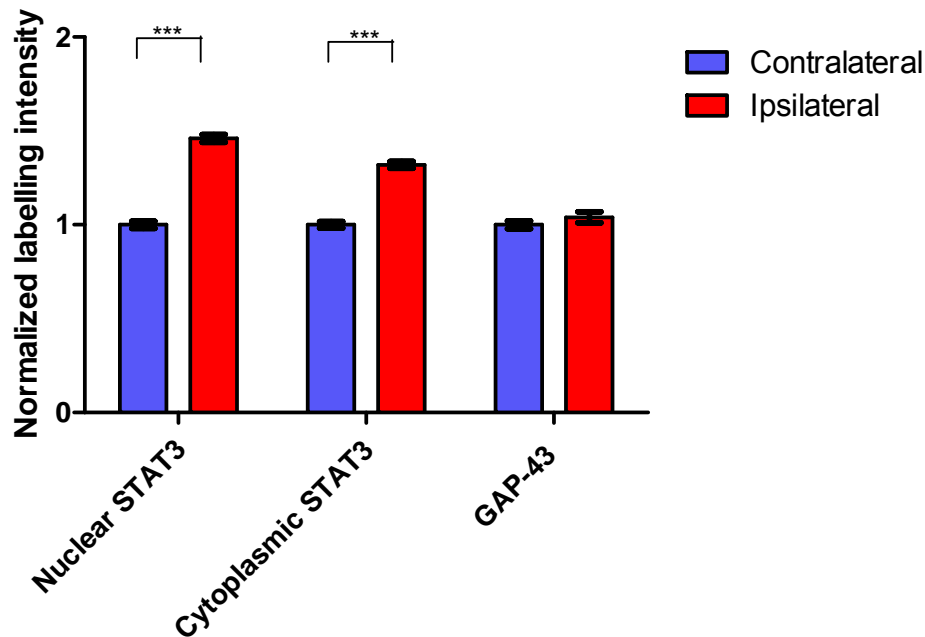
Figure 7-26. Quantification of total STAT3 and GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following dorsal root transection. The mean immunoreactivity $\pm$ SEM of normalized data to control (blue bars) values. Total STAT3 expression increases significantly in the nuclei (***$P<0.001$), and in the cytoplasm of injured neurons (***$P<0.001$). No significant changes are observed in GAP-43 ($P >0.05$). Each bar represents the mean value of 800 or more neurons from 4 different rats.

Scatter plot of DRG neurons from this experiment confirmed that total STAT3 increased in the cytoplasm and nuclei DRG neurons ipsilateral to dorsal transection, especially in the small to medium sized neurons, while GAP-43 expression remained unchanged (Figure 7-27).
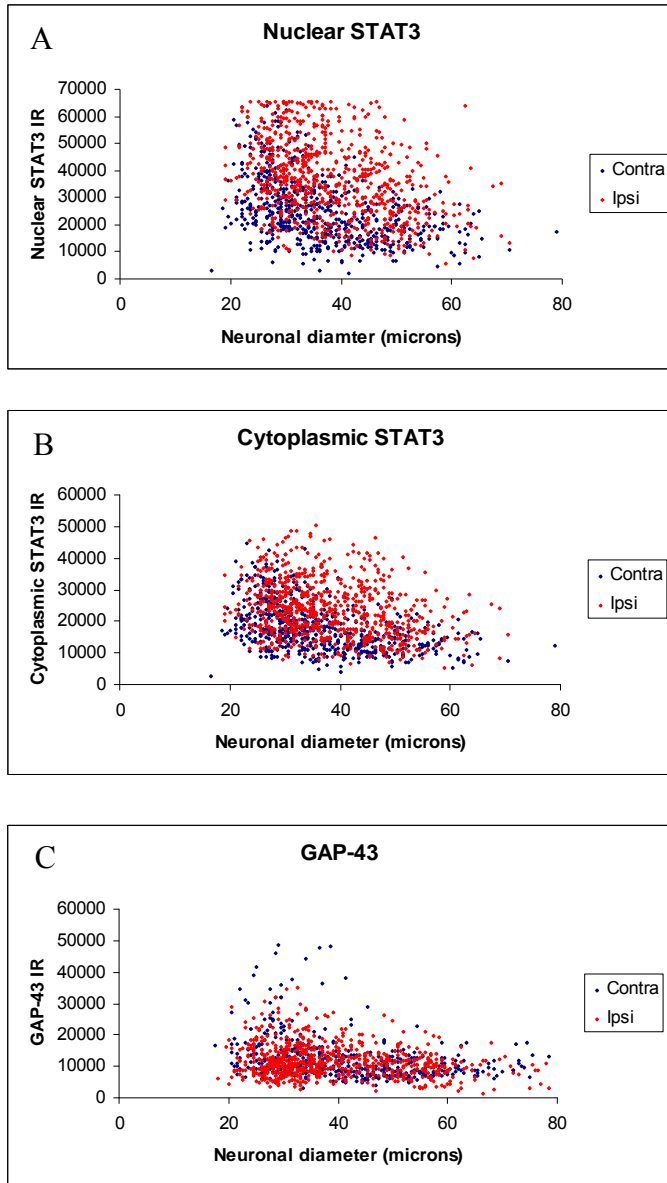
Figure 7-27. Dorsal root transection induces changes in the expression of nuclear and cytoplasmic total STAT3. Representative scatter plot of immunoreactivity of nuclear (A) and cytoplasmic (B) STAT3, and (C) GAP-43 in L5 DRG neurons comparing contralateral (blue diamonds) with ipsilateral (red diamonds).

7.2.4 GAP-43 and pSTAT3 expression seven days after injury.

The activated (phosphorylated) form of STAT3 is translocated to the nucleus. Here we observed an increase in the expression of pSTAT3 in the nuclei of DRG neurons ipsilateral to dorsal root transection (Figure7-28). This increase in pSTAT3 was seen across the entire size population of DRG neurons.
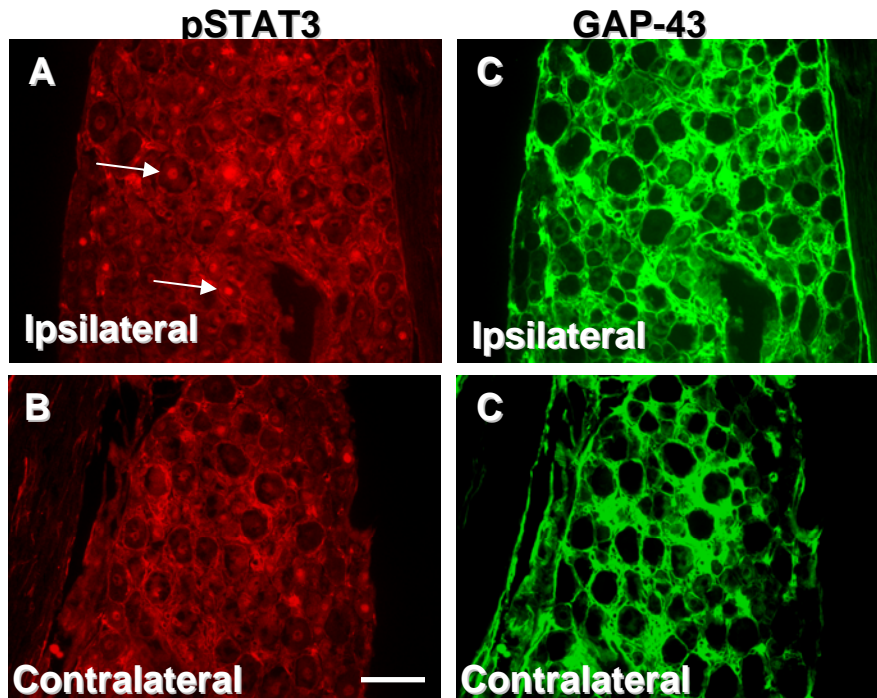
Figure 7-28. Dorsal root transection increases the levels of nuclear pSTAT3 7 days after injry. Micrographs of identical sections of L5 DRG neurons double labeled with pSTAT3 (red, A and B) and GAP-43 (green, C and D). (A) In injured DRG (ipsilateral), nuclear pSTAT3 (red) expression increases compared to contralateral DRG (B). GAP-43 (green) expression does not increase in ipsilateral (C) compared to the contralateral (D). Arrows point to nuclei that are pSTAT3 +. Scale bar represents 100 µm.

Analysis of the data by two-way ANOVA followed by Kruskal Wallis test confirmed that pSTAT3 expression significantly increases (1.11 ± 0.02, $P < 0.001$) in the nuclei of DRG neurons ipsilateral to the dorsal root transection, while no changes are

seen in GAP-43 expression (Figure 7-29). The results here are similar to the pattern

observed in peripheral nerve transection experiments.

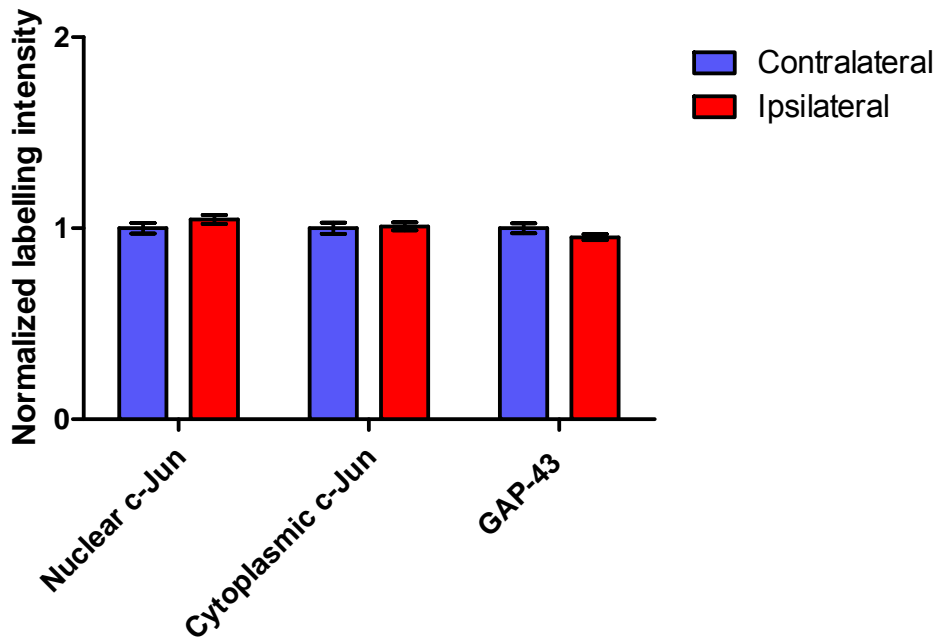## pSTAT3 and GAP-43 (dorsal root transection)
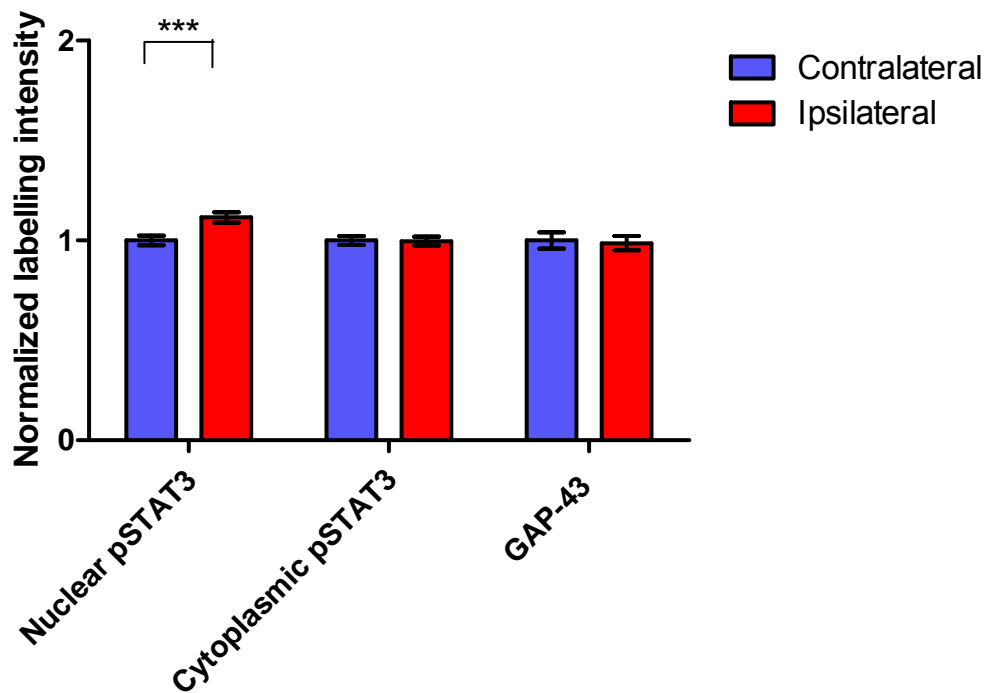
Figure 7-29. Quantification of total pSTAT3 and GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following dorsal root transection. The mean immunoreactivity $\pm$ SEM of normalized data to control (blue bars) values. Phosphorylated pSTAT3 expression increases significantly in the nuclei (***$P<0.001$), but not in the cytoplasm of injured neurons. No significant changes are observed in GAP-43 ($P >0.05$) of DRG neurons that have undergone dorsal root transection. Each bar represents the mean value of 800 or more neurons from 4 different rats.

Scatter plot of DRG neurons from this experiment confirmed pSTAT3

immunoreactivity increases in the nuclei of DRG neurons ipsilateral to dorsal root

transection with no obvious relation to neuronal size, while GAP-43 expression remains

unchanged (Figure 7-30).
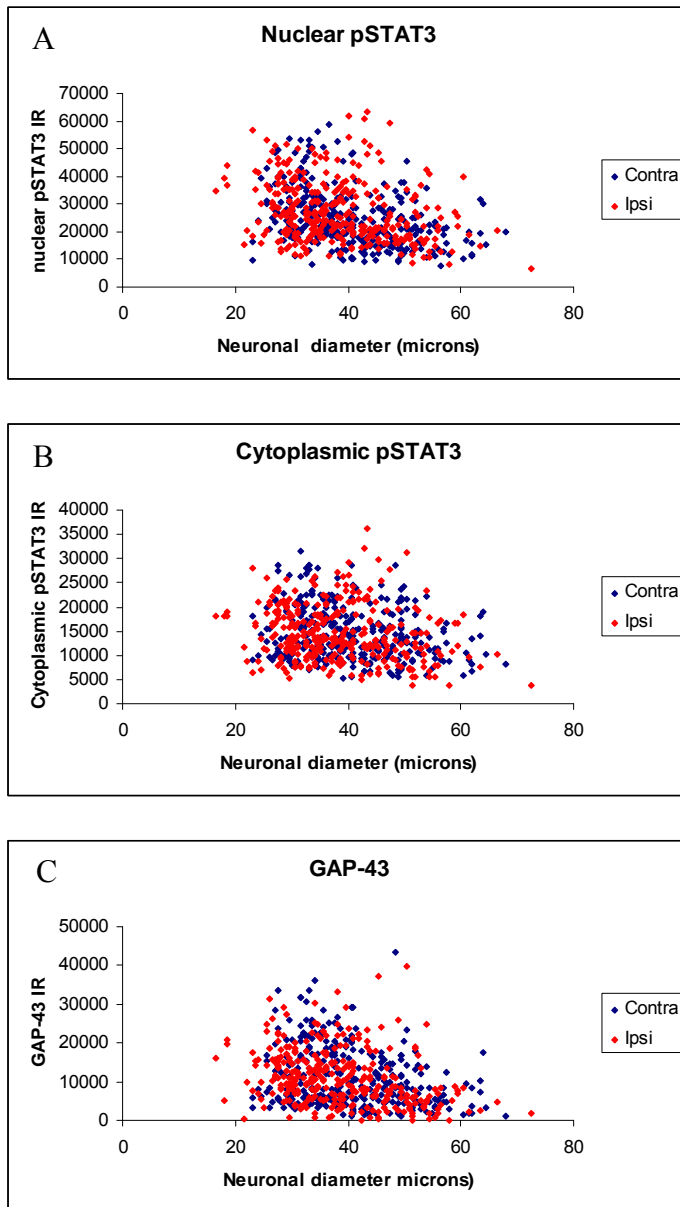
Figure 7-30. Dorsal root transection induces changes in the expression of nuclear pSTAT3. Representative scatter plot is shown of immunoreactivity of nuclear (A) and cytoplasmic (B) pSTAT3, and (C) GAP-43 in L5 DRG neurons comparing contralateral (blue diamonds) with ipsilateral (red diamonds).

Table7-1 summarizes the results that compares the effects of peripheral transection and that of dorsal root transection. The expression of GAP-43, ATF3, and c-Jun was upregulation as a result of peripheral transection. However, STAT3 expression was upregulated as a result of dorsal root transection.

Table 7-1. Summary of results comparing peripheral nerve transection with dorsal root transection.

Protein	Peripheral transection	Dorsal root transection
GAP-43	↑ (red)	±
ATF3 nuclear	↑ (red)	±
ATF3 cytoplasmic	↑ (red)	±
c-Jun nuclear	↑ (red)	±
c-Jun cytoplasmic	±	±
STAT3 nuclear	±	↑ (red)
STAT3 cytoplasmic	±	↑ (red)
pSTAT3 nuclear	↑ (red)	↑ (red)
pSTAT3 cyoplasmic	↓ (blue)	±

This table summarizes the results obtained from peripheral transection vs. dorsal transection. Proteins examined are listed in the far left column. Red arrows indicate an increase, whereas blue arrows indicate a decrease in the expression, as judged by measuring immuofluorescence intensity.

7.3 Modification of normal DRG neuronal injury response by FGF-2 and cAMP.

Previous *in vitro* work from our lab showed that cultured DRG neurons suppressed GAP-43 expression as a result of FGF-2 administration. Additionally, cultured adult rat DRG neurons exposed to membrane-permeable analogs of cyclic AMP

and adenyl cyclase activators downregulated GAP-43 protein expression and mRNA in a dose-dependent manner (Andersen et al., 2000). We were interested in testing whether FGF-2 could downregulate GAP-43 *in vivo*. We asked if a continuous administration of FGF-2 for seven days through surgically implanted minipump at the proximal cut end of the injured sciatic nerve would modulate the expression of GAP-43 in DRG neurons.

7.3.1 Effect of FGF-2 and cAMP on GAP-43 expression.

In order to test if materials available at the proximal cut end of the sciatic nerve could be retrogradely transported, we carried out a pilot study using FluoroGold® (FG) filled minipumps. FG was infused at the nerve lesion site fir 7 days. The majority of injured DRG neurons showed positive FG staining (Figure 7-31). This would indicate that dbcAMP and/or FGF-2, available at a cut axon tip, might also be retrogradely transported to DRG neurons. Alternatively, dbcAMP and/or FGF-2 at the cut end could initiate a biochemical response *in situ* whose products could be retrogradely transported. To our surprise, FGF-2 applied through our model system did not suppress GAP-43 expression in peripherally injured DRG neurons. In contrast FGF-2 increased the expression of GAP-43 in the neurons ipsilateral to the nerve transection, and also, in the contralateral uninjured DRG neurons.

7.3.1.1 Western analysis.

Western blot analysis showed a specific band at the 45 KD level corresponding to GAP-43 (Figure 7-32). This band is only reactive in the injured DRG neurons ipsilateral to transected sciatic nerve. Faint bands are present in the contralateral lanes, confirming

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the basal levels of GAP-43 expression. The contralateral GAP-43 is more visible in both

dbcAMP and FGF-2 lanes, and is almost non-detectable in the contralateral vehicle

treated group. Optical scanning was uses to quantify the amount of increase of GAP-43

immunoreactivity on the ipsilateral side in each experimental condition compared to the

normalized control values. FGF-2 administered *in vivo* via minipump significantly ($P <$

0.05) increases GAP-43 expression in the ipsilateral DRG neurons.
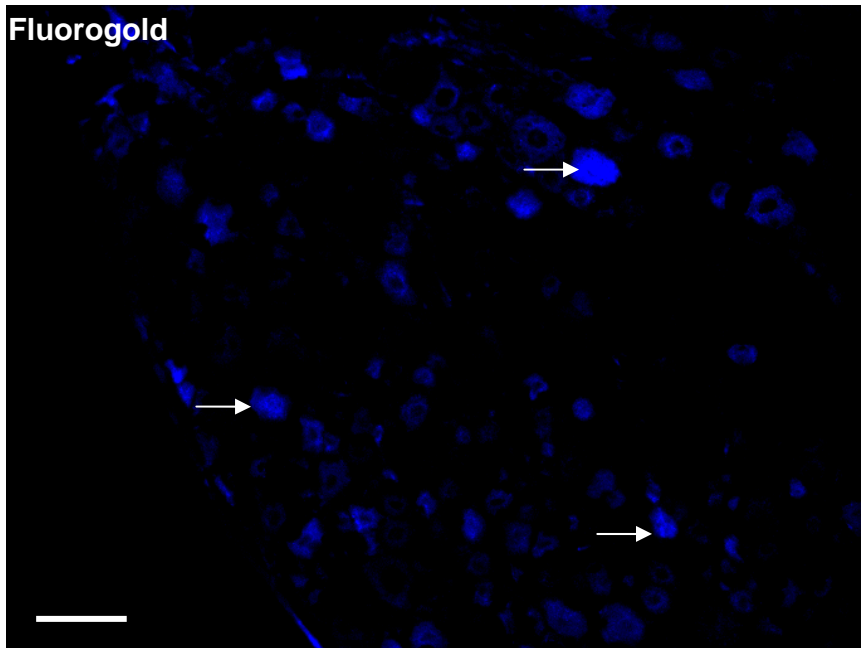
Figure 7-31. Retrograde FluoroGold® (FG) labeling of injured DRG neurons.
Accumulation of FG retrograde tracer in DRG neurons after Fluorogold was delivered to
the cut end of sciatic nerve by osmotic minipump for seven days. The majority of the
neurons retrogradely transport Fluorogold from the minipumps. Some neurons are more
brightly stained than others (arrows). Scale bar represents 100 μm.

$$+ \qquad ++$$

$$1.15 \pm 0.01 \qquad 1.5 \pm 0.01$$

Vehicle dbcAMP   FGF-2

C   Ip   C   Ip   C   Ip
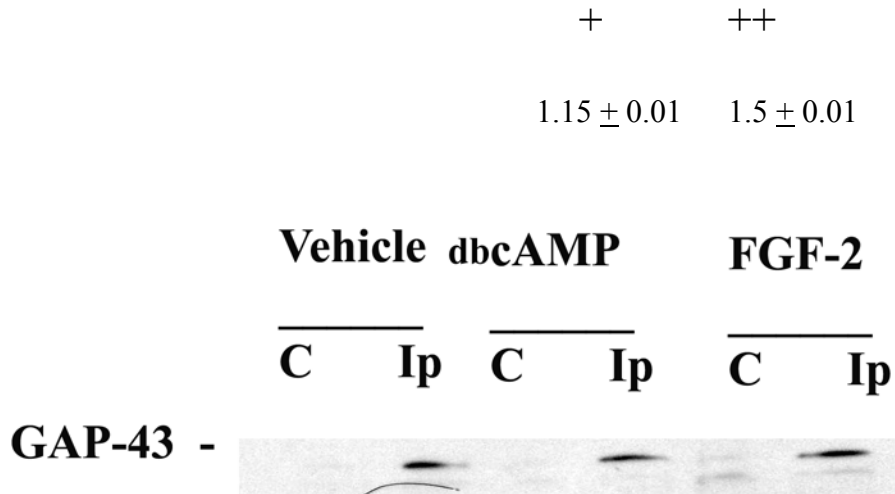
GAP-43 -

Figure 7-32. dbcAMP and FGF-2 minipump infusion increases GAP-43 levels in DRG neurons. As a result of treatment with dbcAMP and FGF-2, the injured DRG neurons upregulated GAP-43 expression 1.15, and 1.5 fold relative to loading control (GAPDH) respectively.

7.3.1.2 Immunohistochemistry.

Figure 7-33 A, B, and C show typical immunohistochemical photomicrographs. These are representatives of the images used in the quantification method, and statistical analysis. The expression of GAP-43 was increased in the ipsilateral vehicle control ganglion. The increase is enhanced as a result of cAMP, and FGF-2 treatment in the small to medium diameter neurons. A careful examination of the photomicrographs showed that almost the entire subpopulation of neurons expressed elevated GAP-43 levels in the case of vehicle treatment. When treated with dbcAMP, the small to medium diameter neurons were more brightly stained with GAP-43, compared with the rest of the neuronal subpopulation of cells. When FGF-2 was introduced, the small to medium diameter neurons were also more brightly stained.

Scatter plot of the quantification data clearly depicted the enhanced expression of

GAP-43 in the small to medium neurons (20-40 µm), when treated with dbcAMP or with

FGF-2 (Figure 34 A, B, and C).
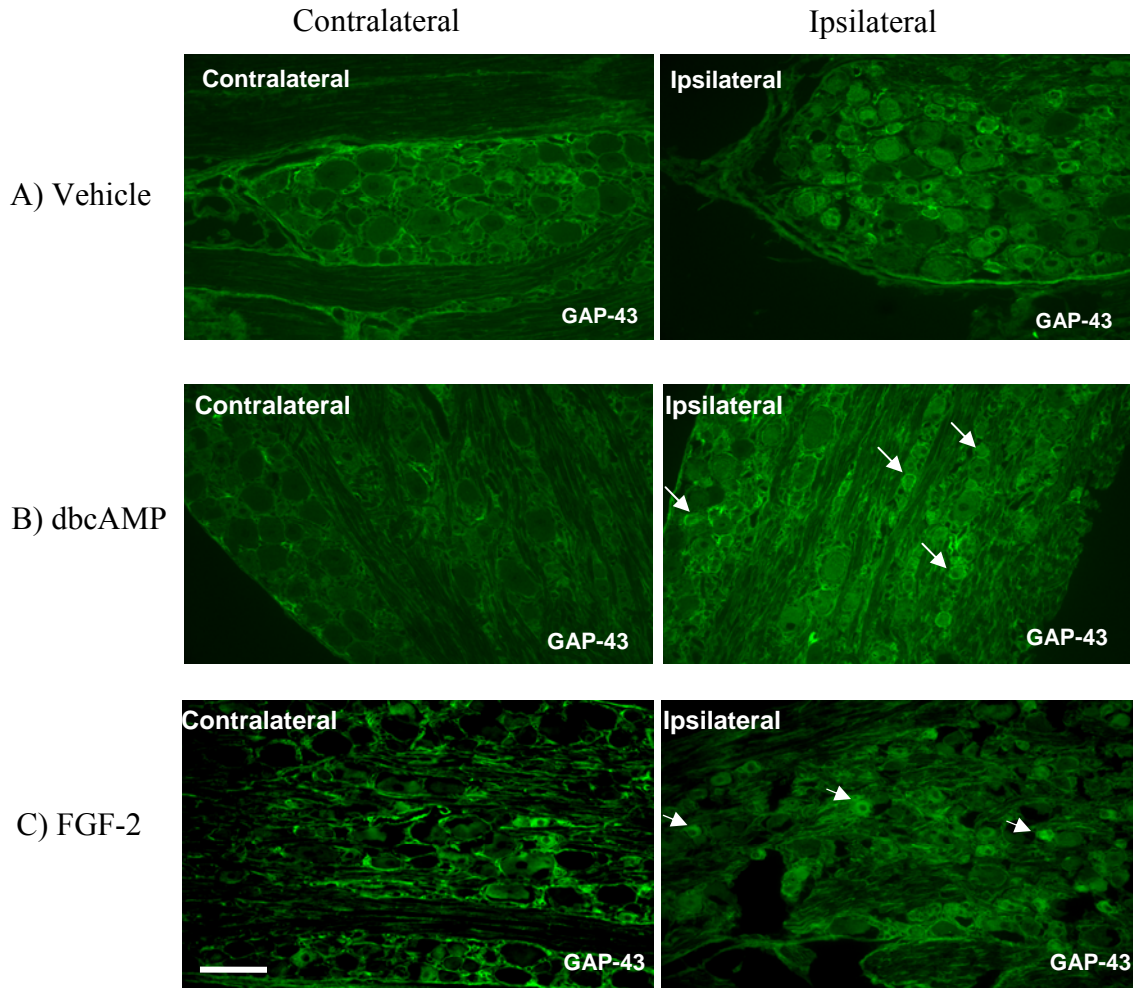
Figure 7-33. FGF-2 and dbcAMP minipump infusions increase GAP-43 levels in DRG neurons. As a result of treatment with dbcAMP and FGF-2, the injured (ipsilateral, right panel) DRG neurons upregulated GAP-43 expression, especially in the small diameter neurons (arrows), when compared to the vehicle ipsilateral (top right), where the expression spans the entire neuronal subpopulation. Scale bar represents 100 µm.
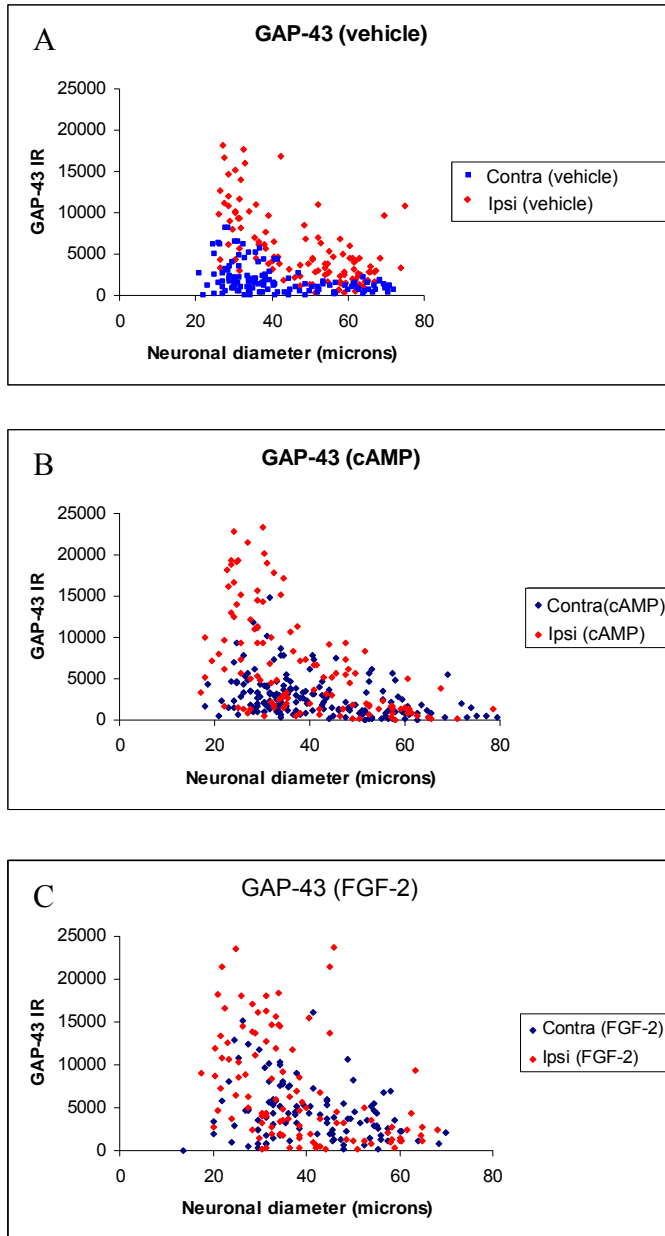
Figure 7-34. FGF-2 and dbcAMP minipump infusions increase GAP-43 levels in DRG neurons. Representative scatter plot is shown for the quantification of the micrographs in Figure 7-33. Plots of (A) Vehicle, (B) cAMP, and (C) FGF-2 confirm the western and histochemical data.

7.3.1.3 Cytoplasmic localization.

Two-way ANOVA followed by Dunn's multiple comparison tests were carried

out on the normalized values of the immunoreactivity. The results show significant

increase in GAP-43 expression as a result of FGF-2 and of cAMP ($P < 0.001$). Ipsilateral

FGF-2 (2.637 $\pm$ 0.05570) and contralateral FGF-2 (1.6150 $\pm$0.032900) values are higher

compared to vehicle values. dbcAMP treated also show higher contralateral and

ipsilateral values (1.1470 $\pm$ 0.021260) and (2.501 $\pm$ 0.06198), respectively (Figure 7-35).
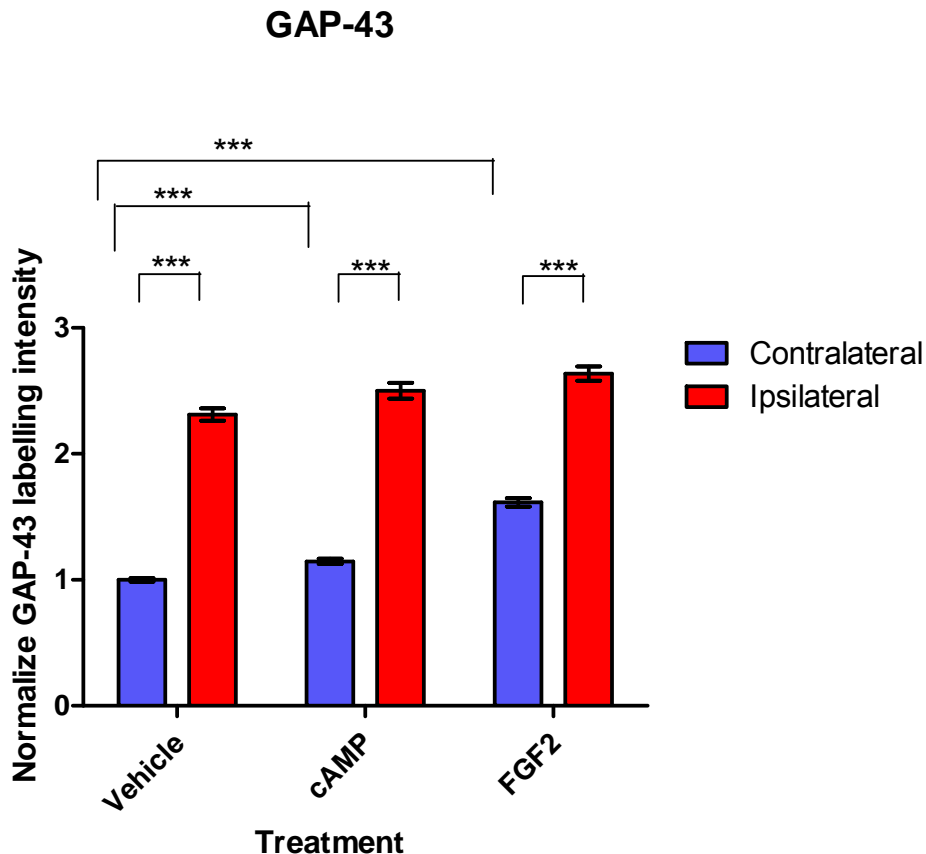
GAP-43

Figure 7-35. Quantification of GAP-43 immunohistochemical staining intensity in L5 DRG 7 d following vehicle, FGF-2 and dbcAMP minipump surgical implantation. The mean immunoreactivity $\pm$ SEM of normalized data to control (contralateral vehicle) (blue bars) values. FGF-2 and dbcAMP cause GAP-43 expression to increase significantly in DRG neurons (***$P < 0.001$) ipsilateral (red) and contralateral (blue) columns. Each bar represents the mean value of 800 or more neurons from 4 different rats.

7.3.2 Effect of FGF-2 and cAMP on ATF3 expression

7.3.2.1 Western analysis.

Western blot analysis showed a specific band at just below the 20 KD level corresponding to ATF3 (Figure 7-36). This band is only reactive in the injured DRG neurons ipsilateral to transected sciatic nerve, and virtually non-existent in the contralateral lanes. FGF-2 administered *in vivo* via minipump did not lower injury induced ATF3 expression in the ipsilateral DRG neurons.
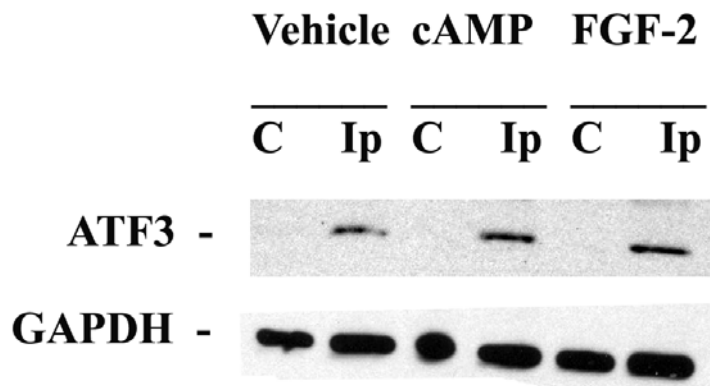
Figure 7-36. dbcAMP and FGF-2 minipump infusion does not suppress ATF3 levels in injured DRG neurons. Western blot analysis for ATF3 in L4 and L5 DRG neurons. No visible suppression of injury-induced ATF3 expression as a result of treatment with dbcAMP and FGF-2. GAPDH is used as internal loading control.

7.3.2.2 Immunohistochemistry.

Figure 7-37 (A) vehicle, (B) dbcAMP, and (C) FGF-2 treated DRG show typical immunohistochemical photomicrographs. These are representative images used in the quantification method and statistical analyses. The expression of ATF3 is increased in both the nucleus and the cytoplasm in the ipsilateral micrographs. dbcAMP and FGF-2 treatment did not alter the dramatic ATF3 induction seen in the vehicle.
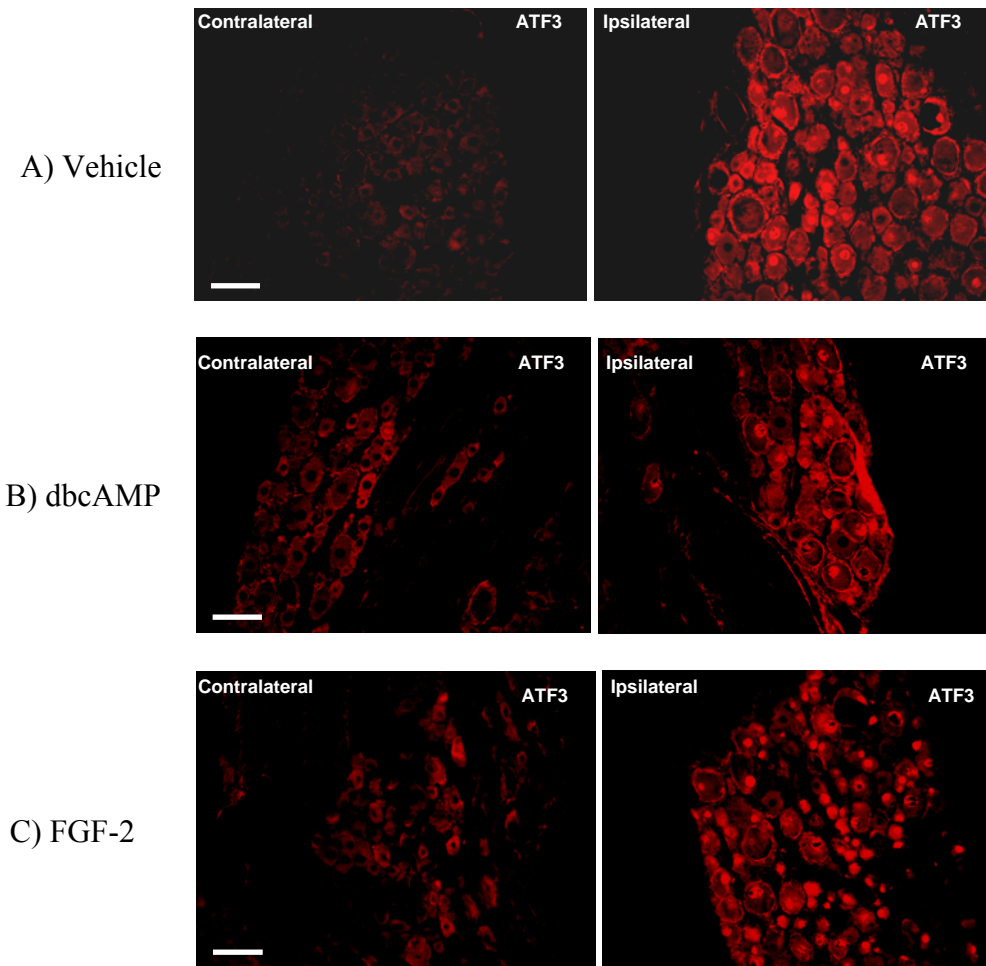
Figure 7-37. FGF-2 and dbcAMP minipump infusions do not suppress the overall increase in ATF3 levels in injured DRG neurons. As a result of treatment with (B) dbcAMP and (C) FGF-2, ATF3 expression in the injured (ipsilateral, right panel) DRG neurons is not suppressed, when compared to vehicle (A) filled minipump, quantified below. Scale bar represents 100 µm.

7.3.2.2.1 Nuclear localization.

Two-way ANOVA followed by Dunn's multiple comparison tests were carried out on the normalized values of the immunoreactivity. The results show significant decrease in nuclear ATF3 expression as a result of FGF-2 and of cAMP treatment when compared to the vehicle control ($P < 0.001$). Ipsilateral FGF-2 (15.05 ± 0.46) is significantly lower compared to normalized vehicle values. dbcAMP treated also show lower ipsilateral

values (15.73 ± 0.53) (Figures 7-38 B, D, and F) lower scatter plots and (7-39 bar

graphs).
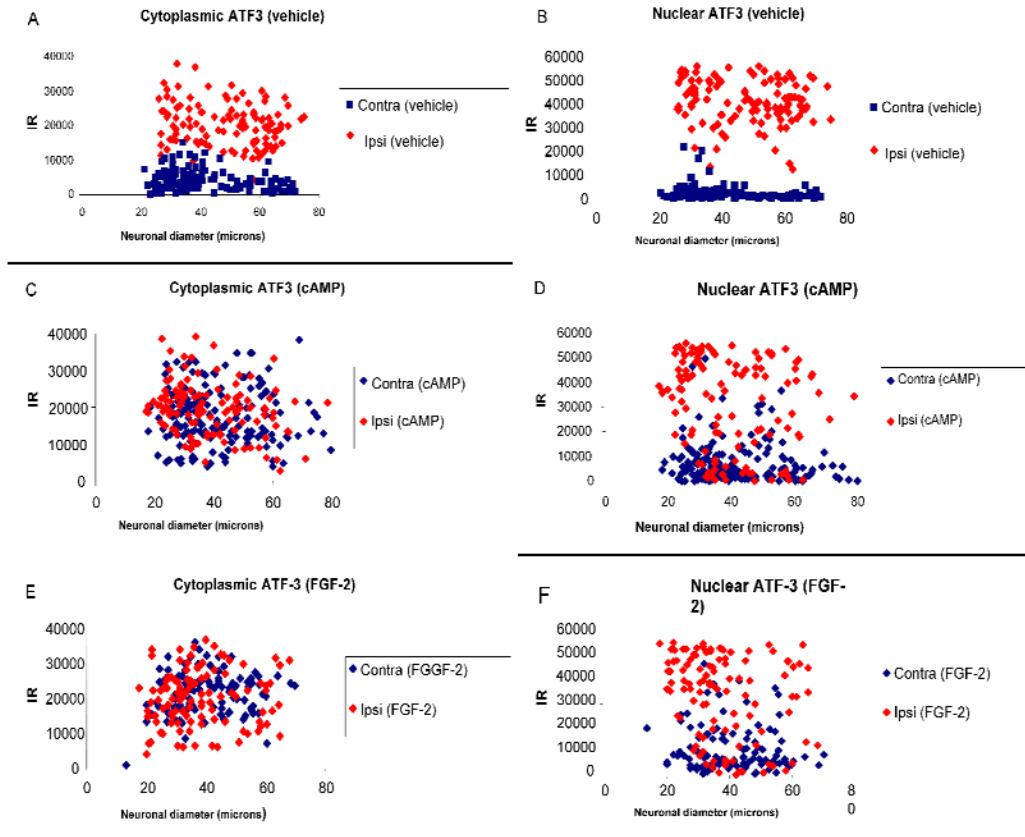
Figure 7-38. FGF-2 and dbcAMP minipump infusions did not repress the overall injury induced ATF3 levels in DRG neurons.   Scatter plot quantification of the immunehistochemistry of cytoplasmic (A, C, and E) and nuclear (B, D, and F) ATF3 following treatment with vehicle (A and B), cAMP (C and D), FGF-2 (E and F).
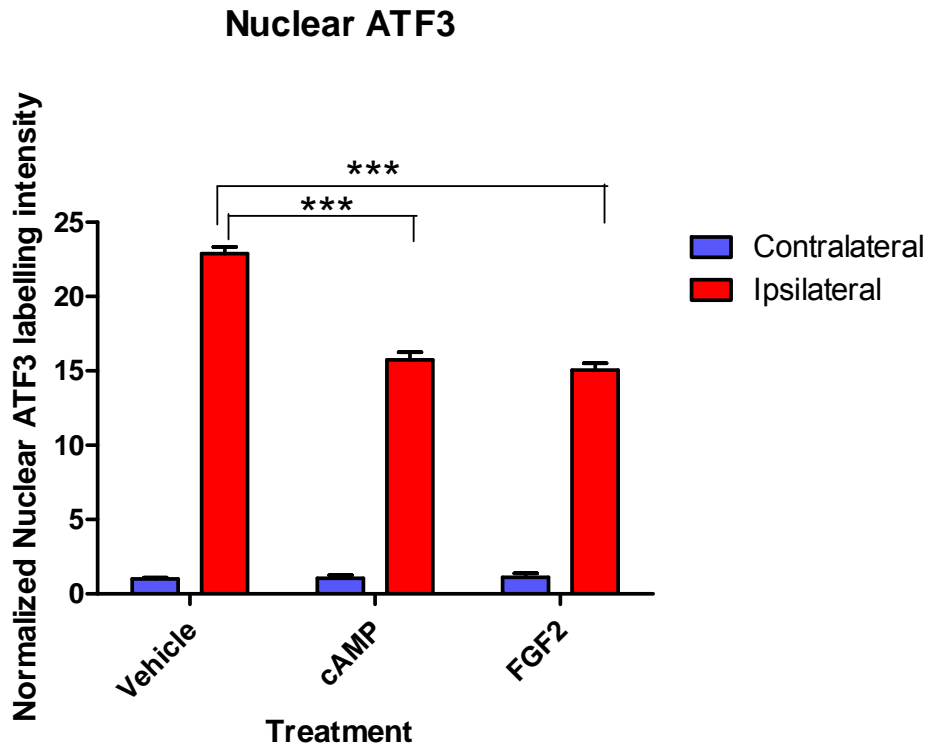
Figure 7-39. FGF-2 and dbcAMP decrease nuclear ATF3 expression ipsilateral to the surgical manipulation. FGF-2 and cAMP cause a significant decrease in the nuclear ATF3 levels (***$P<0.001$). Each bar represents the mean value of 800 or more neurons from 4 different rats.

7.3.2.2.2 Cytoplasmic localization.

Ipsilateral cytoplasmic ATF3 expression as a result of FGF-2 and of cAMP delivery was decreased when compared to vehicle. Ipsilateral FGF-2 ($3.508000 \pm$ 0.102400) was significantly lower, while contralateral FGF-2 (3.703 ± 0.12090) was higher when normalized to vehicle values ($P< 0.001$). dbcAMP treated neurons also showed lower ipsilateral values (2.600000 ± 0.117300) and higher contralateral values (3.182 ± 0.09839), respectively (Figures 7-38 A, C, and E) upper scatter plots and 7-40 bar graphs. Contralateral effects as a result of treatments can be observed.

Cytoplasmic ATF3
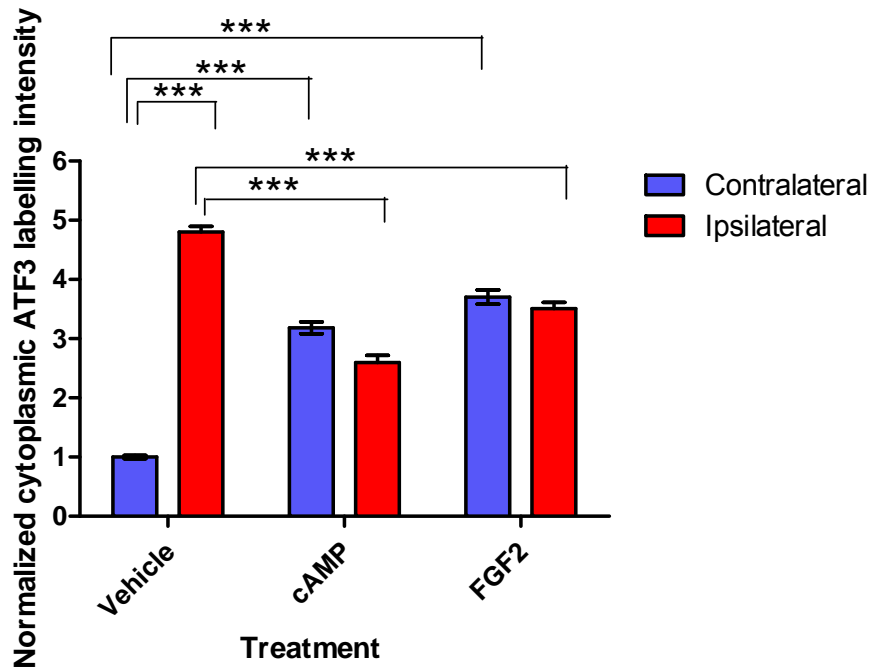
Figure 7-40. FGF-2 and cAMP decrease cytoplasmic ATF3 expression ipsilaterally and increase the contralateral expression.
 FGF-2 and cAMP cause a marked reduction in ipsilateral cytoplasmic ATF3 expression (***$P<0.001$) when compared to vehicle ipsilateral. Ipsilateral (red) and contralateral (blue) data. Each bar represents the mean value of 800 or more neurons from 4 different rats.

7.3.3 Effect of FGF-2 and dbcAMP on c-Jun expression

7.3.3.1 Western analysis

Western blot analysis showed a specific band at just below the 37 KD level corresponding to c-Jun (Figure 7-41). Chronic cAMP and FGF-2 administration via minipump increased c-Jun expression in injured DRG neurons. The basal level of expression did not change as a result of peripheral nerve transection in the vehicle treated animals. However, dbcAMP applied *in vivo* to the proximal cut end of the nerve induced c-Jun expression compared to contralateral. This induction is also seen with FGF-2.

135

FGF-2   dbcAMP   Vehicle

Ipsi Cont   Ipsi Cont   Ipsi Cont
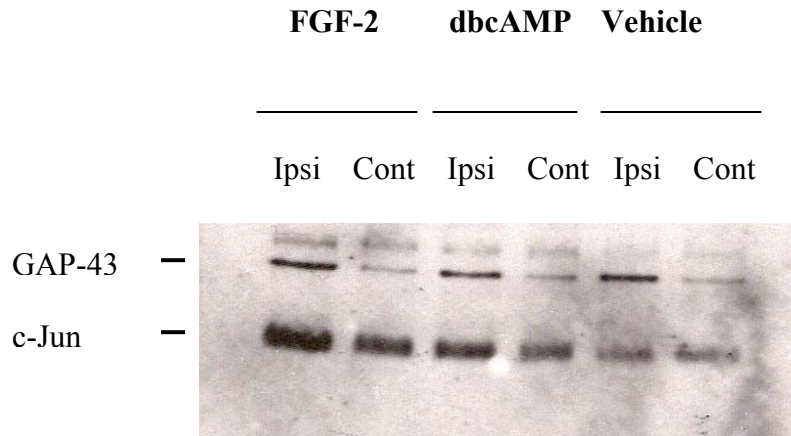
GAP-43

c-Jun

Figure 7-41. FGF-2 and dbcAMP increases c-Jun expression ipsilateral and contralateral to the surgical manipulation. FGF-2 and dbcAMP application cause a noticeable elevation in c-Jun expression. GAP-43 immunolabelling is also included on this blot to confirm the presence of an injury response.

7.3.3.2 Immunohistochemistry

There is a constitutive c-Jun expression in the contralateral DRG neurons. Injury induces an upregulation in c-Jun in the nuclei and in the cytoplasm of injured DRG neurons. This injury-induced upregulation of nuclear c-Jun is further enhanced by dbcAMP and FGF-2 delivered to the cut end of the nerve (Figure 7-42 A, B, and C, and Figure 7-43 B, D, and F, lower scatter plots).

Scatter plots clearly show an increase in nuclear c-Jun as a result of FGF-2 application to the cut end of the sciatic nerve.
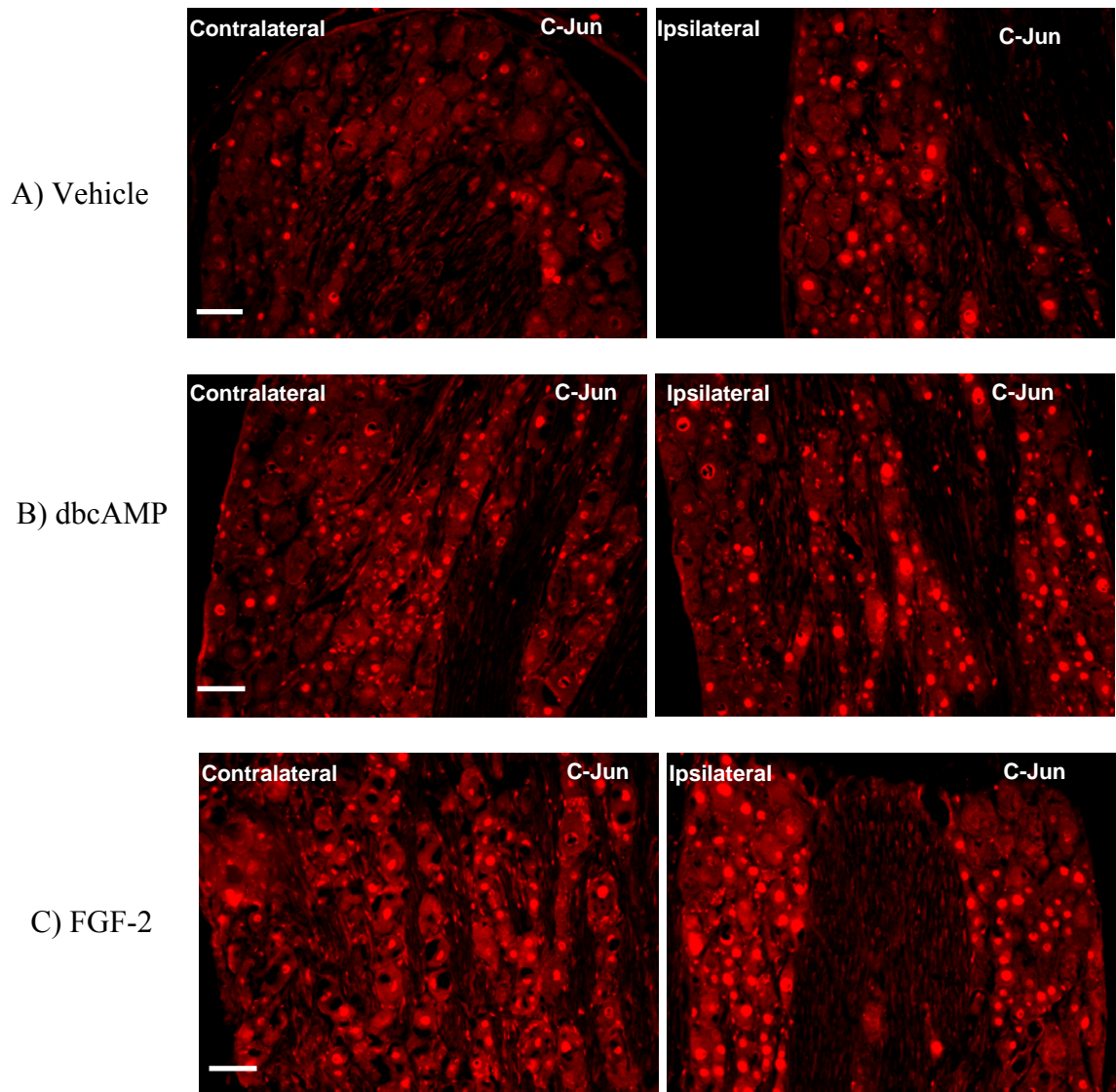
Figure 7-42. FGF-2 and dbcAMP minipump infusions do not repress injury induced c-Jun levels in DRG neurons. There is a constitutive level of cytoplasmic and nuclear expression in contralateral DRG neurons. These images are used in the quantification and statistical analyses below, and statistical analysis (A) vehicle, (B) dbcAMP, and (C) FGF-2. Scale bar represents 100 μm.
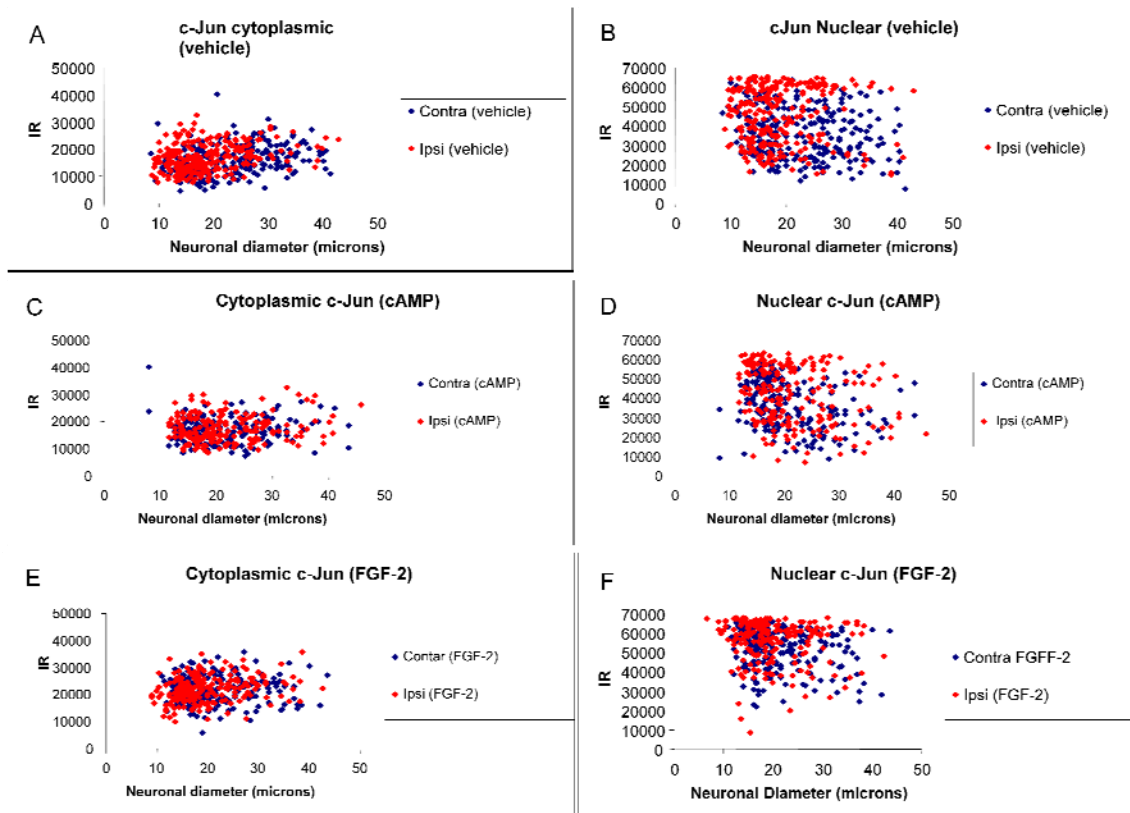
Figure 7-43. FGF-2 and dbcAMP minipump infusions did not repress injury induced c-Jun levels in DRG neurons. Scatter plot quantification of the immunohistochemistry of cytoplasmic (A, C, and E) and nuclear (B, D, and F) c-Jun. Plots of vehicle (A and B), dbcAMP (C and D), FGF-2 (E and F). FGF-2 minipump infusions increase nuclear c-Jun levels in DRG neurons, while cAMP decreases nuclear c-Jun, confirming the Western blot and histochemical dat

7.3.3.2.1 Nuclear localization.

Statistical analysis shows significant decrease in nuclear c-Jun expression in injured neurons as a result of cAMP application (1.20 ± 0.01 vs. 1.27 ± 0.01, $P< 0.001$). In contrast, FGF-2 (1.38 ± 0.01, $P< 0.001$) caused a significant increase compare to the vehicle control (Figure 7-44). Interestingly, FGF-2 applicaion also appeared to cause an increase in nuclear c-Jun on the uninjured contralateral side.
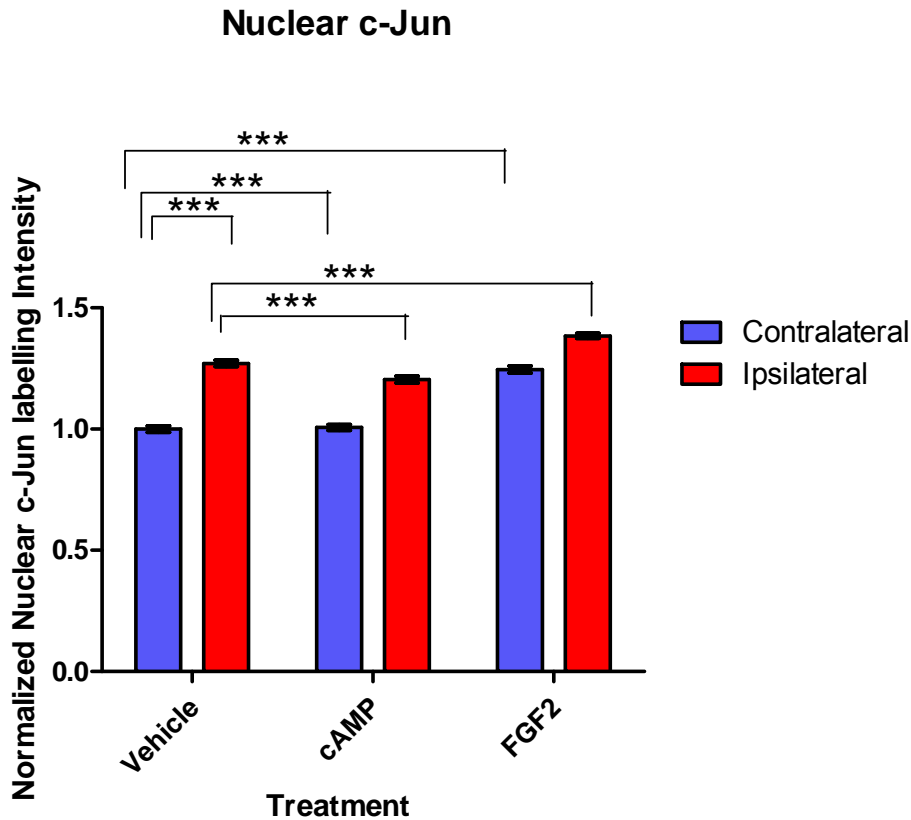
Figure 7-44. dbcAMP decreases nuclear c-Jun, while FGF-2 increases nuclear c-Jun expression. dbcAMP causes a significant reduction in nuclear c-Jun expression (***$P<0.001$) (Ipsilateral (red) and contralateral (blue) data). Each bar represents the mean value of 800 or more neurons from 4 different rats.

7.3.3.2.2 Cytoplasmic localization.

Statistical analysis show significant (P< 0.001) decreases in expression of c-Jun in the cytoplasm of cAMP and of FGF-2 treated DRG neurons. cAMP cytoplasmic value was lowered to (1.082 ± 0.01312), and that of FGF-2 to (1.209 ± 0.0140), Figure 7-43 upper plots, and Figure 7-45).
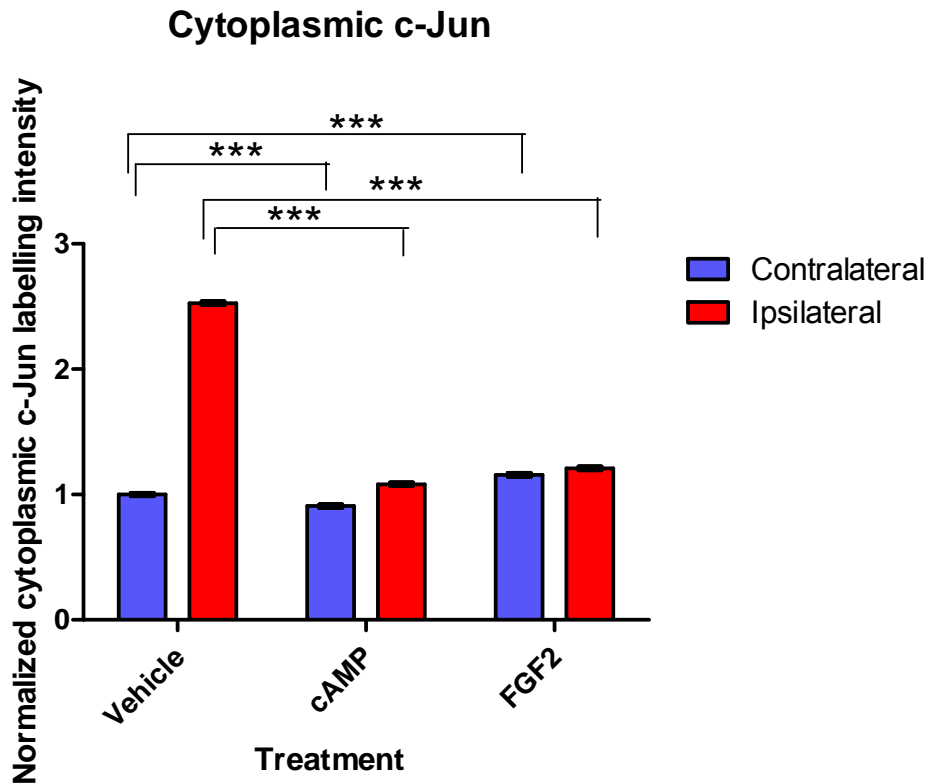
Figure 7-45. Both dbcAMP and FGF-2 decrease cytoplasmic c-Jun. dbcAMP and FGF-2 cause a significant reduction in cytoplasmic c-Jun expression (***$P<0.001$). (Ipsilateral (red) and contralateral (blue) data). Each bar represents the mean value of 800 or more neurons from 4 different rats.

7.3.4 Effect of cAMP and FGF-2 on STAT3 expression

7.3.4.1 Western analysis

Western blot analysis showed a specific band at the 90 KD level corresponding to

STAT3 (Figure 7-46). The blot in Figure 7-46 shows that cAMP and FGF-2

administration *in vivo* via minipump suppressed injury induced STAT3 expression.
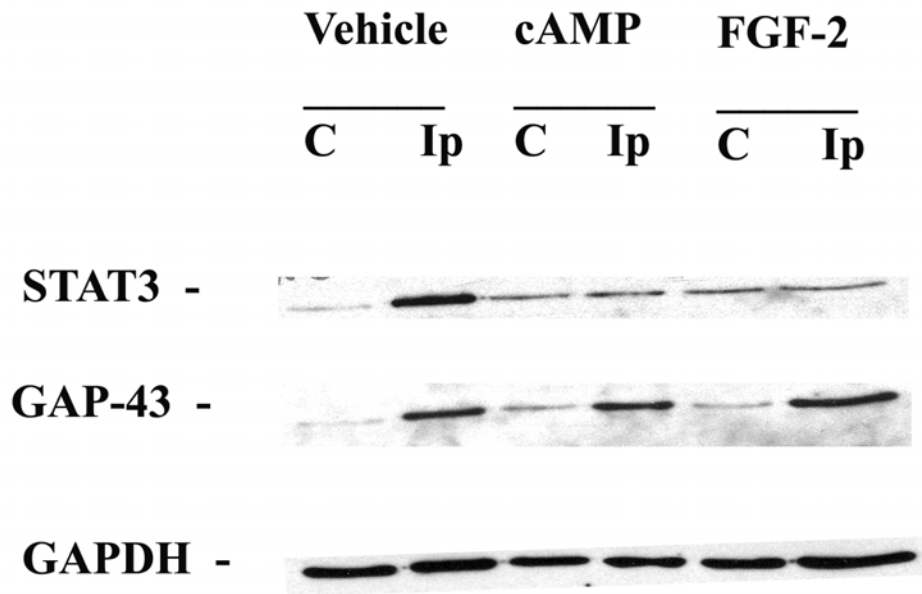
Figure 7-46. FGF-2 and dbcAMP suppress injury induced STAT3 expression. FGF-2 and dbcAMP suppress injury induced STAT3 expression. GAP-43 immunolabelling is also included on this blot to confirm the expression of an injury response, and GAPDH as internal loading control.

7.3.4.2 Immunohistochemistry

There is a constitutive STAT3 expression in the contralateral DRG neurons. Injury induces an upregulation in STAT3 in the nuclei and in the cytoplasm of injured DRG neuron. This injury-induced upregulation of total STAT3 is reduced by cAMP and FGF-2 delivery to the cut end of the nerve (Figures 7-47 A, B, and C, and 7-48 B, D, and F).

Scatter plot of the cytoplasmic and nuclear STAT3 shows an increase in the nuclear STAT3, especially in the nuclei of small neurons (Figure 8 A and B).

7.3.4.2.1 Nuclear localization.

Statistical analysis shows a significant decrease in nuclear STAT3 expression as a result of cAMP but not FGF-2 (0.9233 ± 0.01648, *P*< 0.001) (Figure 7-49).

7.3.4.2.2 Cytoplasmic localization.

Statistical analysis show significant decrease in cytoplasmic STAT3 expression as a result of cAMP (0.94 ± 0.01, ****P*< 0.01), and of FGF-2 (0.90 ± 0.01, *P*<0.05) (Figure 7-50).
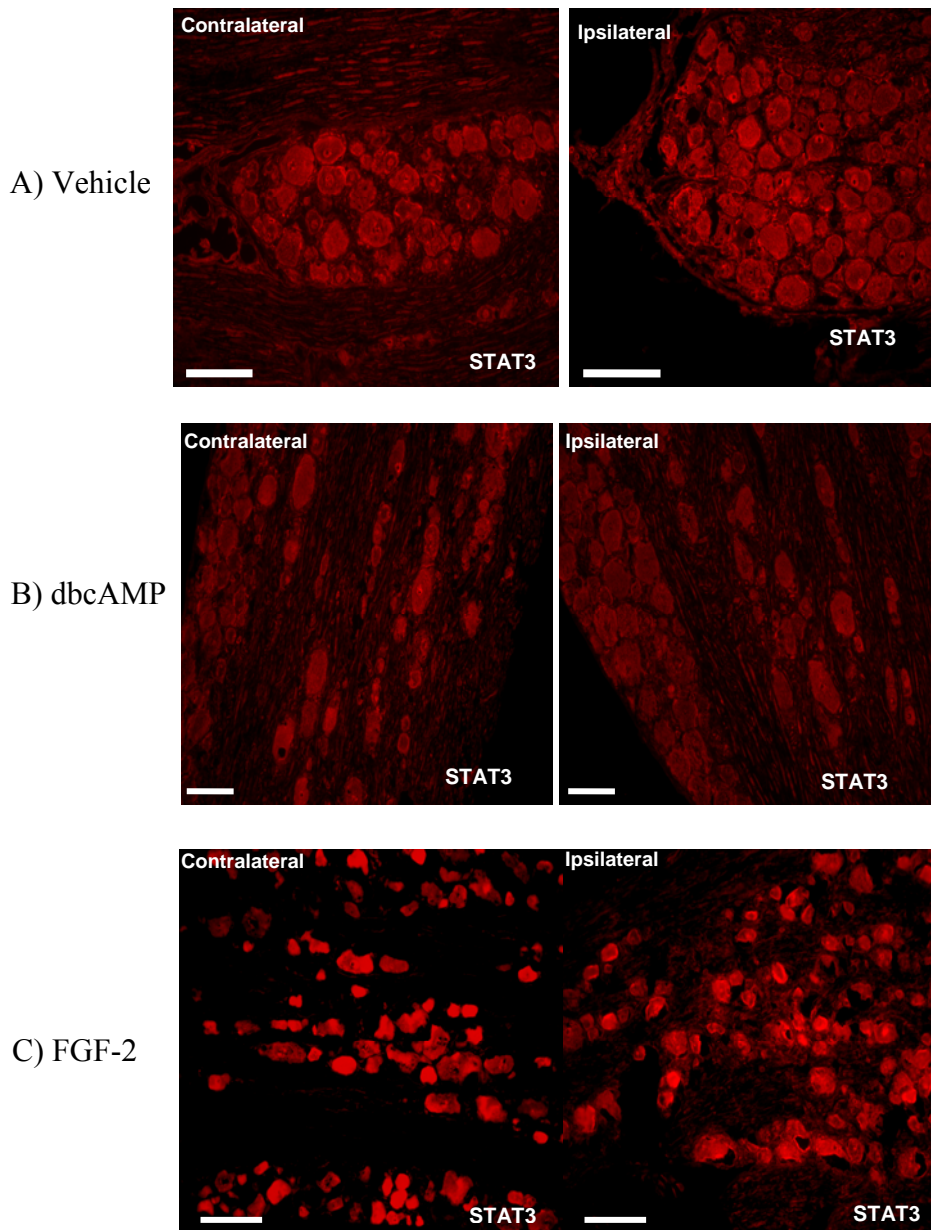
Figure 7-47 A, B, and C shows typical immunohistochemical photomicrographs of total STAT3 expression in response to dbcAMP and FGF-2. There is a constitutive level of cytoplasmic and nuclear expression in contralateral DRG neurons. These images are used in the quantification method, and statistical analysis (A) vehicle, (B) dbcAMP, and (C) FGF-2.

Figure 7-48. FGF-2 minipump infusion does not change nuclear or cytoplasmic total STAT3 levels in DRG neurons, while cAMP decreases nuclear STAT3. Scatter plots quantification of the immunohistochemistry of cytoplasmic and nuclear STAT3. Plots of (A, and B) Vehicle, (C and D) dbcAMP, and (E and F) FGF-2 confirm the immunohistochemical data.
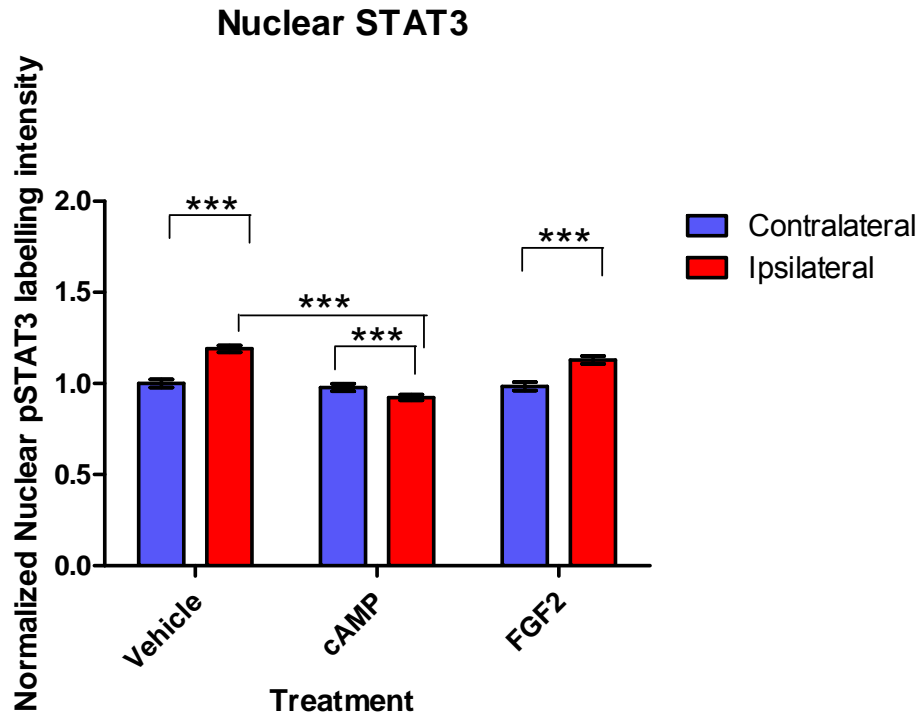
Figure 7-49. dbcAMP decrease nuclear STAT3, while FGF-2 does not change nuclear STAT3 expression. dbcAMP causes a significant reduction in nuclear STAT3 expression compared to injury response alone (***P<0.001), ipsilateral (red) and contralateral (blue) data. Each bar represents the mean value of 800 or more neurons from 4 different rats.
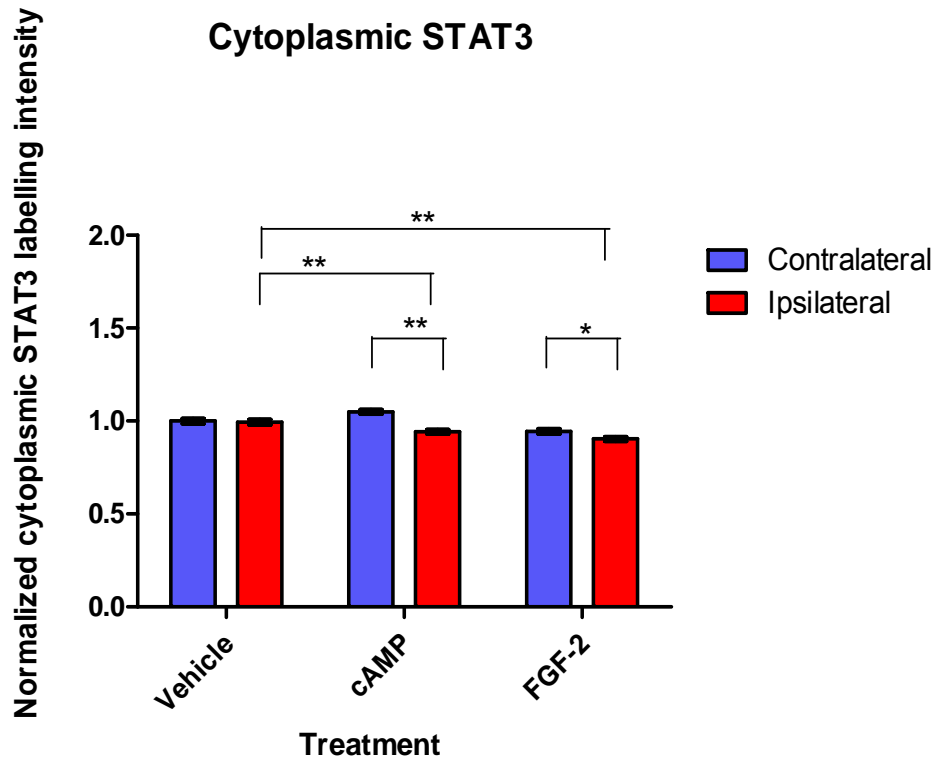
Figure 7-50. FGF-2 and dbcAMP decrease cytoplasmic STAT3 expression. dbcAMP causes a significant reduction in cytoplasmic STAT3 expression compared to injury alone (**$P<0.001$), ipsilateral (red) and contralateral (blue) data. Each bar represents the mean value of 800 or more neurons from 4 different rats.

7.4.5 Effect of cAMP and FGF-2 on pSTAT3 expression

7.4.5.1 Western analysis

Western blot analysis showed a specific band at the 90 KD level corresponding to pSTAT3 (Figure 7-54). The level of pSTAT3 immunoreactivity on these blots did not show a consistant change as a result of chronic dbcAMP or FGF-2 infusion on either the ipsilateral or contralateral sides. No quantification was done on this blot. A sample is shown in Figure 7-51.
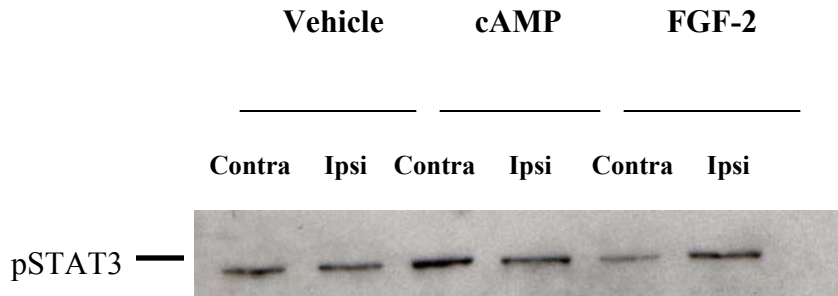
Figure 7-51. FGF-2 and dbcAMP do not show substantial difference on pSTAT3 expression ipsilateral and contralateral to the surgical manipulation. FGF-2 and dbcAMP did not induce a measureable change in pSTAT3 expression.

7.4.5.2 Immunohistochemistry

There is a constitutive pSTAT3 expression in the contralateral DRG neurons. Injury induces an upregulation in pSTAT3 in the nuclei and in the cytoplasm of injured DRG neuron. This injury-induced upregulation of pSTAT3 is further enhanced by cAMP and FGF-2 delivery to the cut end of the nerve (Figures 7-52 A, B, and C, and 7-53 B, D, and F).
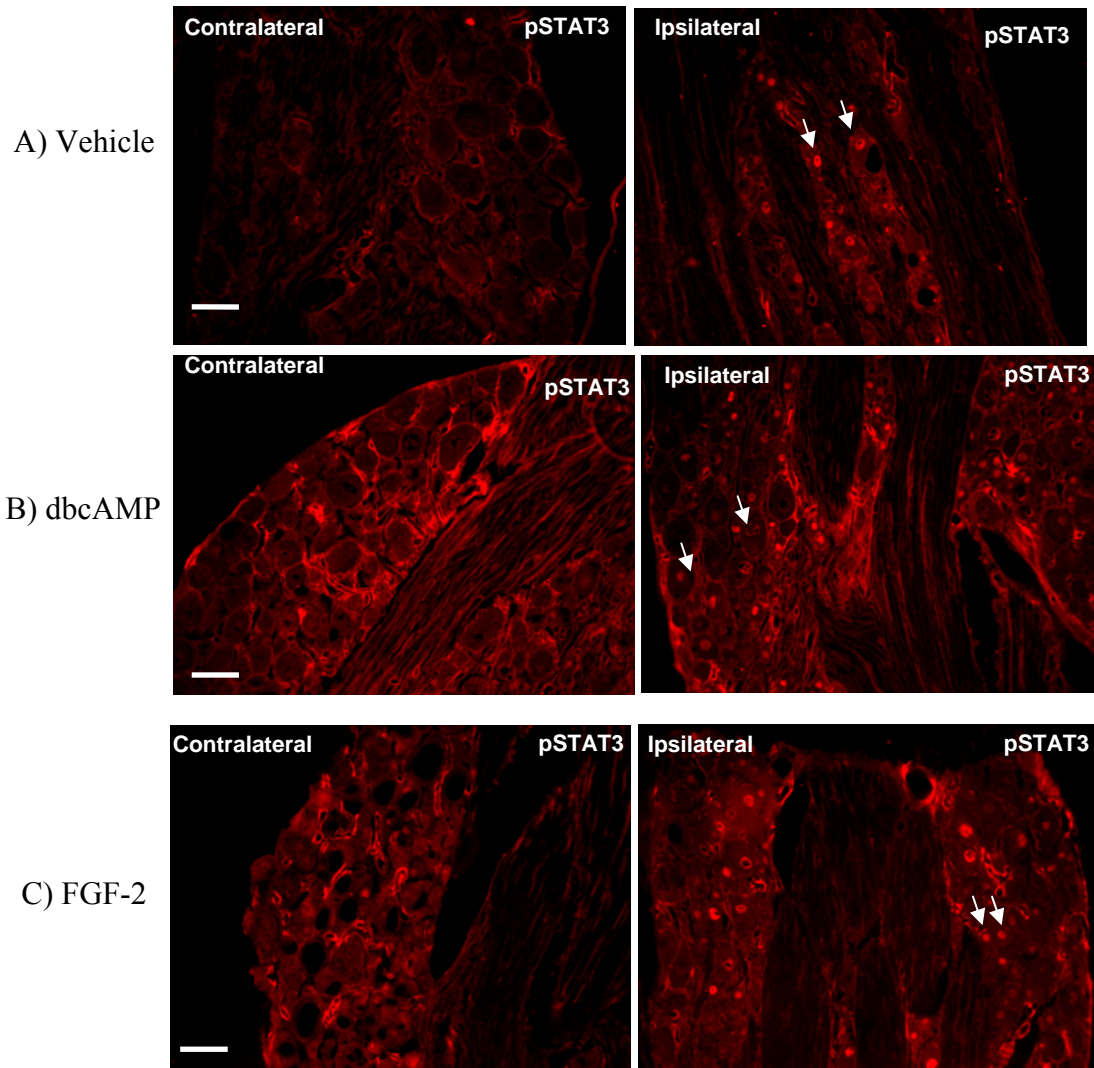
Figure 7-52 A, B, and C shows typical immunohistochemical photomicrographs of total pSTAT3. There is a constitutive level of cytoplasmic and nuclear expression in contralateral DRG neurons. These images are used in the quantification method, and statistical analysis (A) vehicle, (B) dbcAMP, and (C) FGF-2. Arrows point to nuclei that express pSTAT3 as a result of injury.

Figure 7-53. FGF-2 and dbcAMP minipump infusions increase nuclear pSTAT3 levels in DRG neurons, while dbcAMP increases cytoplasmic pSTAT3. Scatter plots quantification of the immunohistochemistry of cytoplasmic and nuclear pSTAT3. Plots of (A, and B) Vehicle, (C and D) dbcAMP, and (E and F) FGF-2 confirm the immunohistochemical data.

7.4.5.2.1 Nuclear localization.

Statistical analysis shows a significant increase in nuclear pSTAT3 expression as a result of FGF-2 (4.395 ± 0.18960, $P < 0.001$), but not dbcAMP (Figure 7-54). Contralateral effects are observed.
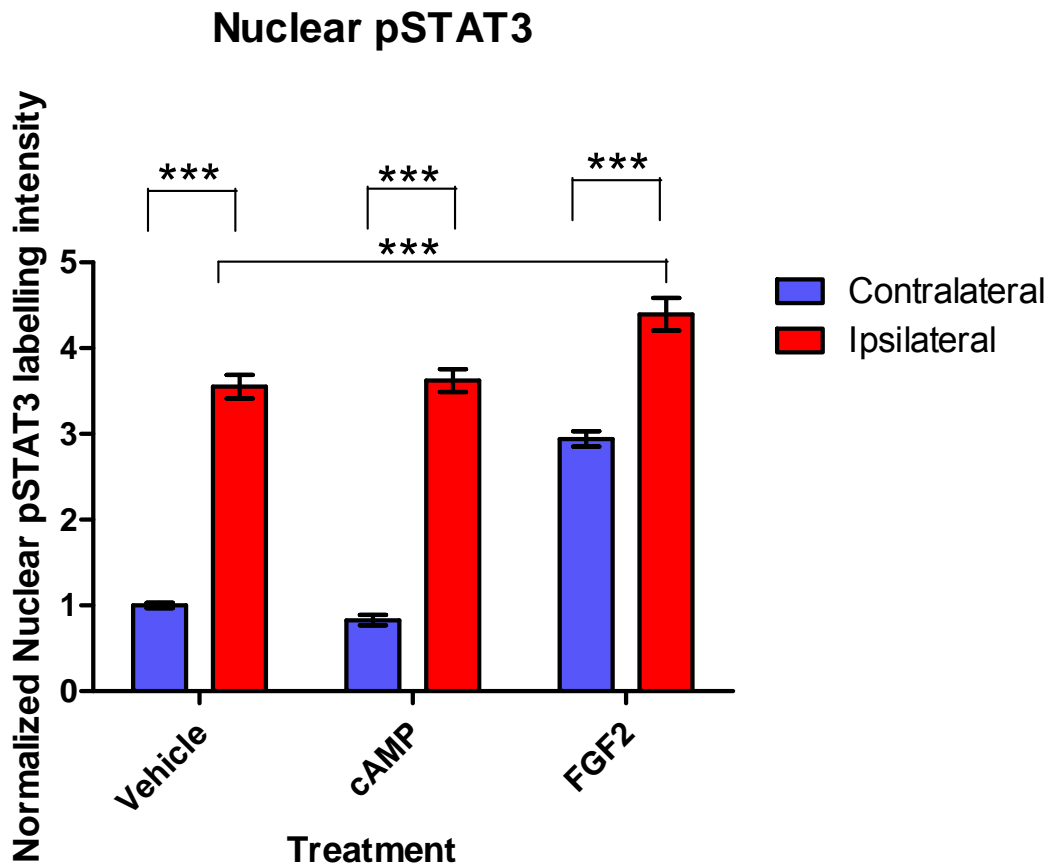
Figure 7-54. FGF-2 increase nuclear pSTAT3, while dbcAMP does not change injury induced nuclear pSTAT3. FGF-2 causes a significant elevation in nuclear pSTAT3 expression (***$P<0.001$), ipsilateral (red) and contralateral (blue) columns.

7.4.5.2.2 Cytoplasmic localization.

Statistical results show significant increase in cytoplasmic pSTAT3 expression as a result of cAMP (3.189 $\pm$ 0.10380, **$P< 0.001$), but not FGF-2 (Figure 7-55).

Cytoplasmic pSTAT3
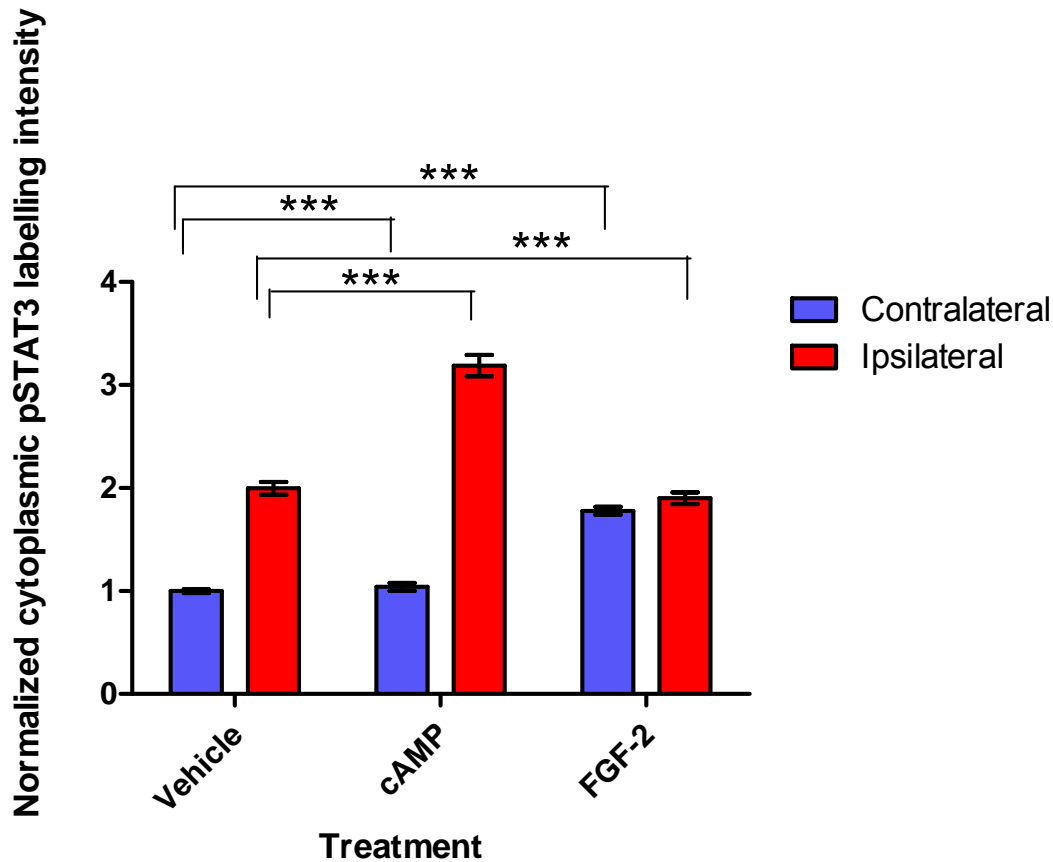
Figure 7-55. dbcAMP increase cytoplasmic pSTAT3, while FGF-2 does not change injury induced cytoplasmic pSTAT3. dbcAMP causes a significant elevation in cytoplasmic pSTAT3 expression (***$P<0.001$), ipsilateral (red) and contralateral (blue) columns.

Table 7-2 summarizes the results of minipump implantation containing vehicle, dbcAMP, or FGF-2. It appears that neither cAMP nor FGF-2 suppressed the injury induced upregulation of GAP-43. However, injury induced upregulation of ATF3 and c-Jun, although not completelt suppressed, seems to be downregulated by cAMP, and or FGF-2. pSTAT3, on the other hand, is upregulated by FGF-2 infusion.

Table 7-2. Summary of the results of minipump implantation.

Protein	Vehicle	dbcAMP	FGF-2
GAP-43	↑	↑	↑
ATF3 nuclear	↑	↓	↓
ATF3 cytoplasmic	↑	↓	↓
c-Jun nuclear	↑	↓	↑
c-Jun cytoplasmic	↑	↓	↓
pSTAT3 nuclear	↑	±	↑
pSTAT3 cyoplasmic	↑	↑	±

This table summarizes the results obtained from minipump implantation containing vehicle, dbcAMp, or FGF-2. Proteins examined are listed in the far left column. Red arrows indicate an increase, whereas blue arrows indicate a decrease in the expression, as judged by measuring immuofluorescence intensity.

CHAPTER 8
8.0 DISCUSSION

8.1 Adequacy of experimental design and techniques.

8.1.1. Peripheral nerve transection versus central transection.

The pseudounipolar neurons of the DRG have two branches, a peripheral branch that is capable of mounting a robust regenerative response after nerve injury, and a central branch extending into the dorsal column of the spinal cord that does not regenerate as well. However, a conditioning lesion introduced to the peripheral branch can cause the lesioned central branch to re-grow into the dorsal column. This unique anatomy of the DRG neurons, the fundamentally opposing effects that injury to either branch can produce, and the easy accessibility to manipulate either branch make this model system an excellent testing grounds to study the molecular events influencing nerve regeneration. In our peripheral versus central root transection model, we introduced the injury on one side of the animal and used the un-operated contralateral side as our internal control because no immunohistochemical difference in GAP-43 expression has been reported between un-operated controls and contralateral controls (Schreyer and Skene, 1991; 1993).

Peripheral sciatic nerve transection at mid-thigh is not expected to induce all L4 and L5 DRG neurons to display the injured phenotype because some of these neurons may innervate proximal structures (Devor et al., 1985) or may extend axonal branches to nerves other than the sciatic (Devor et al., 1984; Langford and Coggeshall, 1981; McMahon and Wall, 1987; Pierau et al., 1982; Taylor and Pierau, 1982).

8.1.2. Chronic infusion technique.

The chronic infusion technique used in this project has been modified from an already reported method (McDonald and Zochodne, 2003). In that report, the authors stressed that two criteria must be met in order to ensure a successful *in vivo* delivery system. Firstly, a successful delivery system must allow for long-term administration and secondly, the model must allow a direct, local delivery (McDonald and Zochodne, 2003). Delivery by repeated injection will necessarily result in uneven, pulsatile dose levels. Moreover, it is well established that stress caused by handling of the animal for repeated injections can create difficulty in interpreting the results.

By using implantable osmotic minipumps we have developed a model that features smooth, continuous flow of known concentrations of the drugs of interest (dbcAMP, and FGF-2), directly delivered to the transected site, and over a well defined period of time (seven days) without the added stress of repetitive injections. The method fits the two criteria of successful delivery. Further, by confining the cut nerve end and the pump catheter within a small plastic chamber, the method employed here assures direct delivery to the cut end of the sciatic nerve. Our current project provides evidence that immunohistochemical analyses of the expression of early transcription factors and growth associated proteins known to be part of the peripheral nerve regeneration response are quantifiable seven days after nerve transection.

FluoroGold filled minipump results showed that the large majority of injured DRG neurons could obtain the tracer dye after delivery from the pump, and retrogradely transport it to their cell bodies. About 30% of DRG neurons showed very intense retrograde FluoroGold labeling, while most of the remainder showed some detectable

labelling. This finding is of importance since it confirms effective delivery and

availability for transport of compounds present in the minipumps. Although we could not

quantify the efficiency of FluoroGold labeling in the absence of a marker for Fluorogold

unlabelled neurons, our results appear to be in concert with those of (Liabotis and

Schreyer, 1995), who reported over 60% retrograde labeling of DRG neurons following

sciatic nerve transection at the mid-thigh level.

8.1.3. Technical limitations

Despite the overall success of the technique, limitations do exist. The selection

process of the neuron size was entirely based on the DAPI counterstain. Given that

injured DRG neurons would exhibit an excentric nuclear position within the cytoplasm,

our different neuronal sizes, may not reflect the actual neuronal size of the injured

neurons.

Moreover, our technique does not entirely preclude some systemic effects that

may be generated as a result of leakage of FGF-2 or dbcAMP from the chamber region.

This may explain some of the contralateral effects that we observed (see below). Another

limitation to our experimental design is the motility of the animals post-operatively.

Normal or pathological behavior following surgery could move the chamber or the

minipump from their ideal surgical positions. Some rats chewed on their paws, possibly

introducing additional inflammatory signals from the periphery.

Our Western blot analyses proved to be a unique challenge, since we attempted to

probe for changes in low-abundance transcription factors in the DRG neurons. The DRG

are small in size, and include neuronal cell bodies, axons, myelin and glial cells. In order

to obtain the adequate amounts of proteins for Western analysis, we needed to perform

the experimental surgical procedures and implants on a large number of animals. This

provided some technical limitations. For future studies using our current technique,

Western analysis could be improved, if a greater number of animals are utilized in each

group.

8.2 New findings.

8.2.1. Peripheral vs. Central root transection.

Our peripheral nerve transection results confirm previously reported *in vivo* work

regarding the induction of GAP-43 and of specific injury-induced transcription factors.

8.2.1.1. GAP-43 expression

The expression of GAP-43, a membrane phosphoprotein, dramatically increases

as a result of peripheral nerve transection in DRG neurons (Schreyer and Skene, 1991;

1993; Woolf et al., 1990) . Elevation of GAP-43 mRNA has previously been detected 24

hours following peripheral nerve transection (Chong et al., 1994). GAP-43 protein

expression is induced at least five-fold as early as two days after sciatic verve transection,

and this increase persists for up to 80 days following injury (Sommervaille et al., 1991).

Our results of GAP-43 upregulation in response to injury ranged between 1.5 to 3 fold.

This induction of GAP-43 is in agreement with the reported literature describing GAP-43

as a protein of the peripheral injury response (Liabotis and Schreyer, 1995; Schreyer and

Skene, 1993).

GAP-43 upregulation did not occur in our dorsal transection experiments, in agreement with the previously reported literature (Chong et al., 1994; Piehl et al., 1993; Schreyer and Skene, 1993). Because the strong effect following peripheral nerve transection was not seen following central root transection, injury associated upregulation of GAP-43 has been proposed to be controlled by a mechanism unique to the peripheral branches. These and other observations support the hypothesis that GAP-43 induction in DRG neurons is caused by loss of controlling element(s), normally originating only from peripheral target tissue (Schreyer and Skene, 1993).

8.2.1.2. ATF3 expression.

We show in this study that ATF3 is also induced in DRG neurons after peripheral, but not central axonal injury. ATF3 is thought to contribute to nerve regeneration by increasing the intrinsic growth state of injured neurons. Our finding that ATF3 is expressed seven days after peripheral nerve transection is in agreement with previously published reports (Seijffers et al., 2006; Seijffers et al., 2007). Our results show that ATF3 expression remained high even fourteen days after peripheral injury, indicating a persistent role in the regenerative response that is closely correlated with GAP-43 expression. Our results showing a lack of ATF3 expression as a result of dorsal root transection are in agreement with reported data (Seijffers, 2006). Another study, however, showed upregulation of ATF3 in L5 DRG neurons (Huang, 2006) after dorsal root transection. This study differs from our experiment in that the authors examined ATF3 expression only 1 day after dorsal transection, and spinal cord hemisection.

Generally, any upregulation of ATF3 expression as a result of dorsal transection appear, therefore, to be short lived.

ATF3 is a transcription factor that is synthesized in the cytoplasm via the normal cellular process of protein synthesis. Upon activation it is translocated to the nucleus where it can produce its biological effects (Isacsson et al., 2005). Our results showed an increase in ATF3 expression in both the cytoplasm and the nucleus of the peripherally transected nerve. The increase in the cytoplasm is indicative of an increase in the synthesis of ATF3. Once synthesized, it is then translocated to the nucleus where we observed it to be abundantly expressed.

8.2.1.2.1 GAP-43 and ATF3 expression 7 d vs 14 d comparison.

When comparing the results of 7 d vs. 14 d peripheral sciatic nerve transection, a continuing increase in the injury response can be readily observed. Nuclear ATF3 expression increased 4 fold in 14 d injured DRG neurons vs. 3 fold increase in the 7 d injured DRG neurons. Cytoplasmic ATF3 expression increased 1.5 fold in the 14 d injured DRG neurons vs. the 7 d injured DRG neurons. GPA-43 expression increased 2 fold in the 14 d injured DRG neurons vs. 1.4 fold increase in the 7 d injured DRG neurons. The increase in our GAP-43 results could be attributed to the increased expression of the small, medium and large diameter subpopulation of injured neurons, in agreement with previously published work (Schreyer and Skene, 1993; Sommervaille et al., 1991).

The increase in our ATF3 expression is also in agreement with previously reported data demonstrating that sciatic nerve transection induced an upregulation of ATF3 immunoreactivity in 82% of L4 DRG cells 14 days post transection (Averill et al.,

2004). These authors attributed the ATF3 increase to a loss of target derived neurotrophic factors such as NGF and GDNF (Averill et al., 2004).

More of the 14 d injured small to medium diameter neurons expressed ATF3 than in the 7 d injury model. This implies that, as part of the regenerative process, injured DRG neurons might differentially in a coordinated fashion express ATF3 depending on the size of the injured neurons.

While there is a clear and robust increase in the expression of GAP-43 and ATF3 as a result of peripheral nerve transection, the expression of both GAP-43 and ATF3 remained unchanged as a result of dorsal root transection. This implies that signals that control the expression of ATF3 and GAP-43 do not originate from the central branch of the DRG; rather, the un-interrupted, continuous flow of factors from the peripheral branch maintains the suppressed state of both GAP-43 and ATF3.

8.2.1.3. c-Jun expression.

We find that c-Jun expression is markedly upregulated, and localized primarily in neuronal nuclei, seven days following peripheral nerve transection, in agreement with previous studies (Jenkins and Hunt, 1991; Jenkins et al., 1993; Leah et al., 1993). Our present results also indicate that c-Jun expression is very similar to GAP-43 expression, although a small number of injured neurons upregulated nuclear c-Jun expression without simultaneously upregulating GAP-43. The observed failure of upregulation of c-Jun expression in DRG neurons following dorsal root transection is also similar to the pattern of GAP-43 expression. Peripheral axotomy may cause, in addition to loss of peripheral target-drived factors, metabolic and morphological changes in the cell body leading to an increase in transcription factors including c-Jun. This 'loss' of peripheral target factors,

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however, appearently does not occur as a result of dorsal root transection, and this has been related to the reduced regenerative capacity of the central axon. Trophic and other target derived elements that are available through the peripheral branch of DRG could ameliorate the upregulation of c-Jun in DRG neurons subjected to dorsal root transection. The contention of the influence of target-derived factors on the expression of regeneration associated or growth associated proteins within the injured DRG neurons is supported further by the conditioning lesion effect on regrowth of central axons in a favorable environment (Richardson and Issa, 1984; Richardson and Verge, 1986; 1987).

The failure to upregulate c-Jun expression following dorsal transection contrasts somewhat with a previous report that a small percentage (18 %) of DRG neurons upregulate c-Jun expression following dorsal root transection (Broude et al., 1997). We feel that our robust c-Jun immunohistochemical technique was adequate to detect low level changes. It is possible that the surgical approach used in the previous study resulted in more inadvertent damage of peripheral axons at the surgical site than ours did.

It is not completely understood whether c-Jun regulates the expression of GAP-43 either directly or indirectly. However, the increased expression of GAP-43 following peripheral nerve transection could be one of the events initiated by c-Jun activation. It would be of interest to determine if there is a direct pathway(s) linking the transcription factor c-Jun to transcriptional regulation of GAP-43 (see below).

8.2.1.4. Coordinated expression of c-Jun and ATF3

ATF3 and c-Jun belong to the same ATF/CREB family of transcription factors and can form a heterodimer (Hai and Curran 1991; Hsu, Laz et al. 1991; Chu, Tan et al.

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1994). Furthermore, c-Jun increases in sensory neurons after peripheral nerve transection (Leah, Herdegen et al. 1991; Broude, McAtee et al. 1997) similar to ATF3 expression in the present study. It is thus tempting to speculate that such heterodimers are a key modulator of gene expression of key growth associated proteins during the regenerative process. ATF3/c-Jun heterodimers are known to bind to different sites activating the expression of a number of genes. In contrast, ATF3 homodimers tend to act as repressors of gene expression (Hai and Hartman, 2001). However, we found that uninjured DRG neurons weakly express c-Jun protein, in contrast to the lack of constitutive expression of ATF3. Thus, in intact DRG neurons, c-Jun but not ATF3 may play a role in the maintenance of normal phenotype in some neurons.

Our results indicate that cytoplasmic c-Jun expression in peripherally axotomized neurons does not change, whereas, cytoplasmic ATF3 expression is upregulated. This supports the idea that the rate of ATF3 protein synthesis could be increased as a result of injury, while the rate of c-Jun protein synthesis may not be affected. The dramatic increase in the nuclear expression of both ATF3 and c-Jun supports the notion that the rate of phosphorylation and translocation to the nucleus of both proteins is enhanced following injury. Given that no increase in protein synthesis of ATF3 is detected, and that basal level of c-Jun expression exists prior to injury, buttresses the argument that c-Jun and ATF3 may not have a common mechanism of transcriptional regulation. Alternatively, once the injury occurs, ATF3 synthesis and activation, in concert with other transcription factors, may control subsequent c-Jun activation.

It is reasonable to consider ATF-3 to be a key regulator of intrinsic growth in DRG neurons because it is induced in all sensory neurons after peripheral nerve injury

(Benn, Perrelet et al. 2002). However, ATF-3 overexpression does not increase c-Jun expression (Seijffers, Allchorne et al. 2006). ATF-3 may act in some neurons in concert with c-Jun to regulate the expression of target genes and thereby promote regeneration. The growth-promoting action of ATF-3 may not be dependent upon c-Jun, but may be dependent on other transcription factors across the entire population of DRG neurons. ATF-3 may be just one of a multitude of factors that contribute to neurite outgrowth, perhaps by orchestrating the gene expression responses in injured neurons. ATF3 regulates the expression of Hsp27 (Lindwall and Kanje, 2005; Nakagomi et al., 2003), one of the injury-induced markers that responds to peripheral, but not central nerve transection (Costigan, 1998, Lewis 1999) (Costigan et al., 1998; Lewis et al., 1999). Our finding that ATF3 is correlated with GAP-43 supports the idea that this transcription factor drives expression of at least some aspects of the intrinsic growth capacity of peripherally injured DRG neurons.

8.2.1.5. Coordinated expression of c-Jun and GAP-43.

Our results showed that while c-Jun expression was, for the most part, colocalized with GAP-43 in injured DRG neurons, a small percentage of injured neurons expressed only c-Jun, but not GAP-43 (Figure 7-10, white arrows). A correlation between c-Jun and GAP-43 expression in the DRG neurons of injured sciatic nerve has been previously reported (Bisby et al., 1995; Broude et al., 1997; Seo et al., 2009). However, in injured , regenerating retinal ganglion cells c-Jun expressing neurons were twice as numerous as GAP-43 expressing neurons (Schaden et al., 1994).   Our results are in agreement with the latter study, since we observed a difference between injured neurons that expressed c-

Jun and those that expressed GAP-43. In the present study we showed that while all

GAP-43 positive injured neurons were also c-Jun positive, not all c-Jun positive injured

neurons were GAP-43 positive. This implies that the molecular regulatory mechanism or

pathway that regulates both c-Jun and GAP-43 may not be active in all injured neuronal

subpopulations. Alternatively, since we did not study the size relationship between the

injured neurons that express either or both c-Jun and GAP-43, it would be tempting to

suggest a differential regulatory mechanism that would be size-dependent. It would be of

interest to investigate the existence of such a regulatory mechanism in our injury model.

8.2.1.6. STAT3 expression.

Our findings with pSTAT3 activation as a result of peripheral nerve transection

are in agreement with previously reported data (Haas et al., 1999; Qiu et al., 2005;

Schwaiger et al., 2000). The earliest detection time of pSTAT3 was reported to be 6

hours post lesion (Lee et al., 2004). The increase in pSTAT3 expression in DRG neurons

in correlation with the elevated expression of GAP-43 suggests that this transcription

factor may contribute to the survival and axonal regeneration of injured sensory neurons.

STAT3 is expressed early following peripheral axon injury, and is activated

persistently until the time that peripheral re-innervation takes place (Schwaiger et al.,

2000). This pattern is very similar to GAP-43 expression where it diminishes once the

normal peripheral target tissues are re-innervated (Schreyer and Skene, 1991). STAT3 is

phosphorylated at Tyr705 residue by JAK kinase in response to cytokine or growth factor

activation of a cell surface receptor. STAT3 is also phosphorylated by PKC at a different

amino acid residue, Ser727, similar to serine phosphorylation of GAP-43 (Tsai et al.,

2007). Following phosphorylation by receptor tyrosine kinases, pSTAT3 then forms homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. Given the similarity of the pattern of expression and the fact that GAP-43 regulation is associated with the presence of AP-1 site, there is a possibility that pSTAT3 homodimers may alone activate the AP-1 site as a result of phosphorylation of PKC or JAK pathways. Alternatively, pSTAT3 can heterodimerize with other transcription factors to potentially influence GAP-43 gene regulation through its AP-1 site.

Cytokines such as IL-6, CNTF and LIF, which are released from Schwann cells and are retrogradely transported to the neuronal cell body after peripheral nerve injury, have been suggested to control STAT3 phosphorylation (Curtis et al., 1993; Curtis et al., 1994). Furthermore, a cooperative transcriptional activity of c-Jun and STAT3 has been documented (Schaefer et al., 1995; Zhang et al., 1999). It is possible that STAT3 could modulate the pattern of transcriptional activity of c-Jun, or ATF3 (Schwaiger et al., 2000).

In contrast to the situation seen following peripheral nerve injury, our studies show for the first time that total pSTAT3 expression is also increased following central root transection of DRG neurons, a pattern that is markedly different from the pattern observed for GAP-43, ATF3 and c-Jun. This implies that pSTAT3 expression is not governed by the same mechanisms that control expression of these three other proteins. It further casts doubt on whether activation of STAT3 is directly involved in regulating transcription of GAP-43 at all.

The discrepancy between the response of STAT3 following central axotomy and the failure of other transcription factors to respond following central root transection

suggests a fundamental difference in the mechanisms governing these responses. The previously described sensitivity of STAT3 to Schwann cell-derived inflammatory cytokines supports the idea that positive inflammatory signals may induce STAT3 transcription and activation when the dorsal root is injured, as well as when the peripheral nerve is injured.

8.2.1.7. Multiple elements of an injury response

The activation of the intrinsic growth capacity of the DRG neurons may involve a number of transcription factors. Our study focused on ATF3, c-Jun, and STAT3. Whether one of these transcription factors acts as an upstream regulator of the others cannot be resolved using the techniques reported here. A recent report (Kiryu-Seo et al., 2008) indicates that the ATF3, c-Jun, and STAT3 transcription factors must first interact with yet another transcription factor, specificity transcription protein-1 (Sp1). This report suggests that Sp1 recruits ATF3, c-Jun, and STAT3 to obtain the requisite synergistic effect. Further, this report also identifies ATF3 as one of the most critical and one of the earliest transcription factors activated after nerve injury (Kiryu-Seo et al., 2008).

MAPKs induce the expression of AP-1 family transcription factors such as Fos and Jun and activate the transcriptional activity of AP-1 site containing genes by phosphorylation of transcription factor activation domains. Homodimers of Jun and heterodimers of Jun/Fos and ATF/CREB bZIP transcription factors all recognize the AP-1 site (Hai and Curran, 1991). The ATF3 transcription factor heterodimerizes with transcription factors that contain leucine zipper domains. These include c-Jun, c-fos and CREB, all upregulated in DRG neurons by nerve injury. ATF3 may also interact with other transcription factors that lack a leucine zipper region, and that are upregulated after

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peripheral injury, such as STAT3. This could be via co-activators or by synergizing with transcription factors that bind to adjacent DNA binding sites (Seijffers et al., 2007). It is well documented that ATF3 is required, but may not be sufficient for the conditioning lesion regrowth of the central branch of the injured DRG neurons (Seijffers et al., 2007).

There are STAT-binding sites in the promoters of many immediate early genes (c-Jun, ATF3, and c-fos) that may potentially bind to the AP-1 site in the GAP-43 gene. In another mechanistic possibility, STAT3 and c-Jun can form a ternary complex that specifically recognizes the AP-1 site (Yoo et al., 2001). Activation of the AP-1 appears to be one of the key regulators of GAP-43 expression. Thus, the activation of STAT3 and/or c-Jun may be required for GAP-43 elevation as a result of peripheral nerve transection, and as part of the DRG neuronal injury response mechanism.

8.2.2. Minipump infusion of FGF-2 or dbcAMP.

Previous work in our laboratory demonstrated that bath-applied FGF-2 or dbcAMP could suppress the upregulation of GAP-43 expression that occurs when adult DRG neurons are excised and maintained in tissue culture (Schreyer, 2004). A key finding of the present study is that chronic infusion of FGF-2 or dbcAMP at the site of peripheral axon transection *in vivo* did not suppress injury induced upregulation of GAP-43 in DRG neurons. In fact, FGF-2 and dbcAMP each modestly enhanced injury-induced GAP-43 expression, in sharp contrast with our *in vitro* findings. The present observations thus argue against a role for extracellular FGF-2 or intracellular cAMP in mediating a chronic repressive signal derived from peripheral targets, and whose absence results in GAP-43 upregulation.

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Previous studies found that microinjection of dbcAMP in lumbar DRG neurons enhances the regeneration of injured central sensory axon branches. The injured axons regrow into the spinal cord lesion, often traversing the injury site, mimicking the effect of a conditioning peripheral nerve lesion (Neumann et al., 2002). Sensory neurons exposed to cAMP analogs *in vivo*, and subsequently cultured *in vitro*, show enhanced growth of neurites and an ability to overcome inhibition by CNS myelin (Han et al., 2004; Qiu et al., 2002a; Qiu et al., 2002b). Thus, activation of cAMP signaling increases the intrinsic growth capacity of injured sensory axons of peripheral nerves. However, it fails to increase the rate of peripheral axon regeneration (Han et al., 2004). Intra-ganglionic injection of dbcAMP also induces IL6 and LIF mRNAs and increases the accumulation of pSTAT3 in neuronal nuclei. This dbcAMP activation of cytokines and pSTAT3 can be partially blocked by a pharmacological inhibitor of JAK 2 kinase (Wu et al., 2007). This indicates that increased cyclic AMP activity on axonal regeneration of primary sensory neurons is mediated by cytokine synthesis within the DRG (Wu et al., 2007), a potential positive signal associated with inflammation.

Cultures of the PC12 cell line which are deficient in GAP-43 can undergo dbcAMP-mediated neurite outgrowth (Baetge and Hammang, 1991). Conversely, PC12 cells that are deficient in dbcAMP-dependent protein kinase can undergo differentiation (Ginty et al., 1991; Scheibe et al., 1991). Therefore, it seems that dbcAMP can increase the intrinsic growth capacity of regenerating peripheral nerves, in the presence and activation of GAP-43, or in the absence of GAP-43. Phosphorylation of GAP-43 itself is not affected by dbcAMP (Van Hooff et al., 1988).

One of the striking findings of our present work is the inductive effect that

dbcAMP and FGF-2 had on the expression of GAP-43, especially causing the injured

small to medium diameter neurons to elevate the expression of GAP-43 (Figure 7-34).

Direct intraganglionic injection of dbcAMP increases tubulin expression in the

DRG (Han et al., 2004). Because Tα1 tubulin and GAP-43 expression have been reported

to be coregulated in response to neurotrophins (Kobayashi et al., 1997) and brief

electrical stimulation in the regenerating femoral nerve (Al-Majed et al., 2004), and

because another tubulin isoform beta3-tubulin (βIII tubulin) gene is induced through an

AP-1 site (Saussede-Aim et al., 2009), a site that is also shared with GAP-43 promoter,

our results of increased GAP-43 expression as a result of dbcAMP are not surprising.

Evidence exists demonstrating the inductive effect of FGF-2 on the expression of

GAP-43 in CNS and PNS neurons. FGF-2 increases the level of GAP-43 mRNA in O2A

progenitor cells *in vitro* (Deloulme et al., 1993). FGF-2 also promotes GAP-43

translocation from the cytosol to the membrane and, at the same time, stimulates GAP-43

phosphorylation (Tejero-Diez et al., 2000). In addition, FGF-2 elevates the synthesis of

GAP-43 in injured retinal ganglion cells, suggesting a pivotal role in injury response and

subsequent axonal growth (Soto et al., 2003). Finally, Schwann cells overexpressing

FGF-2 transplanted in peripherally injured sciatic nerve increased GAP-43 mRNA levels

in DRG neurons (Haastert et al., 2008). These results taken together are in support of our

findings regarding the inductive effect of FGF-2 on the expression of GAP-43.

Another important finding in our current work is that FGF-2 did not suppress

GAP-43 levels in injured neurons. This is also in contrast to *in vitro* results showing a

suppressive activity of FGF-2 on GAP-43 expression (Schreyer, 2004). Our *in vivo*

results agree with published work that shows FGF-2 effectively induces levels of GAP-43 expression through activation of the AP-1 site (Yang et al., 2008). Therefore FGF-2, like cAMP, appears to produce opposing effects on DRG neurons depending on whether they are applied to cut axon ends *in vivo* or by bath application *in vitro*.

We find that cytoplasmic ATF3 levels are reduced as a result of one week of continuous dbcAMP infusion. This may be explained by the fact that ATF3 biosynthesis, but not phosphorylation and nuclear translocation, may be impaired possibly via activation of calcineurin, a cyclosporin-sensitive, calcium-regulated, calmodulin dependent serine/threonine phosphatase (Rusnak and Mertz, 2000). Calcineurin has been shown to be an inhibitor of ATF3 synthesis (Mayer, Dexheimer et al. 2008). Calcineurin is also involved in nuclear import of transcription factors (Polizotto and Cyert 2001), and regulates the K(ATP) channel by inhibiting PKA-dependent phosphorylation of the channel as well as PKA itself (Orie et al., 2009). Such a regulatory mechanism may explain the reduction of the cytosolic ATF3 caused by dbcAMP. Alternatively, ATF3 may heterodimerize with other bZIP and non bZIP family transcription factors. When such protein-protein interaction occurs, it may alter the protein tertiary structure to a form that our antibody does not recognize.

Direct interaction between ATF3 and other transcription factors to regulate gene expression is well documented. ATF3 may be activated independently of cyclic AMP. Cyclic AMP-independent ATF family members have been reported to interact with NFκB and function in the activation of the E-selectin promoter in response to cytokines (Kaszubska et al., 1993). Specifically, the promoter element that ATF3 binds differs by a single nucleotide substitution from the dbcAMP-responsive element consensus sequence,

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and operates in a dbcAMP-independent manner to induce transcription (Kaszubska et al., 1993).

Sequence analysis of the ATF3 gene 5'-flanking region revealed a consensus TATA box and a number of transcription factor binding sites including the AP-1, ATF/CRE, NFκB, E2F, and Myc/Max binding sites. In addition, c-Jun was shown to activate the ATF3 promoter, possibly through the JNK/SAPK pathway (Liang et al., 1996).

The amount of transection-induced GAP-43 expression in DRG neurons is independent of the distance of the injury from the neuronal cell bodies (Liabotis and Schreyer, 1995). ATF3 induction, on the other hand is dependent on the distance between the injury site and the cell body (Tsujino et al., 2000) in DRG neurons. This supports the notion that the molecular pathways regulating GAP-43 could be different and may be independent of the pathways controlling ATF3 expression, despite the fact that both have the AP-1 site common in their regulatory mechanism. In addition, this may explain the reason why cAMP induced GAP-43, and, simultaneously, suppressed ATF3 *in* vivo in our minipump model. Therefore, based on our current findings *in vivo*, cAMP and FGF-2 act to increase the expression of GAP-43, but suppress that of ATF3.

Our experiments show for the first time that dbcAMP delivered at a peripheral nerve transection site suppressed all injury induced transcription factors studied, with the exception of pSTAT3. Increased accumulation of pSTAT3 in the neuronal nuclei as a result of peripheral nerve transection is in agreement with the literature (Lee et al., 2004; Wu et al., 2007). DbcAMP induced pSTAT3 expression in DRG neurons was blocked by a JAK2 inhibitor (Wu et al., 2007). We found that dbcAMP increased the level of

cytoplasmic pSTAT3 expression, indicating that the rate of phosphorylation of this transcription factor is increased. We also found that dbcAMP applied to the proximal cut end of the nerve did not suppress pSTAT3 accumulation in the nucleus.

8.3. Epac Signaling mechanisms.

Recent results indicate the existence of a signaling pathway from cAMP to PKC that is independent of PKA (Parada et al., 2005), but involves the cAMP-activated guanine exchange factor Epac (Hucho et al., 2005). Epac appears to mediate cyclic AMP-dependent axon growth, guidance and regeneration and enhancement of DRG neurite outgrowth *in vitro* (Murray and Shewan, 2008). For example, siRNA mediated knockdown of Epac reduces DRG neurite outgrowth (Murray and Shewan, 2008). Thus, in addition to mediating cAMP-dependent axon growth and guidance, Epac may offer an important target for inducing axon regeneration *in vivo* (Murray and Shewan, 2008). Thus it is possible that cAMP can influence neuronal phenotype either through the Epac system, or through the MAPK pathway, or both.

8.4. Differences between *in vitro* versus *in vivo* conditions.

Our finding that FGF-2 or dbcAMP applied at the injury site can each enhance the upregulation of GAP-43 that follows peripheral nerve transection *in vivo* stands in contrast to previous observations that FGF-2 and dbcAMP can each, or in combination, suppress the upregulation of GAP-43 expression that occurs in adult DRG neurons *in vitro* (Schreyer, 2004) . Standard culture conditions create an artificial and possibly impoverished extracellular environment which causes specific subpopulations of adult DRG neurons to *de novo* synthesize and store transmitters that are not normally

encountered *in vivo* (Schoenen et al., 1989). Thus, culture conditions alone may explain why DRG neurons react differently to FGF-2 or dbcAMP. It is also likely that there is a difference in the electrical activity of DRG neurons maintained (injured) *in vivo* or *in vitro*. Finally, although the DRG neurons in our minipump studies were axotomized and deprived of retrograde signals from their peripheral targets, cultured neurons are deprived of signals from both their peripheral and central (spinal cord and medulla) target fields.

DRG neurons adopt a unique morphology in culture, extending dendrites and or other cellular processes that are absent in *in vivo* conditions. When dissociated DRG neurons are maintained in tissue culture conditions the entire neuron is exposed to materials (dbcAMP, and FGF-2) that are delivered into the culture medium. In our *in vivo* model, FGF-2 and or dbcAMP were specifically delivered to the cut end of the sciatic nerve. This may explain the discrepancy between the effects of these reagents on GAP-43 expression. In normal *in vivo* conditions, potential members of FGF-2 receptor family such as FGFR1, 2 and other members may be localized to the nerve endings where target tissues are innervated. We introduced our injury at the mid-thigh level, breaking the cellular continuity with any FGF-2 receptors that are present at the nerve endings. Similarly, receptors for ligands that cause an increase in intracellular cAMP levels could also be absent at the cut axon stump. This could also potentially explain the difference between FGF-2 and cAMP effects we observed *in vivo* and the previous observations *in vitro*.

A an additional possible explanation of the discrepancy observed between *in vivo* and *in vitro* results may be related to the presence of Sprouty2 as a modulator of FGF-2 influence. As a result of *in vivo* implantation Sprouty2 expression may have increased to

a level that negatively affects FGF-2 signaling through the MEK/ERK pathway. Moreover, it has been reported that local application of FGF-2 increases the rate of infection at the local site of delivery (Kalicke et al., 2007). It is thereofre possible that, as a result of the presence of FGF-2 at the cut site of the nerve, increased rates of infection may have ensued. This could have casued increased secretion of cytokines or other factors that may have influenced the otherwise normal pathways of FFG-2 or cAMP.

8.5 Contralateral effects.

Published data on the occurrence of contralateral effects following unilateral peripheral nerve injury are numerous and it is suggested that these differences can be accounted for by the methodology and type of detection method used (Booth and Brown, 1993; Dubovy et al., 2006; Dubovy et al., 2007). The presence of contralateral effects as a result of ipsilateral manipulations implies the existence of unrecognized signaling mechanisms that link the two sides of the body. In particular, there may be a central mechanism signaling via the system of interneurons within the spinal cord (Koltzenburg et al., 1999). The general circulation may be a second, nonspecific way for delivery of factors from injured nerve to the contralateral DRG which are not directly associated with the injured nerve. In our minipump experiments, we observed some contralateral effects. We suggest that these effects may have originated as a result of some molecules escaping the chamber unit into the systemic circulation.

8.6 Interactions between FGF-2 and cAMP in inducing changes in GAP-43 expression

Activated receptors that influence cAMP production are bound to the heterotrimeric G proteins and receptor occupation can activate the G protein α-subunit. The α-subunit, in turn, hydrolyses GTP to GDP, resulting in the disscociation of the α-subunit. This α-subunit activates adenylyl cyclase, which in turn generates cAMP from ATP. The intracellular increase in cAMP leads to the activaition of protein kinase A (PKA). PKA phosphorylates, among other proteins, the transcription factor CREB, which binds to genes containing a cAMP-response element (CRE). These response elements may include elements that control the expression of immediate early genes, ATF3 being one example. CREB phosphorylation can also induce or suppress the expression of other delayed response genes. PKA has been implicated in the activation of c-Jun transcription factor and other transcription factors may also be activated by PKA.

In addition, cAMP acting through the guanine exchange factor Epac can be an access point for cross-talk to other pathways. For example, the ras/MAPK pathway can be activated by Epac. This MAPK pathway is also subject to extracellular activation by mitogens or growth factors such as FGF-2. Thus, the divergent complexity of cAMP pathways renders cAMP potentially one of the most pivotal biochemical pathways in the process of peripheral nerve regeneration.

FGF-2 can bind to several different FGF receptors (FGFR1-4). This leads to activation of MAPK which, in turn, can induce or suppress the expression of transcription factors that may regulate the injury response. Activation of specific cell signaling pathways is dependent upon the interaction of specific FGF ligands and FGF receptors

and cell type. In addition, some FGF/FGF receptor complexes are translocated to the nucleus where they signal gene expression. Recent work suggests that some FGF isoforms may function as nuclear signaling factors without ever being secreted (Gringel et al., 2004).

The possibilities of cross talk between cAMP and FGF-2 pathways are numerous. In endothelial cells, FGF-2 induced raf/MAPK is inhibited by increasing intracellular cAMP levels, possibly mediated by PKA. Moreover, elevations in cAMP/PKA activity inhibit mitogen-induced cell proliferation. These findings demonstrate that the cAMP/PKA signaling pathway is potentially an important physiological inhibitor of mitogen activation of the MAPK cascade and cell proliferation (D'Angelo et al., 1997).

8.7 Positive and negative signaling that modulates changes in GAP-43 expression

A framework of the steps leading to a successful regeneration based on the conveyance of a positive signal from the injury site has been proposed by Ambron and Walters (Ambron and Walters, 1996), in their studies of the marine mollusk *Aplysia californica*. Successful nerve regeneration depends on transcriptional activation as a result of signals from the site of injury reaching the nucleus. The first stage is proposed to be initiated by action potentials induced by injury. These action potentials reach the cell body and act via calcium and cAMP to switch on early genes. The second stage is initiated at the site of injury, where MAP-kinases are locally activated, then retrogradely transported through the axons back to the nucleus, triggering yet additional changes. The third stage is modulated by signals that originate from growth factors and cytokines released extraneuronally by cells at the site of injury. Finally, as regenerating axons

make contact with the peripheral target, signals from target derived growth factors and signaling cues arrive at the cell body to stop the regenerative process (Ambron and Walters, 1996).

Our observation of an increase of total STAT3 and pSTAT3 expression following central root transection of DRG neurons indicate that the elements that control STAT 3 synthesis and phosphorylation do not originate from the peripheral target tissue. As a result of central root transection, cytokines such as IL-6, CNTF and LIF, which are released from cells a the site of injury, can be retrogradely transported to the neuronal cell body and act as positive signals to upregulate the expression and activation of STAT3.

After peripheral nerve transection many adaptive changes are influenced by altered availability of neurotrophic factors to the injured neurons. Administration of exogenous NGF, for example, counteracts many degenerative changes observed in the axotomized DRG neurons which are nerve growth factor-responsive (Verge et al., 1996). Changes of retrogradely transported neurotrophins other than NGF also cause upregulation of vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and galanin in well defined neuronal subpopulations (Hokfelt et al., 1994; Kashiba et al., 1992).

In our model, ATF3 and c-Jun may represent examples of negative regulation by target derived factor(s). Uninjured neurons do not express ATF3 and very little c-Jun. Once the peripheral target has been disconnected, by peripheral nerve transection, ATF3 and c-Jun are robustly upregulated in the majority of injured neurons. However, central root transection does not affect ATF3 and c-Jun expression presumably because access to retrograde signals derived from the periphery is left intact.

8.8 Possible mechanism of GAP-43 upregulation

The influence of cAMP on transcriptional regulation is mediated by cAMP response elements (CRE) transcription factors. PKA mediated phosphorylation of CRE binding protein (CREB) at serine 133 is required for transcriptional activation (Gonzalez and Montminy, 1989; Gonzalez et al., 1989).

The intricate signal transduction pathways of PKA and PKC represent integrated signaling avenues that provide multiple levels of regulation and remarkable sensitivity in responding to neuronal injury. Cross-talk between these pathways may be the norm rather than the exception, providing the neuron with greater sensitivity in discerning the multiple extracellular signals that are active during the course of the regenerative response. This level of complexity of interactions renders neurons more responsive to endogenous and perhaps exogenous modulators of the neuronal regenerative reponse, and provides exquisite control of gene expression. Peripheral nerve injury causes the release of a number of cytokines which have been documented to play an important role in the initiation, and maintenance of the regenerative response typically seen following peripheral nerve injury. It seems that the JAK/STAT pathway activation could be one of the main mediators in the signal transduction pathway of cytokines in response to our peripheral injury model. However, since we did not observe identical response to that of GAP-43 expression as a result of dorsal root transection, it would seem unlikely that JAK/STAT pathway controls GAP-43 expression. Phosphorylation of STAT3 on position Tyr705 and enhanced nuclear translocation was found within 3 h in injured neurons, lasting longer than 3 months (Schwaiger et al., 2000). Thus, while STAT3 activation might be an important step in the overall sensory neuron response to injury, it

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could influence the expression of growth associated proteins other than GAP-43. Alternatively, STAT3 activation may modify the neuronal responsiveness to other signaling molecules that, under normal circumstances, would not be active. Differential upregulation of certain receptors, for example NP-1, selectively in large diameter neurons as a result of peripheral nerve transection has been demonstrated (Gavazzi et al., 2000). It would also reasonable to suggest that, not only certain signaling pathways may be activated as a result of STAT3 activation, but also selected size of neurons may become more sensitized to specific signaling pathways.

Based on our findings and those of others (Kiryu-Seo et al., 2008) a mechanism leading to elevated GAP-43 expression *in vivo* can be proposed: Immediately following a peripheral nerve injury, specificity transcription protein-1 (Sp1) is activated. STAT3, one of the earliest transcription factors is then phosphorylated. Sp1 recruits STAT3 to initiate JAK/STAT3 signaling pathway leading to upregulation of growth associated proteins other than GAP-43. As the time course of injury proceeds, ATF3 is activated. Sp1 then recruits both STAT3 and ATF3 to activate the program of GAP-43 upregulation. c-Jun then follows, providing additional synergistic effect on the expression of GAP-43 expression (Figure 8-1).
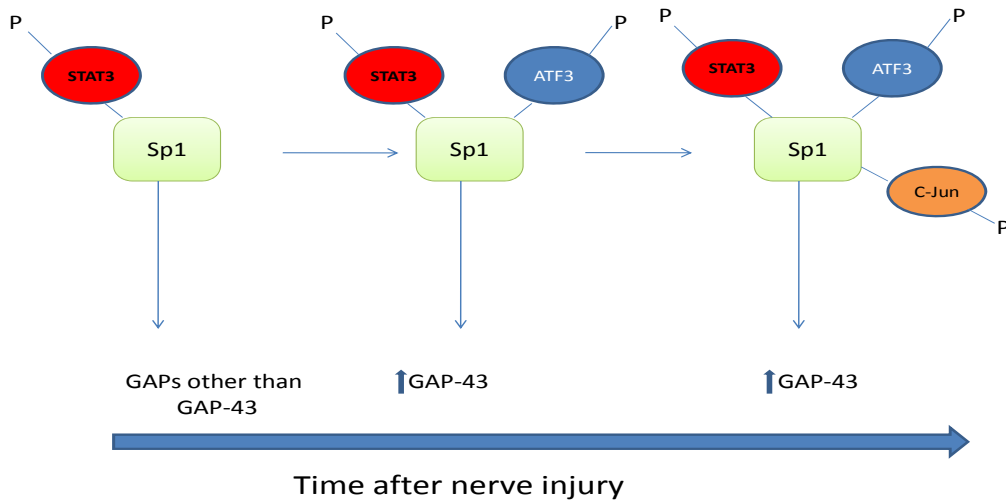
Figure 8-1. Proposed mechanism of GAP-43 upregulation as a result of phosphorylation of STAT3, ATF3, and c-Jun. Phosphorylated STAT3 is recruited first by Sp1, and GAPs are upregulated. When ATF3 is activated, it is also recruited by Sp1 leading to the upregulation of GAP-43. When c-Jun is activated, the synergy between the three transcription factors leads to the upregulation of GAP-43.

CHAPTER 9
9.0 CONCLUSION

GAP-43 expression is upregulated as a result of peripheral, but not central axonal branch injury in DRG neurons. Key transcription factors ATF3, c-Jun, and STAT3 showed a distinct pattern of expression, with ATF3 and c-Jun expression closely resembling GAP-43 expression. STAT3 cytoplasmic expression was the exception, since it did not change as a result of peripheral nerve transection, but was upregulated as a result of dorsal root transection.

These results support a multifactorial scheme of injury response as a result of peripheral and central axon branch injuries. Specifically, peripheral branch injurymay cause a robust regenerative response, because interruption of signals originating from peripheral target tissues control the normally suppressed state of growth associated proteins and genes.   However, the increase in cytoplasmic expression of STAT3 observed as a result of dorsal root transection supports the idea that damage to the DRG central axonal branch may generate positive, inflammatory signals that have fundamentally different effects from those available in the peripheral target tissues.

Chronic infusion of dbcAMP or FGF-2 did not suppress injury induced expressions of GAP-43. This is in sharp contrast to earlier *in vitro* studies that showed dose-dependent and synergistic repressive properties of dbcAMP and FGF-2. Injury induced upregulation of the other transcription factors were, however, repressed as a result of dbcAMP and FGF-2 administration. STAT3 revealed a pattern of response to cAMP and FGF-2 that is similar to that of GAP-43.

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9.1 Future directions.

The injury response of peripheral nerves involves a complex series of metabolic and neuronal cell biologic events that take into account synthesis, sorting and assembly, and transport of specialized growth associated proteins to the site of injury that will eventually lead to successful regeneration. This process must be initiated, maintained, and then terminated once the proper target tissues have been re-innervated. We have investigated one growth factor, and one intracellular messenger. The methodology employed in our work can act as a blueprint for the study of other growth factors and intracellular molecules to be investigated in detail *in vivo.*

The time course for the activation of immediate early genes ATF3 and c-Jun must be more carefully addressed in future studies, perhaps by using a more sensitive technique such as real time polymerase chain reaction in order to clarify several important questions:

What are the key transcription factors expressed as a result of peripheral nerve injury, and which are the first to be expressed? How intricately are those transcription factors coregulated? Is there a "master" transcription factor that governs all subsequent response? Is there a time course for the neuronal size subsets such that one subset responds to nerve injury before another? Each of these questions addresses part of the puzzle of peripheral nerve regeneration, and may provide further insight into the chain of causation of the metabolic response to nerve injury.

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